



E-Abstracts and Proceedings of
The 10th
Biotechnology International
Congress (BIC 2026)



2-3 September 2026

Bangkok International Trade & Exhibition Centre (BITEC), Bangkok, THAILAND

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WELCOME MESSAGE

Dear Esteemed Colleagues and Friends,

On behalf of the Thai Society for Biotechnology (TSB) and the Asian Federation of Biotechnology – Thailand Regional Branch Office (AFOB–Thailand RBO), it is my great pleasure to warmly welcome you to the **10th Biotechnology International Congress (BIC 2026)**, to be held on 2–3 September 2026.

This congress serves as a dynamic platform for knowledge exchange, fostering collaboration, and advancing scientific innovation across the biotechnology community.

The theme of this year's congress, *"Next Generation Biotechnology: from Research to Sustainable Global Impacts,"* underscores the transformative power of next-generation biotechnology in translating cutting-edge research into real-world solutions. It highlights how emerging technologies such as synthetic biology, precision fermentation, genomics, and AI-driven innovations are converging to address pressing global challenges in health, food security, industry, and environmental sustainability.

We are honored to welcome distinguished speakers from Thailand and our valued partner organizations, including Biotechnology and Bioengineering Society of Taiwan (BEST), Korea Society for Biotechnology and Bioengineering (KSBB), Asian Federation of Biotechnology-Malaysian Chapter (AFOB-MC), and Society for Biotechnology Japan (SBJ). Our collaboration with the AFOB Division on Biopharmaceutical and Medical Biotechnology will provide valuable insights into the latest advancements in these rapidly evolving fields.

In addition, we are pleased to encourage the participation of high school students in research project exchanges, fostering early engagement in science and strengthening STEM education at the foundational level.

Let us come together to connect, learn, and be inspired at BIC 2026. I would also like to express our sincere appreciation to VNU Asia Pacific for their invaluable support in co-organizing this event alongside Thailand LAB INTERNATIONAL.

Wishing you a successful congress, fruitful discussions, and a rewarding experience at the **10th Biotechnology International Congress (BIC 2026)** and Thailand LAB INTERNATIONAL, taking place on 2–4 September 2026.

With warm regards,

Associate Prof. Chuenchit Boonchird

President Thai Society for Biotechnology
Vice President AFOB-Thailand



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CONFERENCE SCHEDULE

The 10th Biotechnology International Congress (BIC 2026)

DAY 1: Wednesday, 2 September 2026

ROOM: MR212 - MR213					
Start Time	End Time	Code	Speaker	Topic	Chair/Co-Chair
8.00 AM	9.30 AM	Registration			
9.30 AM	10.00 AM	Opening Ceremony	Welcome Speech: - Mr. Anucha Parnpichat (Assistant Director Competence Center LAB & Health VNU Asia Pacific) - Prof. Takeshi Omasa (President of The Asian Federation for Biotechnology, AFOB) Opening Speech: Assoc. Prof. Chuenchit Boonchird (President of The Thai Society for Biotechnology)		MC: Assoc. Prof. Rujikan Nasanit
10.00 AM	10.30 AM	Plenary I	Prof. Takeshi Omasa (Osaka University)	Mammalian Cells: A Workhorse Platform for Biologics Production	Chair: Prof. Chi-Wei Lan
10.30 AM	11.00 AM	Plenary II	Prof. Jian-Jiang Zhong (Shanghai Jiao University)	Biomanufacturing Technology: Its Evolution and Contribution to the Society	
11.00 AM	11.20 AM	Break			Co-Chair: Assoc. Prof. Rujikan Nasanit
11.20 AM	11.50 AM	Plenary III	Prof. Vip Viprakasit (Mahidol University)	The Cure is in the Code: The Rise of Gene Therapy	
11.50 AM	1.00 PM	Lunch and Visit Exhibition Hall EH102 – EH104			

CONFERENCE SCHEDULE

DAY 1: Wednesday, 2 September 2026

ROOM: MR212																		
Start Time	End Time	Code		Speaker					Topic								Chair/Co-Chair	
Session I																		
1.00 PM	1.20 PM	Keynote I		Prof. Penjit Srinophakun (Kasetsart university)					Life Cycle Assessment of Lignin-Derived Biomaterial for Better Environmental Friendliness								Chair: Prof. Mohd Fauzi Mh Busra Co-Chair: Dr. Phattanon Prasithichoke	
1.20 PM	1.35 PM	Invited I		Prof. Ir Juferi Idris (UiTM Sarawak Branch)					Optimization of Biochar Derived from Gaharu Waste (<i>Aquilaria malaccensis</i>) for the Adsorption of Methylene Blue via the Taguchi Method									
1.35 PM	1.50 PM	Invited II		Prof. Joongoo Lee (POSTECH)					Automated, Molecular Assembly of Reconstituted Cell-Free Systems from in Vitro Produced Components									
1.50 PM	2.02 PM	OP-01		Anuyut Yootoum					Comparative Pretreatment of Coffee Cherry Pulp for Polyhydroxyalkanoate Production by Thermotolerant <i>Inquilinus</i> sp. CF22									
2.02 PM	2.14 PM	OP-02		Mary Segbefia					Sustainable Pretreatment of Durian Rind for Cellulose Nanofiber Extraction as a Bio-Based Reinforcement Material									
2.14 PM	2.26 PM	OP-03		Bana Azad Tawfeeq					Plant-Microbe Interactions for Microplastic Removal Using Mangrove-Associated Herbaceous Plants in a Hydroponic System									
2.26 PM	2.38 PM	OP-04		Hamza Ghilzay					Green Extraction and Isolation of Cellulose Nanofibrils from Kiam (<i>Cotylelobium melanoxylon</i>) Wood Waste									
2.50 PM	3.50 PM	Break & Poster Session (Foyer between MR213 - MR214)																
		PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01
		PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18
		PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35
Session III																		
3.50 PM	4.10 PM	Keynote III		Prof. Mohd Fauzi Mh Busra (Universiti Kebangsaan Malaysia)					Next-Generation Chronic Wound Therapy: Enhancing ColPatch® with Plasma-Functionalised Bioscaffolds								Chair: Prof. Penjit Srinophakun Co-Chair: Assoc. Prof. Nopphon Weeranoppanant	
4.10 PM	4.30 PM	Keynote IV		Assoc. Prof. Datin Ts. Khairani Idah Mokhtar (International Islamic University Malaysia)					Harnessing Nature’s Potential: <i>In vitro</i> Evaluation of Malayan Deer Velvet Antler Extract and Chitosan Scaffolds on Dental Stem Cell-Based Bone Regeneration									
4.30 PM	4.50 PM	Keynote V		Assoc. Prof. Indranil Banerjee (Indian Institute of Technology)					Serum Albumin Supramolecular Complex based NIR-Responsive Multifunctional Phototherapeutic Platform for Chronic Wound Healing									
4.50 PM	5.02 PM	OP-09		Naga Darsshini					Physicochemical Characterization and 2D In Vitro Wound Healing Evaluation of a Novel Tripeptide									
5.02 PM	5.14 PM	OP-10		Fang Lim					Effect of Peptide Sequence Length on the Physicochemical Properties and Biological Performance in Hyperglycaemic Wound Healing									
5.14 PM	5.26 PM	OP-11		Suppanut Sripratum					Cellulase-Assisted Enzymatic Remodeling of Bacterial Cellulose as a Scaffolds for Tissue Engineering Applications									

CONFERENCE SCHEDULE

DAY 1: Wednesday, 2 September 2026

ROOM: MR213																		
Start Time	End Time	Code		Speaker					Topic							Chair/Co-Chair		
Session II																		
1.00 PM	1.20 PM	Keynote II		Prof. Kunal Pal (National Institute of Technology Rourkela)					Functional Mimicry of Solid Shortenings via Synergistic Polymer–Lipid Emulgels: Engineering Structure–Quality Relationships in Steamed Bread Formulations							Chair: Prof. Takeshi Omasa Co-Chair: Assoc. Prof. Nisit Watthanasakphuban		
1.20 PM	1.35 PM	Invited III		Assoc. Prof. Paiboon Tunsagool (Kasetsart University)					Genome-Based Analysis and Anti-Oomycete Activity of <i>Priestia aryabhattai</i> PTKU-123 Against the Durian Pathogen <i>Phytophthora palmivora</i>									
1.35 PM	1.47 PM	OP-5		Fina Amreta Laksmi					Single-Site Polarity Modulation Improves the Catalytic Efficiency and Thermostability of D-allulose 3-epimerase through Flexibility–Rigidity Rebalancing									
1.47 PM	1.59 PM	OP-6		Anusada Pattaravipak					Development of Oil-in-Water Emulsion Formulations to Enhance the Efficiency of Vitamin D3 Encapsulation									
1.59 PM	2.11 PM	OP-7		Pornpavit Phewkum					Application of Ohmic Heating Pretreatment to Accelerate the Aging Process and Modulate the Bioactive Profile of Black Shallots									
2.11 PM	2.23 PM	OP-8		Farida Pattanabhummi					Evaluation of the Protective Effects of an Energy- and Electrolyte-Enhanced Mulberry Beverage Against Delayed Onset Muscle Soreness Through Redox Balance Regulation									
2.47 PM	3.50 PM	Break & Poster Session (Foyer between MR213 - MR214)																
		PP-01	PP-02	PP-03	PP-04	PP-05	PP-06	PP-07	PP-08	PP-09	PP-10	PP-11	PP-12	PP-13	PP-14	PP-15	PP-16	PP-17
		PP-18	PP-19	PP-20	PP-21	PP-22	PP-23	PP-24	PP-25	PP-26	PP-27	PP-28	PP-29	PP-30	PP-31	PP-32	PP-33	PP-34
		PP-35	PP-36	PP-37	PP-38	PP-39	PP-40	PP-41	PP-42	PP-43	PP-44	PP-45	PP-46	-	-	-	-	-
Session IV																		
3.50 PM	4.10 PM	Keynote VI		Prof. Hideki Aoyagi (University of Tsukuba)					Development of an <i>In Vitro</i> Co-Culture Platform for Studying Interactions Between Intestinal Epithelial Cells and the Gut Microbe							Chair: Prof. Kunal Pal Co-Chair: Assoc. Prof. Paiboon Tunsagool		
4.10 PM	4.25 PM	Invited IV		Dr. Tsung-ju Li (Grape King Bio Co, Ltd.)					Liquid Fermentation Tech for Sustainable Health and Industry Advancement									
4.25 PM	4.40 PM	Invited V		Asst. Prof. Nisa Patikarnmonthon (Mahidol University)					Evolution of Nanoparticle Assembly: Bridging Proven Encapsulation with Reconfigurable Microfluidic Platforms									
4.40 PM	4.55 PM	Invited VI		Assoc. Prof. Jittima Luckanagul (Chulalongkorn University)					Designing Impactful Nanomedicine: Bridging Scientific Discovery and Global Translation									
4.55 PM	5.07 PM	OP-12		Ekaterina Grizanova					Evolution of <i>Bacillus thuringiensis</i> Virulence during Passage through the Resistant and Susceptible Host									
5.07 PM	5.19 PM	OP-13		Nichapat Wongsanekham					Encapsulation of Anti-Dengue Virus NS3 Monoclonal Antibody in PEG-PLGA Nanoparticles for Intracellular Delivery									

CONFERENCE SCHEDULE

The 10th Biotechnology International Congress (BIC 2026)

DAY 2: Thursday, 3 September 2026

ROOM: MR212 - MR213					
Start Time	End Time	Code	Speaker	Topic	Chair/Co-Chair
8.00 AM	9.30 AM	Registration			
9.30 AM	10.00 AM	Plenary IV	Prof. Alissara Reungsang (Khon Kaen University)	Sustainable Utilization of Sugarcane Industry Biomass and Residues for Bioenergy and Biochemical Production	Chair: Prof. Hideki Aoyagi Co-Chair: Assoc. Prof. Tatsaporn Todhanakasem
10.00 AM	10.30 AM	Plenary V	Dr. Uracha Ruktanoncha (NANOTEC)	Next-Generation Nanobiotechnology: Transforming Healthcare, Food Systems, and Sustainability	
10.30 AM	10.50 AM	Break			
10.50 AM	11.05 AM	Industry talk I	Dr. Walaiporn Timbuntam (Amano Enzyme Asia Pacific Co., Ltd)	Industrial Enzymes for a Circular Future: Global Market Landscape and Amano Specialty Solutions"	
11.05 AM	11.20 AM	Industry talk II	Ms Sawitree Suklour (Gibthai Co. Ltd.)	From Discovery to Biomanufacturing - Integrated Biotechnology Solutions for Sustainable Innovation	
11.20 AM	1.00 PM	Lunch and Visit Exhibition Hall EH102 – EH104			

CONFERENCE SCHEDULE

DAY 2: Thursday, 3 September 2026

ROOM: MR212																		
Start Time	End Time	Code				Speaker				Topic							Chair/Co-Chair	
Session V																		
1.00 PM	1.20 PM	Keynote VII				Prof. Ivan Dubovskiy (Siberian State University of Engineering and Biotechnology)				Biocontrol of Insects Pests in Russia: from Classic Bioinsecticides to State of Art Technology / or Pest Insects Biocontrol using Microbials and Genetic Biotechnologies							Chair: Prof. Alissara Reungsang Co-Chair: Dr. Phattanon Prasithichoke	
1.20 PM	1.35 PM	Invited VII				Assoc. Prof. Thawatchai Chaijarasphong (Mahidol University)				CRISPR Detection Platforms for Diseases in Aquaculture								
1.35 PM	1.47 PM	OP-14				Nantavadee Bootprom				Development and Evaluation of a Pumpkin Seed-Enriched Functional Cream Cheese for Modulating Risk Factors of Polyendocrine Metabolic Ovarian Syndrome								
1.47 PM	1.59 PM	OP-15				Pincha Kwandee				Fecal Microbiota Alterations Induced by Triphala Extract								
1.59 PM	2.11 PM	OP-16				James Konkov				In Vitro Activity of a Co-Eluted Amphiphilic Crude Mixture Obtained from <i>Priestia aryabhatai</i> PTKU-123 Against <i>Phytophthora palmivora</i>								
2.11 PM	2.23 PM	OP-17				Agnibha Banerjee				Co-Solvent-Driven Structural Engineering of Rice Bran Oil-Based Oleogels Through Multidimensional Characterization: Advancing Solid Fat Mimetics in Food Biotechnology								
2.23 PM	2.35 PM	OP-18				Sampurna Ghosh				Multiscale Investigation of Pectin-Mediated Structural Reinforcement and Shelf-Life Enhancement in Whole Wheat Bread								
2.35 PM	3.35 PM	Break & Poster Session (Foyer between MR213 - MR214)																
		PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01	PP-01
		PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18	PP-18
		PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35	PP-35
Session VI																		
3.35 PM	3.55 PM	Keynote VIII				Prof. Chi-Wei Lan (Yuan Ze University)				Nano-Bio Synergy: Biomass-Derived Carbon Quantum Dots for Enhancing Microbial Metabolism and Biochemical Production							Chair: Prof. Ivan Dubovskiy Co-Chair: Assoc. Prof. Thawatchai Chaijarasphong	
3.55 PM	4.15 PM	Keynote IX				Dr. Sissades Tongsima (BIOTEC)				Pioneering the Computational Genomic Bedrock of Next-Generation Biotechnology								
4.15 PM	4.30 PM	Invited IX				Assoc. Prof. Thanyaporn Wongnate (VISTEC)				Research and Innovation for Sustainable Waste Management in the Bio-Based Economy								
4.30 PM	4.45 PM	Invited X				Dr. Thapakorn Jaroentomeecha (Mahidol University)				Cell-Free Synthetic Glycobiology: Engineering Glycomolecules for Next-Generation Biotherapeutics and Vaccines								
4.45 PM	4.57 PM	OP-19				Sachidanand Singh				In Silico Evaluation of <i>Moringa oleifera</i> and <i>Solanum americanum</i> Phytochemicals to Protect Red Blood Cell Membrane in Haemolytic Anemia: A Network Pharmacology Approach								
4.57 PM	5.09 PM	OP-20				Sivatch Imrattanak				Characterization, Purification and Media optimization of <i>Saccharomyces cerevisiae</i> BHF5D35-2Δade2 for Superoxide dismutase production								
5.09 PM	5.55 PM	Awards and Closing Ceremony															Asst. Prof. Adisak Romsang	

CONFERENCE SCHEDULE

DAY 2: Thursday, 3 September 2026

ROOM: MR213																					
Start Time	End Time	Code			Speaker					Topic							Chair/Co-Chair				
Youth Science																					
1.00 PM	1.15 PM	Invited VIII			Asst. Prof. Adisak Romsang (Mahidol University)					Shaping the Future of Responsible Biotechnology in Thailand through Biosafety Education and Training							Chair: Prof. Joongoo Lee Co-Chair: Dr. Thapakorn Jaroentomeechai				
1.15 PM	1.30 PM	OP-21			Korapak Thaweechaithaworn					SPIDERS DETECH: An AI Innovation for Classifying Spider Species via a LINE Chatbot											
1.30 PM	1.45 PM	OP-22			Noppatsorn Virulbuntheng					A Deep Learning-Based Smartphone Solution for Thai Jasmine Rice Quality Assessment											
1.45 PM	2.00 PM	OP-23			Thanistar Kingnara					Deep Learning-Based Skin Disease Classification Chatbot											
2.00 PM	2.15 PM	OP-24			Jiratchaya Sathitkul					Evaluation of Antioxidant Activity, Total Phenolic Content, and Total Flavonoid Content of <i>Phyllanthus emblica</i> and <i>Garcinia mangostana</i> Extracts for Potential Biotechnological Applications in Herbal Oral Healthcare											
2.15 PM	2.30 PM	OP-25			Kanatpol Sahakanjanakul					Bid by Bit: A Micro: bit-Based Wearable Device for Lower-Limb Motion Monitoring and Rehabilitation											
2.35 PM	3.35 PM	Break & Poster Session (Foyer between MR213 - 214)																			
		PP-01	PP-02	PP-03	PP-04	PP-05	PP-06	PP-07	PP-08	PP-09	PP-10	PP-11	PP-12	PP-13	PP-14	PP-15	PP-16	PP-17			
		PP-18	PP-19	PP-20	PP-21	PP-22	PP-23	PP-24	PP-25	PP-26	PP-27	PP-28	PP-29	PP-30	PP-31	PP-32	PP-33	PP-34			
		PP-35	PP-36	PP-37	PP-38	PP-39	PP-40	PP-41	PP-42	PP-43	PP-44	PP-45	PP-46	-	-	-	-	-			
Youth Science																					
3.35 PM	3.50 PM	OP-26			Aana Singhapong					A Preliminary Study of Liquid Biopsy-Based Detection of Epidermal Growth Factor Receptor Mutations Using a Surface Acoustic Wave Biosensor							Chair: Asst. Prof. Adisak Romsang Co-Chair: Asst. Prof. Nisa Patikarnmonthon				
3.50 PM	4.05 PM	OP-27			Kantapat Harnborvonkij					A Low-Carbon Biochar Module for Nitrogen Reduction, Eutrophication Mitigation, and Sustainable Urban Water Resilience											
4.05 PM	4.20 PM	OP-28			Alin Kasemtanakul					KneeHab Pro: A Wearable Motion Tracking System for Post Knee Surgery Rehabilitation											
4.20 PM	4.35 PM	OP-29			Punika Prempiyakij					AI-Engineered Nanoproteins: A De Novo Approach to Rapidly Suppress Human-to-Human Hantavirus Transmission											
5.09 PM	5.55 PM	Awards and Closing Ceremony (ROOM: MR212)															Asst. Prof. Adisak Romsang				

CONFERENCE SCHEDULE

Poster Session

Foyer between Room MR213 - 214		
Code	Presenter	Topic
PP-01	Chia-Hung Kuo	Gelatin/agar Film Combined with Psidium cattleianum Leaves Carbon Dots for Food Preservation
PP-02	Panyisa Potibut	Population Dynamics of Ammonia-Oxidizing and Nitrite-Oxidizing Bacteria in Outdoor Shrimp Culture Ponds
PP-03	Mongkhon Phanthura	Development of a SYBR Green Real-Time PCR Assay Targeting the <i>amoA</i> and <i>nirB</i> Genes for Monitoring Nitrifying Bacteria in a Recirculating Aquaculture System (RAS)
PP-04	Weera Piyatheerawong	Valorization of Sweet Sorghum Juice for Sustainable Protein Production Through Co-cultivation of Microalgae and Yeast Cells
PP-05	Rattiya Waeonukul	Effect of Maltose Concentration on the Production of Long-Chain Isomaltooligosaccharides by <i>Bacillus subtilis</i> strain AP-1
PP-06	Prattana Ketbot	Production of Manno Oligosaccharides from Soybean Residues by <i>Bacillus tequilensis</i> PLM1
PP-07	Watchaphon Wuttiyan	Genomic Resequencing Reveals Genetic Diversity and Population Structure of Durian in Uttaradit Province, Thailand
PP-08	Noppakorn Thadasawate	Postbiotic Potential of Cell-Free Supernatants from Khao-mak Microbial Isolates: Antimicrobial Activity and Stability
PP-09	Saharit Palasan	Nested Recombinase Polymerase Amplification Coupled with a CRISPR-Cas12a Assay for Sensitive and Specific Detection of <i>Streptococcus iniae</i> in Fish
PP-10	Suparada Suwanlertlum	Rapid CRISPR-Based Detection Platform for Species-Specific Identification of <i>Edwardsiella</i> Bacteria
PP-11	Rungnapa Pichaikarn	Whole Genome Characterization of <i>Streptomyces angustmyceticus</i> as a Potential Biological Control Agent Against Southern Blight Disease Caused by <i>Agrothelia rolfsii</i>
PP-12	David Herawan	Substantial Catalytic Enhancement of D-allulose 3-epimerase from <i>Arthrobacter psychrolactophilus</i> : A Sustainable Chitosan Immobilization Strategy for Production of Low-Calorie Sweetener
PP-13	Yeryeong Lee	Preparation and Characterization of Mealworm-Contained Rice Starch Soft Foods for Older Adults with Dysphagia
PP-14	Mamoru Wakayama	Isolation and characterization of α -1,3-glucanase from alkaliphilic bacterium, <i>Alkalihalobacillus</i> sp.
PP-15	Paphanpaw Lekboonphet	Optimal Conditions for Mycelial Growth of the Ectomycorrhizal Mushroom Het Ra-ngok Lueang (<i>Amanita javanica</i>)
PP-16	Sirilak Baramee	D-Tagatose Production Using Recombinant L-Arabinose Isomerase from <i>Cellulomonas palmitytica</i>
PP-17	Jinnawat Nakmuang	Evaluation of Factors Affecting Phase Separation in Pickering Emulsions Stabilized by <i>Microbacterium esteraromaticum</i> SBS1-7
PP-18	Yuka Oe	Analysis of the Growth of Lactic Acid Bacteria Producing D-Serine under Controlled pH Conditions and the Expression of Genes Related to Serine Metabolism
PP-19	Nanako Mizuno	Identification and Functional Analysis of Serine Racemase Genes in D-Serine-Producing Lactic Acid Bacteria
PP-20	Otono Tsurudome	Roles of Aspartate β -Decarboxylases in Aspartate Metabolism in <i>Acetobacter pasteurianus</i> SKU1108
PP-21	Nang Yee Mon Htoo	Structural Basis for the High Catalytic Efficiency of the Iron-Dependent Xylose Isomerase from <i>Lactococcus lactis</i> IO-1
PP-22	Thunyamas Guntawang	Development of a Paper-Based Test Strip for Colorimetric Detection of Cholinesterase Activity in Canine Serum
PP-23	Apinya Singkhala	Valorization of Soybean Processing Residue as an Alternative Nitrogen Source for Enhanced Dark Fermentative Biohydrogen Production

CONFERENCE SCHEDULE

Poster Session

Foyer between Room MR213 - 214		
Code	Speaker	Topic
PP-24	Nurulhuda Madawa	Isolation and Identification of Thermoacidophilic Bacteria for Accelerated Food Waste Degradation
PP-25	Chonticha Janprungton	Evaluation of Culture-Substrate Compatibility and Hydraulic Retention Time for Enhanced Biohydrogen Production
PP-26	Thiyapan Noppakoon	Valorization of Corn Tassel Agro-Residues: Polyphenol Recovery and Cosmetic Potential via Conventional and Ultrasound-Assisted Extractions
PP-27	Thanpitcha Jenkit	Antioxidant and Antimicrobial Activities of Crude Extracts from <i>Phellinus</i> sp.
PP-28	Wasupol Phakamas	In Vitro Generation of Multispecific Cytotoxic T Lymphocytes Targeting GD2, PRAME, and PD-L1
PP-29	Thanakorn Tangsereesuk	Cloning, Heterologous Expression, and Purification of Sphingomyelinase of <i>Staphylococcus epidermidis</i>
PP-30	Sidra Bibi	Optimizing Mycelium Pellet Morphology and Its Impact on Bioactive Compound Production via a Taguchi Image Analysis Framework
PP-31	Worraanong Leewattanasuk	Nucleotide Variations in the Middle Region of Melanocortin 4 Receptor Gene and Their Potential Association with Obesity Risk in the Thai Population
PP-32	Nitikul Teankrajang	Expression and Purification of Recombinant <i>SpCas9-HF1</i> -Plus Protein in <i>Escherichia coli</i> lysYI [®] for High-Precision Gene Editing
PP-33	Tung Anh Hoang	A Multipurpose Antibody Format for Flexible Assay Development Using Cell-Based and Cell-Free Production
PP-34	Yujin Jeong	Thermo-Responsive Morphological Evolution and Rheological Behavior of Wax/Butter-Based Phase Change Microparticles
PP-35	Hwain Lee	Skin-Adhesive Illite-Containing Amphiphilic Hydrogel Composite Patches for Wound Care
PP-36	Jarunluk Keerativoranant	Investigating the Role of <i>Fusobacterium nucleatum</i> in Colorectal Cancer and the Inhibitory Effect of <i>Dendranthema morifolium</i> as a Natural Antibacterial Agent
PP-37	Thanabodee Worrakul	AI-Based Oral Lesion Detection System with Integrated Online Chatbot
PP-38	Saowaphak Duangram	Deciphering the Tissue-Specific Molecular Imprint of Oral Mesenchymal Stem Cells: A Comparative RNA-Seq Analysis
PP-39	Panisa Ngiampaisal	Transcriptomic Effects of Propylene Glycol on Oral Mucosal Barrier Integrity
PP-40	Siwat Viriyaoydiyiem	PANI Smart Sutures
PP-41	Poonnapa Warunyurattana	Development of a Graphene-Modified Quartz Crystal Microbalance Biosensor for Non-Invasive Prostate Cancer Detection
PP-42	Tiarn Sripongtanakul	<i>mecA</i> and <i>dnaG</i> Gene Knockout in <i>Staphylococcus aureus</i> via CRISPR-Cas9 System as a Model for Methicillin-resistant <i>Staphylococcus aureus</i> inhibition
PP-43	Pititada Preechapongmit	An AI-Based System for Facial Expression Classification: A Comparative Study of Model Training Across Various Emotions
PP-44	Punyada Vanitchanont	Development of an AI-Based LINE Chatbot for Preliminary Classification of Common Pediatric Skin Conditions
PP-45	Wasitpol Akkawattananukul	Computational Evaluation of Understudied Second-Shell Residues Surrounding the Catalytic Triad of PETase
PP-46	Panisara Kraichotchai	Deep Learning-Based Classification of Oral Diseases

Mammalian Cells: A Workhorse Platform for Biologics Production

Takeshi Omasa^{1,2*}, and Noriko Yamano-Adachi^{1,2}

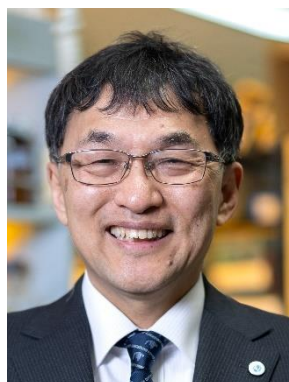
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ABSTRACT: Mammalian cell lines are indispensable platforms for the industrial-scale production of pharmaceutical proteins (biologics) because of their ability to perform accurate protein folding, assembly, and post-translational modifications. Among these, Chinese hamster ovary (CHO) cells have become the gold standard for the production of therapeutic proteins, owing to their high reliability and robustness. In our recent work, we established novel cell lines derived from lung and ovary tissues of the Chinese hamster (*Cricetulus griseus*), thereby expanding the repertoire of host cells available for recombinant protein manufacturing. These cell pools were gradually adapted to serum-free conditions through stepwise reduction of serum concentrations. The resulting serum-free cell pools were designated Chinese hamster lung (CHL)-YN (Riken BioResource Bank: RCB5004) and Chinese hamster ovary (CHO)-MK, respectively. Both CHL-YN and CHO-MK cell lines exhibit rapid proliferation, with doubling times of less than 10 hours. In this presentation, we introduce the characteristics of these two cell lines and discuss their potential impact on recombinant protein manufacturing^{1,2}.

Keyword: Antibody drug, Chinese hamster ovary (CHO) cells, Cell engineering, Cell culture engineering.



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Biomanufacturing Technology: Its Evolution and Contribution to the Society**Jian-Jiang Zhong^{1*}**¹*State Key Laboratory of Microbial Metabolism, Department of Bioengineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dong-chuan Road, Shanghai, 200240, China***Correspondence to: E-mail: jjzhong@sjtu.edu.cn*

ABSTRACT: Biomanufacturing technology originates from the penicillin fermentation in the 1940s under the intimate collaboration of microbiologists and chemical engineers. It has evolved rapidly with the fast development of biology, engineering and interdisciplinary boundaries, including synthetic biology in recent years. In this presentation, recent work in biomanufacturing technology including the strain breeding and fermentation production of ethanol, antibiotics and enzymes will be briefly introduced. Considering the significance of the natural and health-enhancing functional foods in preventing diseases and promoting human health, studies on the edible medicinal mushroom *Ganoderma lucidum* (Lingzhi) and the medicinal plant ginseng, both of which have been used for over 2000 years as oral administration medicines in East Asia to treat various diseases and improve human health, are to be presented in detail, which produces bioactive triterpenoids ganoderic acids (GAs) and ginsenosides, respectively. An example of synthetic biology-based biomanufacturing of GAs by engineering baker's yeast is further shown. We design an GA biosynthetic pathway, and have identified key P450 enzymes catalyzing the triterpenoid structure modification and reprogrammed the GA biosynthetic network in the yeast *Saccharomyces cerevisiae*. For example, the P450 enzymes CYP512W2 (oxidization), CYP512W6 (hydroxylation) and CYP512A13 (catalyzing the conversion of carbon-carbon conjugated double bonds on lanostane skeleton) were identified and analyzed. By engineering the key enzymes, a higher titer of GAs was achieved in the yeast fermentation. In addition, we reveal the catalytic mechanism of related CYPs. Our work provides new insights into the post-modification of triterpenoids. In addition, we investigated bioactivities of GAs, e.g., the inhibition of GA-T on hepatocellular carcinoma both *in vitro* and *in vivo* was revealed. Our work creates an efficient biomanufacturing platform for functional food key components - GAs, and it is of great impact on the development of GAs-producing yeast based dietary nutrition for human health.

Keyword: Biomanufacturing technology, Human health, Synthetic biology, Bioactive compounds, Catalytic mechanism of enzymes



Dr. JJ Zhong is a distinguished professor at Shanghai Jiao Tong University, China. He has published over 270 papers in peer-reviewed international journals. He was awarded the Cheung Kong Scholar Professorship from the Ministry of Education (MOE), National Science Fund for Distinguished Young Scholars by National Natural Science Foundation of China, the inaugural award of "Young Asian Biotechnologist Prize" by the Society for Biotechnology (Japan), the 1st-class Prize for Advancement of Science & Technology by MOE for twice (in 2004 & 2014), and the "Research Exchange Award" from the Korean Society for Biotechnology and Bioengineering. He is on the editorial board of 10 SCI-cited international journals and was an invited speaker in about 90 international conferences.

The Cure is in The Code: The Rise of Gene Therapy

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ABSTRACT: Cell and gene therapy have transitioned from conceptual innovation to transformative clinical reality, redefining the therapeutic landscape for both inherited and acquired diseases. This presentation provides a comprehensive overview of the evolution of gene-based interventions, from early viral vector strategies to current precision genome editing platforms, including CRISPR/Cas systems and next-generation delivery technologies. Emphasis is placed on the translation of these advances into real-world clinical applications, particularly in monogenic disorders such as thalassemia, where gene addition and gene editing approaches have demonstrated curative potential by restoring effective erythropoiesis and eliminating transfusion dependence. Beyond hematologic diseases, the field has expanded rapidly into immunotherapy, where genetic modification of immune cells—most notably chimeric antigen receptor (CAR) T cells and emerging CAR-NK platforms—has revolutionized cancer treatment. These strategies are now being extended to autoimmune diseases, offering the possibility of durable immune reprogramming. As the burden of cancer and immune-mediated disorders continues to rise globally, including in Thailand, such approaches represent a paradigm shift toward highly personalized and potentially curative therapies. Critical challenges remain, including scalability, cost, long-term safety, and equitable access, particularly in low- and middle-income settings. The integration of regulatory innovation, such as adaptive frameworks and “sandbox” models, alongside regional manufacturing capabilities, will be essential to bridge the gap between discovery and accessibility.

Keyword: Gene therapy, Genome editing, Thalassemia, CAR-T cells, Precision medicine



Prof. Vip Viprakasit, MD, DPhil (Oxon) is a Professor of Pediatrics at Mahidol University and a leading hematologist at Siriraj Hospital, Bangkok. He directs one of Southeast Asia's largest thalassemia centers, with expertise in red cell disorders, genomics, and gene therapy. His work spans clinical care, translational research, and precision medicine. He has received numerous national and international awards for clinical excellence and research innovation including the Outstanding Scientist Award and Outstanding Researcher Award of the year and is widely recognized for advancing curative therapies and fostering global collaborations in hematology and gene therapy.

Sustainable Utilization of Sugarcane Industry Biomass and Residues for Bioenergy and Biochemical Production

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ABSTRACT: Thailand is among the world's largest sugarcane and sugar producers and generates enormous volumes of residues — bagasse, sugarcane leaves, filter cake, filter press, and bagasse ash — together with downstream by-products such as molasses and hydrogenic effluent. Much of this material is still disposed of by open-field burning or landfilling, contributing to PM_{2.5}, greenhouse-gas emissions, and the loss of valuable carbon and nutrients. This keynote presents an integrated biorefinery strategy, executed under Thailand's Bio-Circular-Green (BCG) Economy framework, that converts these residues into bioenergy, biochemicals, and biomaterials. Hydrothermal carbonization (HTC) of bagasse and sugarcane leaves under dual-optimized conditions produced hydrochar with HHV of 23.4–23.6 MJ/kg, an 18–23% upgrade over raw biomass. Its quality was comparable to lignite, but with sulfur below 0.05%. The hydrochar also exhibited ammonium adsorption of 23.76 mg/g. Integration with solar drying reduced energy cost by 83% and CO₂ emissions by approximately 80%. Metal-modified (Fe, Ni) biochar from the same residues enhanced dark fermentative biohydrogen yield to 108.77 mL-H₂/g-glucose, a 58.77% increase over the control. Fermentation of sugarcane leaf hydrolysate by *Actinobacillus succinogenes* TISTR 1994 yielded 18.87 g/L succinic acid (yield 0.59 g/g, sugar utilization 95.3%), with transcriptomic analysis revealing an inverse relationship between Reductive-TCA gene expression and product output. Sugarcane-waste-derived pellets enabled simultaneous CO₂ and H₂S removal from real biogas, with an IRR of 51% and a 2-year payback. Downstream, freshwater microalgae *Coelastrella* sp. KCU-P1 and *Acutodesmus* sp. KCU-P2 valorized hydrogenic effluent into polyhydroxybutyrate (up to 6.35% of dry cell weight) while removing 38–53% COD, 14–20% nitrogen, and 56–69% phosphorus. Together, these results demonstrate a cascading biorefinery that transforms every fraction of the sugarcane industry residue stream into bioenergy, biochemicals, and biomaterials — supporting Thailand's transition toward carbon neutrality and the UN Sustainable Development Goals.

Keyword: Sugarcane residues, Biorefinery, Biohydrogen, Hydrothermal carbonization, Bio-Circular-Green economy



EDUCATION

Ph.D. Water Resources, Iowa State University, USA (2000)

M.Sc. Pharmaceutical Sciences (Microbiology), Mahidol University, Thailand (1991)

B.Sc. Biotechnology, Khon Kaen University, Thailand (1989)

RESEARCH INTERESTS

Biohydrogen, biomethane, and biohythane production from lignocellulosic biomass; bioremediation and phytoremediation; waste treatment and utilization; biorefinery technologies for circular bioeconomy

KEY ACHIEVEMENTS

245+ publications (Scopus) 300+ (Google Scholar) | 7,300+ citations (Scopus) 9,000+ citations (Google Scholar) | h-index 49 (Scopus) h-index 53 (Google Scholar) | Stanford Top 2% World Scientist (2020–2025)

16 petty patents | 70+ funded research projects as PI | 8 book chapters

TRF/TSRI Senior Research Scholar (2016–2022)

SELECTED HONORS & AWARDS

National Distinguished Researcher Award (NRCT, 2020) | NRCT Research Awards (2016, 2023, 2026)

Toray Science & Technology Prize (2022) | ASAIHL Research Excellence Award (2023)

AAAE Researcher/Innovator of the Year (2024) | Associate Fellow, Royal Institute of Thailand (2020–present) | High-Caliber Researcher (NRCT, 2024)

Next-Generation Nanobiotechnology: Transforming Healthcare, Food Systems, and Sustainability

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ABSTRACT: In the era of rapid global transitions, next-generation nanobiotechnology drives paradigm shifts across healthcare, food systems, and environmental sustainability. As a key engine for deep-tech development, we pioneer the translation of bio-based nano-delivery platforms into high-impact socioeconomic solutions. Our research focuses on utilizing sophisticated nanocarriers such as nanostructured lipid carriers (NLCs), liposomes, and transfersomes to amplify the bioavailability, controlled release, and targeted efficacy of natural bioactive compounds. In advanced nanomedicine, NANOTEC has successfully developed cannabidiol (CBD)-loaded lipid nanoparticles that stabilize the gut-brain-bone axis and enhance neuroplasticity, alongside oral and nasal andrographolide-loaded nanocarriers designed to mitigate systemic neuroinflammation and acute lung injury. In oncology, dual-folate/biotin-decorated liposomes deliver methylnaphthazarin (MNZ) with enhanced specificity against receptor-overexpressing cancer cells. Bridging nanotechnology with dermatological care, CBD-loaded hydrogel scaffolds, transfersomal *Centella asiatica* extracts, and novel dihydroxyresveratrol (DHO)-loaded NLCs offer highly permeable, safe topical solutions that accelerate wound healing and treat hyperpigmentation. Beyond therapeutics, NANOTEC reshapes precision nutrition by valorizing regional agricultural assets to combat metabolic syndromes. The encapsulation of *Kaempferia parviflora* (Black Ginger) methoxyflavones within tailored NLCs dramatically upgrades oral bioavailability to manage obesity and dyslipidemia, while the standardized extraction of gymnemic acid derivatives from *Gymnema inodorum* (Chiang Da) provides scientifically validated anti-diabetic activities for functional food ingredients. Crucially, we strictly adhere to green chemistry principles by utilizing biodegradable matrices and ultrasound-assisted technologies, ensuring that the full lifecycle of these advanced materials minimizes environmental footprints and directly supports the Bio-Circular-Green (BCG) economy model. In conclusion, the integration of next-generation nanobiotechnology into healthcare and food systems represents a monumental step toward achieving sustainable global impact. By transforming volatile, poorly soluble natural molecules into highly stable, targeted nano-formulations, we successfully translate lab-scale innovations into tangible real-world applications that unlock the commercial potential of biodiversity and address global challenges.

Keyword: Nanobiotechnology, Nano-delivery systems, Thai herbal valorization, Healthcare innovation, Sustainable food systems.

	EDUCATION 2002: Ph.D. Advanced Drug Delivery, University of Nottingham, England 1998: B.Sc. Pharmaceutical Sciences, Khon Kaen University, Thailand Research Areas Lipid and polymeric nanoparticles, Nano-encapsulation, Advance drug delivery system KEY ACHIEVEMENTS AWARDS/ACHIEVEMENTS 1. 2025: The Alumni of Honor Award for Research and Innovation for Society from Khon Kaen University. 2. 2025: World's Top 2% Scientist 2025 in the field of Pharmacology & Pharmacy, based on citation impact in 2024, (Stanford University & Elsevier database). 3. 2020-2024: being listed as top 2% of the most cited scientist in the field of Pharmacy & Pharmacology, Elsevier database. 4. 2018: UK alumni Award Professional Achievement from the British council and UK Embassy 5. 2017 – present: selected to be Fellow of the Royal Society of Chemistry, UK (FRSC) 6. 2017 – present: appointed to be Honorary Professor by University of Queensland, Australia 7. 2015 – present: appointed to be Adjunct Professor of Asian Institute of Technology, Thailand 8. 2011: For Women in Science fellowship Award from UNESCO-L'Oreal Foundation. 9. The Young Scientist Award of 2010 from the Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King, Thailand 10. Selected to be 1 of “66 Young Leaders Shaping Thailand's Future of 2012” by the Bangkok Post newspaper which celebrated its 66 years in operation 11. Selected as one of ten prestigious Top careers for 2020 “This's My Future” in 2009 organized by Thaikom foundation 12. Selected to be 1 of 10 “prestige Women of the Year 2006” by Prestige magazine, The nation, and Bangkok Business. 13. Selected to be one of Thai Young leader representatives to join STS forum, Japan 2014
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Life Cycle Assessment of Lignin-Derived Biomaterial for Better Environmental Friendliness

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ABSTRACT: Lignin is an important component in biomass, alongside cellulose and hemicellulose. In ligno-cellulosic ethanol and nanocrystalline cellulose production, lignin is removed and disposed of in wastewater. The black color of lignin is difficult to remove, but it is biodegradable and antimicrobial. Therefore, in this study, lignin was recovered from the wastewater system through precipitation and used to produce lignin-derived biomaterials, which were combined with polyvinyl alcohol (PVA) for mulch films and with polylactic acid (PLA) and polybutylene adipate-co-terephthalate (PBAT) for packaging plastics. It was found that incorporating lignin improved the mechanical properties and biodegradability of the biomaterials. The biomaterials' characteristics were analyzed using SEM, FTIR, XRD, and TGA. Mechanical properties such as tensile strength, elongation at break, and Young's modulus were measured, and biodegradation tests were conducted. A life cycle assessment using SimaPro software and the ReCiPe 2016 Midpoint method indicated both biomaterials had a positive environmental impact and improved biodegradability. The main environmental concerns were global warming, terrestrial ecotoxicity, and marine and freshwater ecotoxicity related to solvent and chemical use. Results showed that adding small amounts of lignin enhanced biodegradability and reduced the environmental footprint of the biomaterials, highlighting their potential for sustainable UV protection material in agriculture and biomedical packaging.

Keyword: Lignin, Antimicrobial activity, Biomaterials, Mulch film, Life Cycle Assessment



Professor Dr. Penjit Srinophakun graduated in Chemical Engineering from the University of Queensland, Australia, in 1996. Then, she returned to work at the Chemical Engineering Department, Faculty of Engineering, Kasetsart University, Thailand. In 2005, 2010, and 2011, she was appointed as the director of the KU-biodiesel Project, director of the Center of Excellence for Jatropha, and the Vice President of the Asian Federation of Biotechnology (AFOB). During 2011-2016 and 2020-2022, she was elected President of the Thai Society for Biotechnology (TSB). Currently, she is more active in research focusing on bioenergy and biorefinery, ligno-cellulosic ethanol, biomaterial, techno-economic feasibility, and Life Cycle Assessment.

Functional Mimicry of Solid Shortenings via Synergistic Polymer–Lipid Emulgels: Engineering Structure–Quality Relationships in Steamed Bread Formulations

Anannya Sahoo^{1#}, Debmalya Banerjee^{1#}, Agnibha Banerjee^{1#}, Bala Chakravarthy Neelapu¹, Sivaraman Jayaraman¹, Maciej Jarzębski², Prakrit Sukyai^{3*}, and Kunal Pal^{1*}

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ABSTRACT: The role of traditional shortenings is vital in modulating the stability of gas cells, water retention, and crumb structure in bakery products. Thus, replacing them with alternative structuring agents becomes significantly challenging. In the present study, novel synergistic polymer-lipid emulgels based on cassia gum powder (CGP), psyllium husk polysaccharide (PHP), and rice bran oil (RBO) were prepared to serve as functional shortening mimics and were further utilized in the formulation of a steamed bread (SB) system. Structural features and quality attributes of formulated SBs were evaluated using various techniques, such as surface microscopy, electrochemical impedance spectroscopy (EIS), moisture analysis, and colorimetric analysis. It was found that substituting the SB formulations with polymer-lipid emulgels induced physicochemical alterations, especially in terms of structural and quality parameters, largely dictated by the emulgel composition. Specifically, both too low and too high amounts of oil in the emulgel formulations drastically affected the crumb architecture. In this context, among the formulations, SB-CI2 (SB formulation prepared with 20% RBO) exhibited an optimal structure-quality equilibrium, as evidenced by a consistent porous crumb structure, high moisture content, and desirable impedance profiles and color attributes. The balanced polymer-lipid network of SB-CI2 promoted gas retention, trapping of the water molecules, and the formation of a coherent matrix, thereby facilitating the development of an organized crumb structure and improved quality characteristics. Notably, SB-CI2 could mimic the functionality of conventional shortenings while incorporating only a lower amount of actual oil, translating to 80% less oil incorporated into the SB matrix. These results revealed that these synergistic polymer-lipid emulgels could serve as tools for structuring the quality attributes of SB formulations and as an alternative for developing healthier bakery products with lower oil content while preserving the structural attributes.

Keyword: Polymer-lipid emulgels, Steamed bread, Solid shortening mimics, Structure–quality relationships, Reduced-oil bakery products.



Professor Kunal Pal, Professor in the Department of Biotechnology and Medical Engineering at the National Institute of Technology Rourkela, India, received his Ph.D. in Materials Science and Engineering from IIT Kharagpur and completed his postdoctoral research at Toronto Metropolitan University, Canada. His research interests include biomaterials, drug delivery, tissue engineering, food processing technology, and AI-driven healthcare. He has published more than 240 research articles in international journals and conference proceedings.

Next-Generation Chronic Wound Therapy: Enhancing ColPatch® with Plasma-Functionalised Bioscaffolds

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ABSTRACT: Chronic diabetic and full-thickness skin wounds remain significant clinical challenges, characterised by impaired cell migration, persistent inflammation, and heightened susceptibility to infection. This presentation integrates two complementary research streams that advance collagen-based biomaterials from mechanistic preclinical investigation to early clinical translation. The first study centres on a three-dimensional acellular ovine tendon collagen type I (OTC-I) bioscaffold, crosslinked with genipin and functionalised via post-carvone plasma polymerisation (GNPppCar). In a streptozotocin-induced diabetic murine model, GNPppCar markedly accelerated wound closure, promoted re-epithelialisation and granulation tissue formation, and reduced inflammatory cell infiltration relative to controls. In vitro assays using human dermal fibroblasts and keratinocytes further demonstrated enhanced cell viability, migration, and proliferation. Mechanistic analyses revealed activation of focal adhesion kinase and PI3K/Akt signalling pathways, underpinning improved cellular attachment and survival. The second study translates this biofunctional scaffold strategy into clinical application through the dermal regeneration template, ColPatch®. In a prospective pilot study of patients with full-thickness skin defects, ColPatch® was well tolerated, with no adverse events reported over a six-month follow-up period. Consistent wound progression and improved scar quality were observed, supporting its safety, feasibility, and therapeutic potential in human use. Collectively, these findings establish a translational continuum from advanced scaffold biofunctionalisation to clinical implementation, underscoring the promise of plasma-enhanced collagen biomaterials as next-generation therapies for chronic wound regeneration.

Keyword: Full-thickness wound, Collagen type I, Plasma polymerization, Plastic surgery, Reconstruction, Skin substitutes



Prof. Dr. Mohd Fauzi Mh Busra (Fauzi MB) is a Full Professor of Advanced Regenerative Medicine (Advanced Biomaterials & Tissue Engineering) at Universiti Kebangsaan Malaysia (UKM) and a leading researcher in advanced biomaterials, tissue engineering, and regenerative medicine, with a focus on collagen-based biomaterials and other natural-based resources for wound healing and tissue repair. He is affiliated with the Department of Tissue Engineering and Regenerative Medicine at Faculty of Medicine, UKM and has contributed extensively to high-impact publications on biomaterial scaffolds and regenerative therapies. He is also actively involved in translational research and commercialization, notably leading the development of ColPatch®, a collagen-based implantable biomaterial for wound management that has demonstrated strong biocompatibility and potential as a therapeutic medical device. The product has progressed from laboratory research toward clinical application and commercialization pathways, reflecting his role in bridging fundamental science with clinical innovation. In addition to teaching and supervising postgraduate students, he has led million dollar research grants and multidisciplinary collaborations, contributing significantly to advancements in biomedical science and regenerative medicine in Malaysia.

Harnessing Nature's Potential: *In Vitro* Evaluation of Malayan Deer Velvet Antler Extract and Chitosan Scaffolds on Dental Stem Cell-Based Bone Regeneration

Khairani Idah Mokhtar^{1*}, Azalea Nur-Qistina Azami², Basma Ezzat Mustafa¹, Norzaity Mohd. Kenali³, Munirah Sha'ban², Azlina Ahmad⁴, Ismail Zainol⁵

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ABSTRACT: In the pursuit of sustainable healthcare, next-generation biotechnology is increasingly exploring natural bio-resources as viable alternatives to synthetic drugs for tissue repair. Deer velvet antler (DVA), or *tanduk rusa*, is traditionally used for its health benefits, yet fundamental research on its effects on dental stem cells remains essential for scientific validation. This presentation explores the scientific translation via fundamental approach of DVA; a traditional Asian remedy, into a potential biological stimulus for regenerative medicine. This study aimed to evaluate the scientific merit of water-extracted DVA from the Malayan deer (*Rusa timorensis*) as a natural stimulus for bone development. Specifically, it investigated the impact of DVA on the biological activity of stem cells from human exfoliated deciduous teeth (SHED) and the combined influence of DVA and chitosan scaffolds in driving osteogenic differentiation. The research employed multidisciplinary approach: cell viability and proliferation were assessed via MTT and alamarBlue assays, while osteogenic potential was quantified through alkaline phosphatase (ALP) activity, calcium mineralization (Alizarin Red-S), and scanning electron microscopy (SEM). Molecular modulation was determined using RT-qPCR for key markers including *RUNX2*, *OSX*, and *OCN*. DVA water extract was non-cytotoxic across all tested concentrations, with 3.125 ug/ml identified as the optimal concentration for cell proliferation. At the functional level, DVA significantly enhanced ALP activity and mineral deposition in SHED. While chitosan scaffolds effectively supported cell proliferation, they were found to inhibit early-stage differentiation. Crucially, the addition of DVA "rescued" this effect, promoting functional osteogenesis and the upregulation of osteogenic gene *RUNX2*. These findings establish DVA as a potent natural osteoinductive factor capable of driving osteoblast differentiation even within suboptimal scaffold environments. By integrating bio-resources with biocompatible materials, this research highlights a sustainable biotechnological framework for bone tissue engineering, bridging the gap between traditional research and future medical impact.

Keyword: DVA-water extract, SHED, Chitosan scaffold, Osteoblast differentiation



Assoc. Prof. Ts. Dr. Khairani Idah Mokhtar is a researcher at the Kulliyah of Dentistry, International Islamic University Malaysia (IIUM), specializing in Developmental Biology with interest in craniofacial genetics, dental stem cells and natural products. Her research work focuses on the fundamental developmental processes of craniofacial structures, particularly secondary palate development and craniofacial abnormalities, and the application of dental stem cells and natural product extracts for tissue regeneration. On top of craniofacial abnormality research, a key pillar of her research is the exploration of bioactive natural products, such as flaxseed extract and Malayan deer antler velvet to modulate the osteogenic differentiation of stem cells from human exfoliated deciduous teeth (SHED). She has secured multiple high-impact projects under the National Fundamental Research Grant Scheme (FRGS) and collaborating on transdisciplinary studies involving natural products, tissue regeneration and craniofacial abnormalities. Her scholarly output includes over 50 publications, and publications of seminal books such as *Dental Stem Cells: Culturing for the Future*. Her research excellence is recognized through her appointment as a National Panel Assessor for FRGS and her status as a Professional Technologist (Ts.). She has successfully translated her research expertise into academic leadership by assisting in the establishment of research-based Master of Dental Sciences and PhD programs in Kulliyah of Dentistry, while supervising numerous postgraduate students; both PhD and Master students, to completion.

Serum Albumin Supramolecular Complex based NIR-Responsive Multifunctional Phototherapeutic Platform for Chronic Wound Healing

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342030, India*

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ABSTRACT: Chronic wounds such as diabetic foot ulcers and infected lesions exhibit delayed healing driven by antibiotic-resistant infection, ischemia, tissue degradation, and fibroblast senescence. Traditional therapies often fail because they lack a comprehensive mechanism of action. To address this, we developed a near-infrared (NIR)-responsive phototherapeutic supramolecular complex composed of bovine serum albumin (BSA), indocyanine green (ICG), and Cu (II) to provide a multifunctional treatment for chronic infected wounds. This platform was designed to simultaneously eradicate bacteria via photothermal and photodynamic effects under 808 nm laser exposure, stimulate angiogenesis through copper-mediated signaling, and activate fibroblasts to accelerate tissue regeneration. Three BSA variants (A7030, A3059, A3294) based on different fatty acid, globulin and protease content were screened to identify an optimal carrier for ICG and Cu (II). The biophysical characterization of 1:1 BSA–ICG complexes showed a pronounced red shift and broadening of ICG absorption and fluorescence for BSA-A7030, indicating improved excitation flexibility and emission properties. The BSA-ICG-Cu (II) complex was then synthesised via a green chemistry approach with BSA-A7030. SEM analysis revealed bead-and-fibril microstructures, and spectroscopy (UV–vis–NIR, fluorescence, circular dichroism) confirmed stable complexation. Under 808 nm NIR irradiation, the complex produced mild hyperthermia and strong photodynamic antibacterial effects in vitro against *Staphylococcus aureus*, *Escherichia coli*, and methicillin-resistant *S. aureus* (MRSA). The complex was further treated with human dermal fibroblasts, which showed enhanced migration and increased VEGF expression, and HUVEC angiogenesis assays displayed improved tube formation. In a *S. aureus*–infected diabetic mouse wound model, NIR-irradiated treatment accelerated wound closure and achieved >90% bacterial burden reduction by day 7 in comparison to control. These findings indicate that the BSA-ICG-Cu (II) complex is a promising NIR-responsive platform combining antibacterial, proangiogenic, and fibroblast-activating activities for chronic wound healing.

Keywords: Diabetic foot ulcer, Phototherapy, Indocyanine Green, Angiogenesis, Serum albumin



Dr. Indranil Banerjee is an associate professor in the Department of Bioscience and Bioengineering, Indian Institute of Technology Jodhpur and a Fellow of the Royal Society of Chemistry. He has been working in the domain of tissue engineering, regenerative medicine, biomaterials, and therapeutic delivery systems for the last 15 years. He has so far published 90 papers, edited 4 books, and having h-index of 44. He received Institute Research Excellence Award in 2022 from IIT Jodhpur. Beyond academia, Dr. Banerjee is an active entrepreneur.

Development of an *In Vitro* Co-Culture Platform for Studying Interactions Between Intestinal Epithelial Cells and the Gut Microbe

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ABSTRACT: Conventional co-culture systems are often complex, lack versatility, and fail to adequately reproduce the intestinal lumen environment. In this study, we developed a novel co-culture system that enables intermittent sampling of culture media, growth monitoring, aerobic–anaerobic co-culture, simple operation, and high-throughput evaluation. By separating the growth spaces of aerobic cells and anaerobic bacteria using liquid paraffin and sealing the anaerobic compartment with mineral oil, co-culture of microbe and MDCK epithelial cells was successfully achieved. The barrier function and viability of MDCK cells, as well as bacterial growth, were comparable to those in monocultures. Enhanced metabolic activity under sealed anaerobic conditions was also confirmed. This versatile system provides a practical platform for studying host–microbe interactions. I will also introduce the activities of the Society for Biotechnology, Japan.

Keyword: Aerobic-anaerobic culture, Co-culture device, Epithelial cell, Host–microbe interaction.



Prof. Hideki Aoyagi received his M.S. degree in Environmental Science in 1990 and his Ph.D. in Agricultural Sciences in 1993, both from the University of Tsukuba, Japan. He is currently a Professor at the Institute of Life and Environmental Sciences, University of Tsukuba. Now, he is vice president of the Society for Biotechnology, Japan. He specializes in cell cultivation engineering, biochemical engineering, and plant tissue culture. His research key words are useful metabolites production, bioreactors, microbes, unculturable microbe, plant cells, protoplasts, insect, animal cells, and various cell culture techniques.

Pest Insect Biocontrol using Microbial and Genetic Biotechnology Approaches

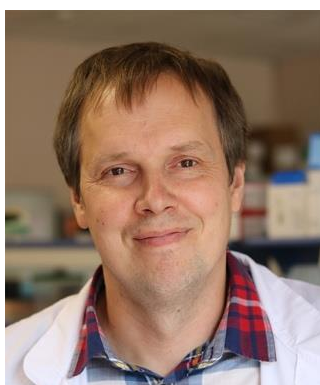
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ABSTRACT: Entomopathogenic bacteria *Bacillus thuringiensis* and fungi of the genus *Metarhizium* are actively used to create insecticidal preparations for plant protection. They account for the majority of the market of biological plant protection products in Russia. In addition to the basis of bioinsecticides, *B. thuringiensis* bacteria are the main source of genes for the transgenesis of agricultural plants, and entomopathogenic fungi of the genus *Metarhizium* are beginning to be actively used as biofertilizers. The talk will present the mechanism of action and ways to increase the effectiveness of biological preparations based on bacteria and fungi. Modern developments in the genetic transformation of entomopathogens and the use of RNA interference, botanicals, nanoparticles will be considered as strategies to increase the activity of biological products. In addition, the ways of formation of insect pest resistance to *B. thuringiensis* bacteria and promising approaches to delay the formation and overcoming of resistance will be shown. The possibility of using entomopathogenic microorganisms and their consortia as multifunctional and growth-stimulating drugs in agriculture will be discussed. The study was supported by the Russian Science Foundation (grant № 26-16-00228).

Keyword: Biological preparations, RNA interference, Pest control, Biofertilizers.



Ivan M. Dubovskiy, D. Sc.

Head of Research Center of Biological Plant Protection,
 Siberian State University of Engineering and Biotechnology

H-index (WoS): 24

WoS ResearcherID: B-8252-2008

Research Interests:

Insect immunity, experimental evolution, resistance, insect infections, *Bacillus thuringiensis*, *Metarhizium*, microevolution, antioxidant systems, genes expressions, biological control

Scientific societies:

Russian Microbiological Society (since 1997), Russian Entomology Society (since 2000), Russian Biotechnology Society (since 2006), Russian Parasitology Society (since 2008), Society for Invertebrate Pathology (since 2018)

Scientific awards:

Dubovsky I.M. awarded the prize of the governor of the Novosibirsk region (for outstanding scientific achievements, 2012) and the city administration of Novosibirsk (Best young researcher, 2018). President of Russian Foundation award grants for young scientists 2011, 2013, 2017 years. Certificate of Honor from the Ministry of Science and Education of the Russian Federation 2023 year. Gratitude from the Governor of the Novosibirsk region 2026 year.

Editorial board member:

Journal of Fungi (WoS, Q1); All Life (WoS, Q2); Bulletin of NSAU (Novosibirsk State Agrarian University) (RSCI); Frontiers in Fungal Biology (WoS)

Science popularization:

TikTok's "Clear Science" channel is a finalist for the Fidelity to Science Award in 2022. Diploma YouTube channel "Ivan Dubovsky" for 100,000 subscribers. Participants (2021, 2022) and the winner (2025) of the Film Science contest. YouTube (@DubovskiyLab) and TikTok (@labbio_nsau) "Harm the pest". Total number of subscribers in social media (TikTok, YouTube, VK) more than 1,000,000 people.

Key publications

- Butt T.M., Coates C.J., Dubovskiy I.M., Ratcliffe N.A. Entomopathogenic fungi: new insights into host-pathogen interactions. *Advances in Genetics*. 2016, 94:307-364.
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Nano–Bio Synergy: Biomass-Derived Carbon Quantum Dots for Enhancing Microbial Metabolism and Biochemical Production

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ABSTRACT: This study presents a novel nano–bio synergistic platform utilising biomass-derived carbon quantum dots (CQDs) as sustainable functional agents to enhance microbial bioprocesses. CQDs were synthesised from complex biomass feedstocks, including microalgal residues and spent brewer's grains, via facile carbonisation, enabling the valorisation of waste into high-value nanomaterials. Functioning as nanoscale electron mediators within bioelectrochemical systems, CQDs effectively stimulate microbial metabolism and cellular bioenergetics. Experimental results demonstrate that CQD supplementation significantly increases intracellular ATP levels, promotes cell growth, and enhances polyhydroxybutyrate (PHB) accumulation, achieving up to 68.13% PHB content without genetic modification. Furthermore, the study elucidates the critical role of CQD surface chemistry, where hydroxyl and carboxyl functional groups contribute to redox activity and antioxidant behaviour, thereby influencing microbial physiology. Nitrogen doping via urea improves CQD yield, dispersion, and structural uniformity, although with altered bioactivity. Overall, this work highlights the integration of nanotechnology and microbial biotechnology, supported by emerging AI-driven biorefinery frameworks, as a promising and eco-friendly strategy for sustainable waste valorisation and efficient biochemical production within a circular bioeconomy.

Keyword: Carbon Quantum Dots, Nano–Bio Synergy, Microbial Bioprocessing, Bioelectrochemical Systems, Circular Bioeconomy.



Professor John Chi-Wei Lan is a leading scholar in biochemical engineering and sustainable biotechnology, currently serving as Professor and Chair of the Department of Chemical Engineering and Materials Science and Chair of the Graduate School of Biotechnology at Yuan Ze University, Taiwan. He also serves as the President of the Biotechnology and Biochemical Engineering Society of Taiwan (BEST), where he promotes international collaboration and academic exchange in biochemical engineering and industrial biotechnology. Professor Lan received his Ph.D. in Biochemical Engineering from the University of Birmingham, United Kingdom, in 2000. He subsequently conducted postdoctoral research at Academia Sinica, Taiwan, and later held research positions at Yamaguchi University in Japan and the Industrial Technology Research Institute (ITRI) in Taiwan. Since joining Yuan Ze University in 2007, he has played an important role in advancing research and education in biochemical engineering and sustainable bioprocess technologies. His research focuses on biorefinery technologies, circular bioeconomy systems, electro-fermentation, and the development of bio-based materials. His work aims to integrate microbial biotechnology, biomass valorization, and advanced bioprocess engineering to develop sustainable platforms for converting renewable resources and industrial residues into high-value biochemical products. Through international collaborations across Asia and beyond, his research contributes to the advancement of low-carbon biotechnology and net-zero bioeconomy strategies. Professor Lan has published extensively in leading international SCI journals, including *Bioresource Technology*, *Renewable and Sustainable Energy Reviews*, and *Biochemical Engineering Journal*. His work has achieved an h-index of 33 and reflects sustained impact in the field of sustainable biotechnology. He is the recipient of several prestigious honors, including the Young Asian Biotechnologist Prize from the Society of Biotechnology Japan and the Y. S. Hsu Foundation Chair Professor Award in 2025. In addition to his academic contributions, he is also an inventor holding multiple patents and the co-founder of Lans Brewing Cooperation, demonstrating his commitment to translating biotechnology innovation into real-world industrial applications.

Next-Generation Genomic Infrastructure: Translating Population Research into Sustainable Global Impact

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ABSTRACT: Implementing equitable precision medicine requires overcoming the global bottleneck of analyzing population-scale genomic data. The Genomics Thailand (GeTH) initiative addresses this challenge through a centralized computational ecosystem. The GeTH framework manages 50,000 whole-genome sequences targeting rare diseases, hereditary cancers, and infectious diseases. These data establish the Thailand Genomic Reference (ThaiGeR). Functioning similarly to gnomAD, ThaiGeR captures the local genomic variant landscape to correct the diagnostic blind spots inherent in global reference databases. Translating this population-scale data into clinical utility relies on v@pp, a domestically engineered clinical decision support system. Functioning as a sovereign alternative to commercial platforms, v@pp integrates the post-processing of sequencing data directly with local diagnostic guidelines and the ThaiGeR allele frequency database. This integration streamlines the prioritization of etiologic mutations. Applying this system to carefully phenotyped clinical cohorts allows regional hospitals to achieve diagnostic yields of 38.9% for singletons and 52% for trios across targeted rare disease classifications. Sustaining this ecosystem requires a secure high-performance computing architecture. Centralizing these computational resources eliminates redundant institutional investments and provides equitable access to advanced bioinformatic pipelines across the healthcare system. Building upon this established foundation, the infrastructure now scales to support the Thai PanGenome (T2T) project. This next phase utilizes long-read sequencing to resolve complex structural variants and address a 5% genomic divergence from standard references like GRCh38. This comprehensive model demonstrates that rigorous computational engineering provides a concrete blueprint for precision health capacity. The resulting framework positions Southeast Asia to leverage next-generation biotechnology for sustainable global impact.

Keyword: Computational genomics, Sustainable infrastructure, Next-generation biotechnology, Precision medicine, Genomics Thailand



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Effect of Carbonization Time on the Physicochemical Properties and Methylene Blue Adsorption of Biochar from Gaharu Waste (*Aquilaria malaccensis*)

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ABSTRACT: Growing concerns over pollution, resource depletion, and waste management have increased interest in biochar as an environmentally friendly adsorbent. However, the production of biochar from gaharu waste (*Aquilaria malaccensis*) remains largely unexplored, despite its availability in Malaysia. This study investigated the effect of carbonization time on the yield, physicochemical properties, and adsorption capacity of biochar derived from gaharu waste. Gaharu wood chips were oven dried and subjected to slow pyrolysis in a nitrogen atmosphere at 800 °C with carbonization times ranging from 30 to 150 min. The resulting biochar was characterized using proximate analysis, Brunauer–Emmett–Teller surface area analysis, and scanning electron microscopy (SEM). The adsorption performance was evaluated using methylene blue as a model pollutant. The results showed that increasing the carbonization time reduced the biochar yield but enhanced the fixed carbon content and pore development. The highest adsorption capacity was achieved at a carbonization time of 150 min, with a surface area of 153.11 m²/g and methylene blue adsorption capacity of 73 mg/g. These findings demonstrate that gaharu waste is a promising biomass source for biochar production and highlight the importance of optimizing pyrolysis conditions to balance the adsorption performance and energy efficiency.

Keyword: Pyrolysis, Biochar, Gaharu waste, Methylene Blue, Adsorption capacity

1. INTRODUCTION

Environmental degradation driven by unsustainable industrial and resource practices continues to intensify, contributing to pollution, climate change, and inefficient waste management. These challenges highlight the need for sustainable strategies that minimize environmental impact while promoting resource utilization [1]. Biochar has gained attention as a functional material for environmental applications due to its ability to adsorb a wide range of pollutants and its role in converting biomass into value added products [2]. However, its performance is highly dependent on feedstock characteristics and production conditions, which govern its physicochemical properties [3].

Although biochar has been extensively produced from various biomass sources such as coconut husks [4], empty fruit bunches [5], corn straw [6], and sewage sludge [7], the exploration of less conventional feedstocks remains limited. The gaharu industry, particularly in Malaysia, generates a substantial amount of residual woody material due to the low essential oil yield of approximately 0.09% [8]. This underutilized waste is often discarded, creating environmental concerns and indicating its potential for value added conversion.

Despite its availability, the use of gaharu waste, particularly from *Aquilaria malaccensis*, for biochar production has not been systematically investigated. This gap limits the understanding of its potential as a functional adsorbent. Therefore, this study aimed to investigate the production of biochar from gaharu waste (*Aquilaria malaccensis*) via slow pyrolysis, focusing on the effect of carbonization time on biochar yield and physicochemical properties, as well as its adsorption capacity for Methylene Blue.

2. MATERIALS AND METHODS

2.1 Preparation of raw materials

The gaharu waste was obtained from a wood oil industry in Kuala Nerang, Kedah, Malaysia. The waste was collected in the form of wet solid chips. The samples underwent oven drying (Mettler, Germany) at a temperature of 105 °C. Once dried, the biomass was stored in airtight plastic containers.

Physicochemical characterization of the raw biomass was performed prior to pyrolysis. The ultimate analysis was conducted via CHNS/O analyser (Thermo Fisher Scientific, USA) following the ASTM D-5291 method. Proximate analysis was carried out to evaluate the moisture content, volatile matter, fixed carbon and ash using a thermogravimetric analyzer (TGA/SDTA851e, Mettler Toledo, Switzerland). The calorific value was determined using a C5000 IKA bomb calorimeter in accordance with the ASTM D2015-00 standard method.

2.2 Pyrolysis experiment

Pyrolysis experiments were conducted using a laboratory scale muffle furnace (Carbolite, UK). The temperature was increased to 800 °C at a constant heating rate of 15 °C/min and maintained for five different carbonization times (30, 60, 90, 120, and 150 min). After pyrolysis, the biochar samples were cooled to room temperature and weighed to determine their yield. The biochar yield was calculated using Equation (1):

$$\text{Yield (\%)} = \frac{\text{Weight of biochar}}{\text{Weight of biomass}} \times 100 \quad (1)$$

2.3 Biochar characterization

The produced biochar was characterized in terms of its proximate composition and pore structure. The surface morphology was examined using a Hitachi Tabletop Microscope TM3030Plus (Hitachi High Technologies, USA). The surface area and pore characteristics were determined using a 3Flex Surface Characterization Analyzer (Micromeritics, USA) based on nitrogen adsorption at 77 K under atmospheric pressure. The Brunauer–Emmett–Teller method was used to determine the specific surface area of the biochar.

2.4 Methylene blue adsorption capacity

For the adsorption experiment, 0.05 g of biochar was added to 100 mL of 40 mg/L methylene blue solution. The mixture was stirred for 3 h to reach equilibrium. After adsorption, the solution was analyzed using a DR 2800 UV–Vis spectrophotometer (Hach, USA) at a wavelength of 665 nm. The adsorption capacity at equilibrium, q_e , was calculated using Equation (2):

$$q_e = \frac{(C_0 - C_e)V}{w} \quad (2)$$

where q_e is the adsorption capacity (mg/g), C_0 is the initial methylene blue concentration (mg/L), C_e is the equilibrium concentration (mg/L), V is the solution volume (L), and w is the mass of biochar (g).

3. RESULTS AND DISCUSSION

3.1 Biomass Characterization

The physicochemical characteristics of gaharu waste, as presented in Table 1, indicate its strong potential as a biochar precursor. Proximate analysis showed a high volatile matter content (69.68%) and a moderate fixed carbon fraction (21.84%), suggesting that the biomass is highly reactive during thermal treatment. The elevated volatile matter promotes extensive devolatilization during pyrolysis, which facilitates the development of the pore structure [9]. The relatively high fixed carbon content provides a suitable basis for the formation of stable carbon frameworks in the resulting biochar. The ash content (8.48%) lies within a moderate range, indicating the presence of inorganic constituents that can influence the thermal decomposition process. These values are consistent with those reported in the literature, supporting the reliability of the results.

Table 1. Physicochemical characteristics of gaharu waste and other biomass

		This study	Other studies	References
Proximate Analysis (%)	Volatile Matter	69.68	60.51-74.28	[11–14]
	Fixed Carbon ^a	21.84	6.49-24.80	[11–14]
	Ash	8.48	1.14-33.00	[11–14]
Ultimate Analysis (%)	C	41.34	27.61-47.77	[11–14]
	H	6.30	3.39-7.50	[11–14]
	O ^a	48.70	26.92-46.0	[11–14]
	N	1.06	0.35-4.64	[11–14]
	S	–	1.30	[12]
	H/C	1.83	1.58	[14]
	O/C	0.88	0.65	[14]
High Heating Value (MJ/kg)		18.178	13.84-19.35	[12–14]

^aby differences

Ultimate analysis revealed that the biomass was primarily composed of oxygen (48.70%) and carbon (41.34%), with smaller contributions from hydrogen (6.30%) and nitrogen (1.06%). These results demonstrate that the high carbon content of gaharu waste make it a promising candidate for use as a raw material for biochar preparation as an adsorbent. This is further supported by the O/C ratio (0.88) and H/C ratio (1.83), which indicate a low degree of aromaticity and a highly functionalized structure. For the efficient use of biomass in a thermal process, a higher H/C ratio is more desirable than the O/C ratio [10]. The properties of gaharu waste in this study are consistent with those reported for other biomass materials and meet the characteristics of good-quality biomass. In addition, the relatively high heating value (18.178 MJ/kg), which is comparable to values in the literature (Table 1), further confirms its suitability for thermochemical conversion processes.

3.2 Characteristics of biochar at different carbonization times

The results showed that the biochar yield decreased progressively with increasing carbonization time (Figure 1). At 30 min, the yield was the highest at approximately 42 wt%, but it declined to approximately 18 wt% at 150 min. This trend suggests that prolonged carbonization leads to greater thermal degradation of biomass, resulting in the release of volatile compounds and a reduction in the solid yield [15]. In contrast, a shorter holding time of 30 min allows less interaction among biomass particles, leading to heat transfer limitations and resulting in a higher yield [10].

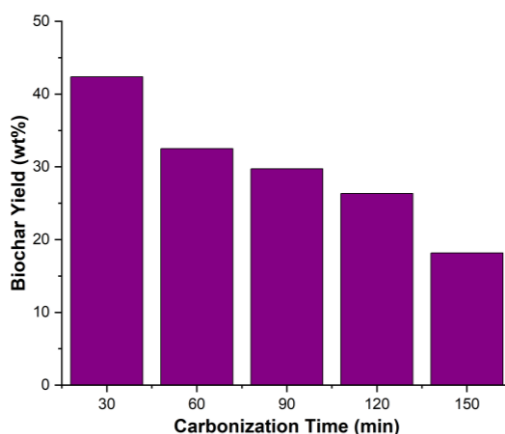


Figure 1. Yield of biochar.

The proximate analysis of biochar samples shows clear compositional changes with increasing carbonization time (Table 2). The fixed carbon content increased gradually from 31.36% at 30 min to 45.03% at 150 min, indicating progressive carbon enrichment and structural stabilization of the biochar. As the holding time increases, the pyrolysis process becomes more thorough, enhancing the carbonization level of the biomass and thereby elevating the fixed carbon content [16]. In contrast, volatile matter decreased consistently from 17.41% to 6.61%, reflecting the continuous release of volatile compounds during pyrolysis. This observation is consistent with the yield of biochar results discussed earlier. These findings indicate that prolonged carbonization enhances the carbon content and reduces the volatile constituents, contributing to the formation of a more stable and carbon-rich biochar structure.

Table 2. Proximate analysis for biochar samples

Samples	Fixed Carbon (wt.%)	Volatile Matter (wt.%)	Ash (wt.%)
30 min	31.36	17.41	51.23
60 min	36.27	13.98	49.75
90 min	36.98	13.30	49.72
120 min	38.84	11.59	49.57
150 min	45.03	6.61	48.36

Table 2. presents the BET surface area analysis, which indicates a gradual development of the pore structure as the carbonization time increases. The surface area increased from 121.34 m²/g at 30 min to 153.11 m²/g at 150 min, indicating enhanced porosity formation during the thermal treatment. A similar trend was observed for the pore volume, which increased substantially from 0.13 to 0.41 cm³/g, suggesting the continuous generation and expansion of pore networks. The pore diameter also increased from 2.56 to 7.22 nm, indicating a transition towards larger mesoporous structures. This observation is in agreement with the study by Tianhao et al. [17], which indicates that prolonged carbonization time contributes to an increase in both the pore volume and diameter of biochar.

The most notable increase in pore development occurred between 30 and 60 min, implying rapid devolatilization and the formation of initial pore channels during the early stage of carbonization. Beyond 90 min, the increase in surface area becomes more gradual, whereas the pore volume continues to expand, suggesting that pore widening and structural rearrangement dominate at later stages [18]. This behaviour is consistent with the removal of volatile components, which creates voids and enhances internal surface accessibility. The results indicate that an extended carbonization time promotes the evolution of a more developed and accessible pore structure, which is favourable for applications requiring a high adsorption capacity.

Table 3. Porous structure properties of biochars obtained at different carbonization times

Samples	BET Surface Area (m ² /g)	Pore Volume (cm ³ /g)	Pore Diameter (nm)
30 min	121.34	0.13	2.56
60 min	145.56	0.21	4.78
90 min	146.73	0.22	5.18
120 min	152.75	0.35	6.56
150 min	153.11	0.41	7.22

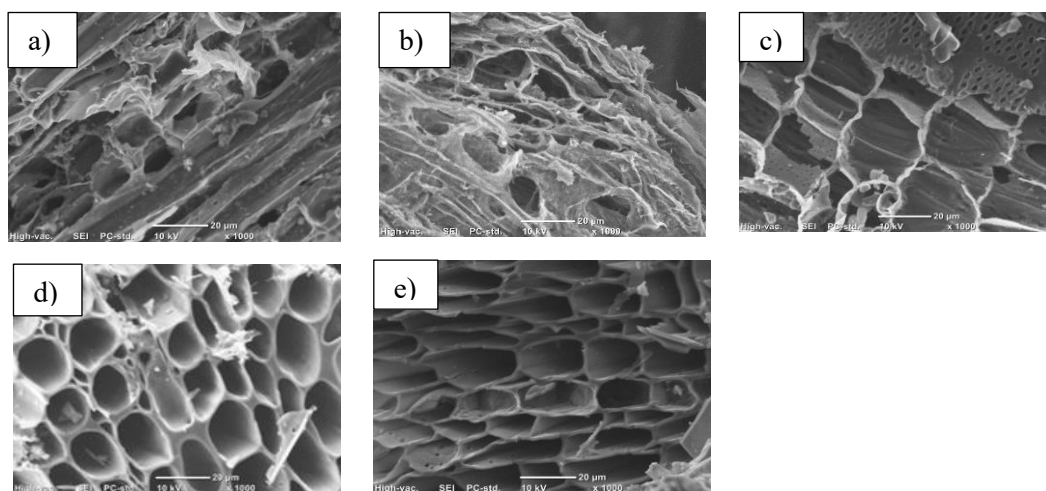


Figure 2. SEM images of biochar at different carbonization times a) 30 min b) 60 min c) 90 min d) 120 min e) 150 min.

The scanning electron microscopy (SEM) images revealed significant morphological changes in the biochar structure with increasing carbonization time (Figure 2). At 30 min, the biochar surface appeared relatively compact and irregular, with limited visible porosity, indicating incomplete decomposition of the biomass structure. At 60 min, the formation of cracks and small pores became evident, suggesting the onset of thermal degradation and volatile release. As the carbonization time increased to 90 min, a more defined pore structure was observed, with the development of interconnected channels. At 120 min, the biochar exhibited a well-developed porous morphology with a honeycomb-like structure, indicating extensive devolatilization and structural rearrangement. By 150 min, the pores became larger and more open, reflecting further pore widening and the removal of residual volatile components. These morphological changes are consistent with the BET surface area results, which show an increase in the surface area, pore volume, and pore diameter with longer carbonization times.

3.3 Methylene Blue Adsorption Capacity Analysis

The adsorption capacity for methylene blue increased notably with prolonged carbonization time, advancing from approximately 54 mg/g at 30 min to approximately 73 mg/g at 150 min (Figure 3). This significant improvement in adsorption efficiency was largely due to the gradual development of the biochar's pore structure throughout the carbonization process. For the effective adsorption of methylene blue, the pore widths of biochar must surpass its molecular longitudinal dimension (1.43 nm) [19], emphasizing the critical role of pore accessibility. As evidenced by the BET analysis, both the surface area and pore volume expand with increased carbonization time, thereby offering a more extensive surface for methylene blue adsorption. In addition, the SEM images revealed the formation of more developed and interconnected pore networks at longer carbonization times, which facilitated the diffusion and retention of methylene blue molecules.

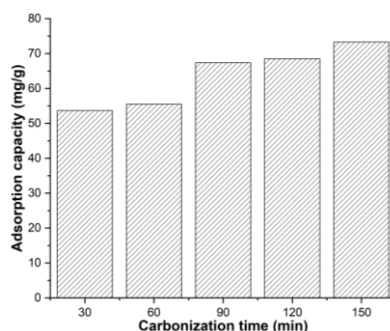


Figure 3. Effect of carbonization time on methylene blue adsorption capacity.

3.4 Comparison with other adsorbents

The findings of the current study were compared with previously reported biochar-based adsorbents for methylene blue removal, as shown in Table 4. Gaharu waste biochar exhibits a competitive adsorption capacity (73 mg/g), higher than sewage sludge (22.37 mg/g) and wheat straw (10.75 mg/g), and close to fallen leaves (78.60 mg/g). Although banana biochar shows the highest capacity (430 mg/g), it is associated with a very high surface area (938.63 m²/g), which may involve higher preparation cost. In contrast, gaharu biochar achieves balanced performance with moderate surface area (153.11 m²/g) and acceptable yield (18.00 wt%). These results suggest that gaharu waste biochar is an effective and cost-efficient adsorbent, with performance influenced not only by surface area but also by surface properties.

Table 4. Adsorption capacity of methylene blue using biochar-based adsorbents derived from various biomass source.

Adsorbents	Temperature (°C)	Time (min)	Yield (wt%)	BET surface area (m ² /g)	Adsorption capacity (mg/g)	References
Gaharu waste	800	150	18.00	153.11	73.00	This study
Sewage sludge	550	120	43.61	25.00	22.37	[20]
Fallen leaves	500	120	–	27.30	78.60	[21]
Wheat straw	500	180	–	12.49	10.75	[22]
Date Seed	550	180	20.94	104.20	42.60	[11]
Banana	800	180	–	938.63	430.00	[23]

4. CONCLUSIONS

In this study, the effect of carbonization time on the properties and adsorption performance of biochar was successfully investigated. The results showed that increasing the carbonization time significantly influenced the physicochemical characteristics of the biochar. The biochar yield decreased with longer carbonization times, whereas the fixed carbon content increased and the volatile matter decreased, indicating enhanced carbonization. The development of the pore structure was confirmed by BET and SEM analyses, which showed increases in the surface area (153.11 m²/g), pore volume (0.41 cm³/g), and pore diameter (7.22 nm), along with the formation of more defined and interconnected pores. These structural improvements contributed to the enhanced adsorption performance of biochar. The adsorption capacity of methylene blue increased from lower values (54 mg/g) at shorter carbonization times (30 min) to the highest value (73 mg/g) at 150 min, demonstrating that extended carbonization improves adsorption efficiency. These findings suggest that carbonization time plays a critical role in optimizing biochar properties, with longer carbonization times producing a more stable, porous, and effective adsorbent material.

5. ACKNOWLEDGEMENT

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Professor Ir. Dr. Juferi Idris currently serves as the Deputy Rector of Research and Industrial Linkages at Universiti Teknologi MARA (UiTM) Sarawak Branch, Campus Samarahan, Malaysia. He is a professor in the field of bioprocess and has been actively involved in advancing research, innovation, and academic collaboration in these areas. Throughout his career, he has contributed to postgraduate supervision, research development, and international scientific engagement, as well as fostering partnerships with regional and global institutions. His work focuses on translating research into impactful and sustainable applications in line with current global needs. In recognition of his academic standing and contributions to the field, he was invited to serve as a Distinguished Speaker at the 10th Biotechnology International Congress (BIC 2026) in Bangkok, Thailand, an international platform that highlights leading experts driving next-generation biotechnology towards sustainable global impact.

Expanded Ribosomal Synthesis of Non-Standard Cyclic Backbones *In Vitro***Joongoo Lee^{1*}**¹*Department of Chemical Engineering, POSTECH, Pohang, 37673, Republic of Korea****Correspondence to:** E-mail: jgoolee@postech.ac.kr

ABSTRACT: The ribosome polymerizes L- α -amino acids into polypeptides, catalyzing peptide bond formation through aminolysis. This process is facilitated by entropy trapping within its peptidyl transferase center (PTC). In this research, we harness this capability to synthesize polymers containing cyclic motifs in the backbone. We design 26 non-canonical monomers (ncMs) with two distinct substrates: dicarboxylic esters and hydrazinoesters, each containing bifunctional moieties that undergo ring-closing reactions through multiple aminolysis reactions. Using a cell-free system that enables the consecutive incorporation of these ncMs into a growing peptide, we discover that the ribosome can produce 5- and 6-membered cyclic backbones, which have never been reported. We also demonstrate that the formation of such cyclic backbones within the ribosome is tunable by altering the substituents of dicarboxylic esters. This discovery expands the range of non-standard backbones that can be synthesized by the ribosome and motivates future research towards expanding ribosome-mediated chemistries for biopolymer synthesis.

Keyword: Ribosome, Cell-free systems, Cyclic backbones, Non-canonical amino acids.

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Selected journal publications

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Genome-Based Analysis and Anti-Oomycete Activity of *Priestia aryabhatai* PTKU-123 Against the Durian Pathogen *Phytophthora palmivora*

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ABSTRACT: *Phytophthora palmivora* is a major oomycete pathogen causing destructive root and stem rot in durian, highlighting the need for sustainable disease management. This study investigated the genomic characteristics and anti-oomycete potential of *Priestia aryabhatai* PTKU-123, isolated from durian orchard soil in Thailand. Among 159 bacterial isolates, PTKU-123 showed the strongest inhibition of *Ph. palmivora*, achieving 80.00% inhibition in dual culture and 78.22% using cell-free supernatant. Whole-genome sequencing revealed a 5.15-Mb circular chromosome with 5,086 coding sequences and 237 carbohydrate-active enzymes. Genome mining identified biosynthetic gene clusters associated with paeniodin, surfactin, schizokinen, carotenoids, and type III polyketide synthase, suggesting diverse bioactive potential. Crude extracts strongly suppressed *Ph. palmivora*, with 2 mg/mL achieving 82.18% inhibition, and retained activity during storage at 4 and 28 °C for 14 days. These findings demonstrate the anti-oomycete activity and genomic potential of PTKU-123, supporting its further development as a microbial resource for sustainable management of *Ph. palmivora* in durian production.

Keyword: Biological control, Durian, Genome mining, *Phytophthora palmivora*, *Priestia aryabhatai*



Assoc. Prof. Paiboon Tunsagool, Ph.D. is an associate professor at the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand. He received his Ph.D. in Biochemistry from Prince of Songkla University. His expertise includes metabolomics, proteomics, metagenomics, microbial biotechnology, and multi-omics, with applications in agriculture, food, and biotechnology.

Liquid Fermentation Tech for Sustainable Health and Industry Advancement

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ABSTRACT: As climate pressure reshapes global food and health industries, next-generation biotechnology must convert environmental constraints into platforms for both human well-being and economic resilience. AI-assisted liquid fermentation has emerged as a cornerstone of this transition, enabling precise, low-footprint bioproduction at industrial scale. Grape King Bio Ltd. presents an integrated model in which this technology simultaneously advances functional health products, reduces resource intensity, and aligns operations with TCFD and SBTi climate frameworks. The strategy is built on a closed-loop system that pairs AI-driven precision fermentation with waste valorization and digital process management. Submerged fungal and probiotic cultivation supports functional supplements and meat alternatives that lower greenhouse gas emissions and land use compared with livestock-based production. Residues from high-value supplement fermentation are repurposed into nutrient-dense animal feed for local farmers, while real-time digital monitoring reduces batch deviation and energy consumption, contributing to a total reduction of 227,371 tonnes CO_{2e} in 2024. To further compress the environmental footprint, academic partnerships have introduced seawater into selected fermentation processes and coupled wastewater treatment with microbial fuel cells, removing organic pollutants while recovering electricity. By linking microbial science, digital engineering, and circular resource design, liquid fermentation offers a replicable bridge from laboratory research to sustainable global impact, delivering health-promoting products, decarbonized industrial growth, and a practical blueprint for next-generation biotechnology coupled with AI.

Keyword: AI-assisted fermentation, Liquid fermentation, Functional foods, Circular bioeconomy, Net-zero biomanufacturing



Dr. Tsung-Ju Li is a scientist working across microbe-derived nutraceuticals, AI-driven R&D, and FDA regulatory strategy, with a track record of 20+ SCIE papers, 8 patents, and 2 FDA clearances which including a New Dietary Ingredient notification and a Phase II trial approval for a MASH botanical drug. He recently developed Agentome5, an agentic pipeline that traces a strain's fermentation metabolites from secretome through to predicted clinical outcomes. Recent recognition includes the 2024 BEST Industrial Elite Award and the 2024 Nutra-Ingredients Asia Ingredient of the Year for *Hericium erinaceus*. He is Assistant Department Manager at Grape King Bio Ltd.

Evolution of Nanoparticle Assembly: Bridging Proven Encapsulation with Reconfigurable Microfluidic Platforms

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ABSTRACT: The utility of therapeutic agents—ranging from small-molecule antibiotics to monoclonal antibodies—is frequently compromised by rapid degradation and restricted intracellular access. This talk highlights the progression of our research in poly (ethylene glycol)-*block*-poly(lactide-co-glycolide) (PEG-PLGA) nanocarriers designed to overcome these barriers. Initially, we established a modified ion-pairing method to encapsulate the antibiotic ciprofloxacin, achieving sustained bactericidal activity against *Enterococcus faecalis* while maintaining high cellular viability. This platform was subsequently adapted for the intracellular delivery of anti-dengue monoclonal antibodies, where the resulting nanoparticles facilitated efficient uptake in hepatocytes and significantly suppressed viral replication by inhibiting apoptotic pathways. To transition these laboratory-scale successes toward broader translation, we conclude by introducing a reconfigurable polydimethylsiloxane (PDMS) microfluidic system. By utilizing 3D-printed modular components as a framework, this platform allows for the rapid assembly of diverse channel geometries to optimize nanoparticle formation. This modular approach provides a reproducible and scalable solution that enhances nanoparticle monodispersity and ensures controllable particle size, offering a high-fidelity, customizable pathway for the next generation of topical and intracellular drug delivery systems.

Keyword: PEG-PLGA, Nanoparticles, Microfluidics, Encapsulation, 3D-Printing, PDMS.



Nisa Patikarnmonthon, Ph.D., is a Faculty Member at the Department of Biotechnology, Faculty of Science, Mahidol University, Thailand. Her research lies at the intersection of molecular nanotechnology and biomaterial engineering. Dr. Patikarnmonthon's laboratory has established validated strategies for the nanoencapsulation of biotherapeutics, ranging from small-molecule antibiotics to monoclonal antibodies. Beyond drug delivery, her work extends to the development of advanced scaffolds for 3D cell culture and the engineering of alginate beads as microcarriers. Her current innovation involves integrating these biomaterial strategies with microfluidic systems to enhance precision, scalability, and therapeutic efficacy in nanomedicine.

Designing Impactful Nanomedicine: Bridging Scientific Discovery and Global Translation

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ABSTRACT: Biopolymer-based nanomaterials are emerging as transformative platforms to address critical challenges in the stability, delivery, and efficacy of bioactive compounds. Building upon our work on functionalized hyaluronic acid (f-HA) systems, this presentation highlights the rational design of novel biopolymers and their combinatorial nano-assemblies as enabling technologies for impactful nanomedicine. Through molecular engineering and modular assembly strategies, these biopolymers can be tailored into versatile architectures—including nanogels, and hybrid supramolecular systems—capable of encapsulating diverse bioactives such as growth factors, cannabinoids, curcumin, and plant-derived antioxidants. These nano-assemblies demonstrate high encapsulation efficiency (>80%), enhanced molecular stability, and improved biological performance through controlled release, targeted delivery, and increased cellular uptake. Importantly, this work advances design principles that bridge scientific discovery with global translation, emphasizing biocompatibility, scalability, manufacturability, and regulatory readiness. Case studies of combinatorial systems illustrate synergistic functionalities, including enhanced tissue penetration, prolonged activity, and responsive behavior under physiological conditions. Collectively, these innovations represent a paradigm shift from passive delivery systems to active, intelligent nanomaterials, accelerating next-generation nanomedicine toward global healthcare impact.

Keyword: Biopolymers; Nanomedicine; Nano-assemblies; Drug delivery; Translational biotechnology



Jittima Amie Luckanagul is an academic researcher and innovation leader in biomaterials and nanomedicine. Her research focuses on the design of advanced biopolymer-based nanomaterials for drug delivery, gene delivery, and biomedical applications. She has extensive experience in translating research into real-world impact, including founding a biotech startup and leading strategic initiatives to advance university research toward global competitiveness. Her work integrates scientific excellence with innovation ecosystem development, aiming to bridge deep-tech research with industrial and clinical applications.

CRISPR Detection for Major Diseases in Aquaculture

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ABSTRACT: CRISPR-Cas systems have revolutionized the biology by providing a highly programmable and precise platform for finding and manipulating nucleic acids. Since 2017, this technology has been increasingly adapted for nucleic acid detection, resulting in diagnostic tools that are rapid, accurate, and uniquely suited for onsite applications in resource-limited settings. This capability is crucial for the aquaculture industry, where early and proactive detection remains the most robust strategy for preventing economic losses due to infectious diseases. This presentation details the development of CRISPR-based diagnostic assays designed for the detection of critical pathogens in economic shrimp and fish, including *Enterocytozoon hepatopenaei* (EHP), infectious hypodermal and hematopoietic necrosis virus (IHHNV), yellow head virus (YHV) genotype 1, and tilapia lake virus (TiLV), among others. Furthermore, we explore innovative strategies for enhancing these platforms, including the integration of DNAzymes for colorimetric visualization, the use of photocontrolled protocols for reaction regulation, and the use of smartphone-based readouts. By combining precision with portability, CRISPR tools offer a sustainable solution for disease management in global aquaculture.

Keyword: CRISPR-Cas, Molecular Detection, Aquaculture, Diseases



Thawatchai Chaijarasphong, Ph.D., is an Associate Professor in the Department of Biotechnology at Faculty of Science, Mahidol University. He holds a B.Sc. from Stanford University and a Ph.D. in Chemistry (Chemical Biology) from University of California at Berkeley. Currently, he leads the CRISPR Technology Research Group at the Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp). His research specializes in developing CRISPR-based tools to detect and combat major aquaculture pathogens, including YHV genotype 1, EHP, and TiLV, among others. His work on CRISPR applications in aquaculture appears in journals such as *Aquaculture* and the *Journal of Fish Diseases*.

Shaping the Future of Responsible Biotechnology in Thailand through Biosafety Education and Training

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ABSTRACT: Biosafety and biosecurity have become critical foundations for ensuring that rapidly advancing biotechnology is developed and applied responsibly. Modern recent biotechnology raises concerns related to laboratory risk management, environmental protection, and the potential misuse of biological materials associated with dual use research. International biosafety governance is guided by global frameworks developed by organizations such as World Health Organization. This presentation highlights the role of biosafety education and training in supporting responsible biotechnology development in Thailand. As biotechnology activities expand across universities, research institutes, and industry, developing a workforce with strong awareness of biosafety principles, biosecurity considerations, and responsible research practices becomes increasingly important. Particular attention will be given to biosafety challenges associated with emerging technologies such as engineered microorganisms, synthetic biology, and advanced genetic modification, as well as the integration of biosafety and biosecurity principles into biotechnology curricula and professional training aligned with international standards, national regulations, and institutional policies. Examples of biosafety capacity building initiatives from Faculty of Science at Mahidol University will be presented, including hybrid and onsite training workshops covering laboratory practices in Biosafety Level 1 and 2 laboratories, together with specialized programs for Biosafety Level 3 containment facilities. These initiatives have strengthened biosafety awareness, improved laboratory safety practices, and enabled trained participants to establish or enhance biosafety management systems within their institutions. Collectively, these efforts demonstrate how strategic biosafety education and capacity building can support responsible research practices, strengthen national biosafety infrastructure, and help shape a sustainable and globally competitive biotechnology ecosystem in Thailand.

Keyword: Biosafety and Biosecurity, Responsible Biotechnology, Biotechnology Education and Training, Laboratory Biosafety, Capacity Building



Dr. Adisak Romsang is an assistant professor in Department of Biotechnology, Faculty of Science, Mahidol University, Thailand. He is actively involved in biosafety leadership and education as Chairman of MUSC-IBC. He has served as a key trainer in numerous biosafety workshops, supporting biosafety capacity building across Thailand. His research focuses on microbial virulence and antimicrobial resistance in pathogenic bacteria, integrating biosafety principles with responsible research. He has been recognized with the Mahidol University Outstanding Teacher Award (2024).

Research and Innovation for Sustainable Waste Management in the Bio-based Economy

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ABSTRACT: Sustainable waste management is a critical challenge in advancing circular and bio-based economies, particularly in regions with abundant agricultural, food, and industrial organic residues. This work presents integrated research and innovation strategies that transform organic waste streams into renewable energy, value-added biochemicals, and bio-based resources through advanced anaerobic microbial technologies. Our research combines biocatalysis, enzyme engineering, metabolic engineering, and synthetic microbiology to develop efficient and scalable systems for waste valorization. Key efforts include microbial strain discovery, enzyme optimization, and the design of synthetic and mixed microbial consortia for enhanced organic waste degradation. These platforms enable the production of biohydrogen, methane, volatile fatty acids, and medium-chain fatty acids, as well as the bioconversion of methane and carbon dioxide into chemical precursors. A notable achievement is the isolation of *Enterococcus faecalis* VT-H1 from palm oil mill effluent, which exhibits exceptional hydrogen-producing capacity and supports the development of immobilized-cell systems successfully implemented from laboratory to pilot-scale food waste treatment. To support circular bioeconomy applications, digestates are further utilized as biofertilizers and plant biostimulants. One flagship innovation is the SUZDEE (Sustainable Zero Waste Digestant for Well-Being) system, a community-scale platform that converts food waste into bioenergy and agricultural inputs, with demonstrated applications in households, schools, and municipalities across Thailand. Industrial translation is further achieved through collaborations in wastewater treatment and resource recovery, alongside commercialization via GreenGen Biotechnology. Overall, this work highlights how interdisciplinary integration of microbiology, bioprocess engineering, and circular bioeconomy concepts can deliver scalable, low-carbon solutions for sustainable waste management and real-world impact.

Keyword: Sustainable waste management, Bio-based economy, Circular bioeconomy, Waste valorization, Bioprocess innovation



Assoc. Prof. Dr. Thanyaporn Wongnate is a faculty member at the School of Biomolecular Science and Engineering, Vidyasirimedhi Institute of Science and Technology (VISTEC), Thailand. She obtained her Ph.D. in Biochemistry from Mahidol University, Thailand, and pursued postdoctoral research at the University of Michigan, Ann Arbor, USA. Her research lies at the interface of biocatalysis, mechanistic enzymology, microbial biotechnology, and circular bioeconomy. She develops microbial and enzymatic platforms for the sustainable conversion of organic waste and industrial by-products into renewable energy, biofuels, and value-added biochemicals. Her work spans enzyme engineering, anaerobic fermentation, synthetic and mixed microbial systems, and translational waste valorization technologies. She has led several interdisciplinary projects in biohydrogen production, biogas systems, green biotransformation, and sustainable waste management, with strong emphasis on real-world implementation and industrial relevance. She is the recipient of notable honors including the Taguchi Award for Outstanding Researcher (2025) and the L'Oréal-UNESCO For Women in Science Award (2019).

Synthetic Glycobiology Decoding Context-Driven O-Glycan Interactions on Mucins

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ABSTRACT: Mucins are large O-glycoproteins that form the protective mucus barrier at all wet body surfaces, where their dense and patterned O-glycans mediate critical interactions with the microbiota and the immune system. Yet how the presentation of O-glycans on mucins – their structures, clustering, and protein backbone context – encodes biological specificity remains poorly understood. Here, we present a synthetic glycobiology framework that addresses this challenge by combining precision genetic glycoengineering of mammalian cells with rationally designed glycoprotein reporters called Glycocarrier to systematically dissect context-driven interactions on mucins. Using Glycocarrier platform in glycoengineered HEK293 and CHO cells, we produce libraries of mucin-like O-glycodomain reporters bearing defined, near-homogeneous O-glycan structures for interrogation of glycan-binding interactions. Applying these reagents, we discovered that a small microbial mucin-binding module, designated X409, employs a novel recognition mechanism based on clustered saccharide patches (CSPs) – composite epitopes formed by rows of inner core monosaccharides across adjacent O-glycans. X-ray crystallography and NMR analyses revealed that X409 engages the GalNAc and galactose residues of neighboring core 1 O-glycans simultaneously, and this multivalent binding mode is unique to the clustered O-glycan context found on mucins but rarely on other glycoproteins. Importantly, this binding mode is inherently resistant to trimming of outer glycan residues by microbial glycosidases, providing a potential mechanism for sustained mucin recognition in the glycan-scavenging environment of the gut. We further show that this CSP-recognition principle extends to host immune receptors and bacterial toxins, some of which exploit repeated O-glycan cluster motifs for selective ligand engagement. Together, these findings establish that mucin O-glycans encode biological specificity through higher-order contextual arrangements, and the synthetic glycobiology toolkits provide new avenues for decoding glycan functions and engineering glycan-based biotechnologies.

Keywords: Synthetic glycobiology, Mucin O-glycosylation, Cell-based glycan array, Glycoengineering, Glycocarriers



Thapakorn Jaroentomeechai currently leads the SynGlyco Lab at the Department of Biotechnology, Faculty of Science, Mahidol University, Thailand. His research integrates synthetic biology with glycobiology to develop programmable platforms for glycoprotein engineering, focusing on cell-free protein synthesis, mammalian cell glycoengineering, and mucin O-glycobiology. He was an EMBO Postdoctoral Fellow at the Copenhagen Center for Glycomics with Prof. Henrik Clausen at the Copenhagen Center of Glycomics, and previously trained with Prof. Matthew DeLisa and Prof. Michael Jewett, where he co-pioneered cell-free glycoprotein synthesis (CFGpS) platform. He also has industry experience at Biogen in the Antibody Discovery group and has authored over 25 peer-reviewed publications.

Comparative Pretreatment of Coffee Cherry Pulp for Polyhydroxyalkanoate Production by Thermotolerant *Inquilinus* sp. CF22

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ABSTRACT: Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers recognized as sustainable alternatives to petroleum-based plastics within the framework of a circular bioeconomy. This study investigated the valorization of coffee cherry pulp (CCP), a major agro-industrial residue generated during wet coffee processing, as a low-cost carbon source for PHA production by the thermotolerant bacterium *Inquilinus* sp. CF22. To enhance sugar recovery from lignocellulosic biomass, two alkaline pretreatment methods were comparatively evaluated: 5% KOH at 90 °C for 1 h in a water bath and 4% NaOH at 121 °C for 25 min under autoclave conditions. Pretreatment with 4% NaOH resulted in a higher total sugar concentration and was therefore selected for subsequent experiments. Enzymatic hydrolysis of the pretreated CCP generated a sugar-rich hydrolysate containing 68.00 g/L total sugars, predominantly glucose (59.69 g/L), along with smaller amounts of arabinose, xylose, and sucrose. Fermentation conditions were optimized using a one-factor-at-a-time (OFAT) approach by evaluating the effects of hydrolysate concentration, nitrogen source, nitrogen concentration, initial pH, and shaking speed on cell growth and PHA accumulation. The optimal conditions consisted of 10 g/L CCP hydrolysate (containing 9.74 g/L total reducing sugars) supplemented with 0.5 g/L (NH₄)₂HPO₄ at an initial pH of 7.2, a cultivation temperature of 45 °C, and a shaking speed of 200 rpm. Under these conditions, the maximum dry cell weight and PHA concentration reached 1.64 ± 0.07 g/L and 0.93 ± 0.02 g/L, respectively, corresponding to a PHA content of 56.7 ± 1.20 wt%. These findings highlight the potential of CCP as a renewable substrate for sustainable PHA production and support its application in agro-industrial waste valorization and bioplastic production within a circular bioeconomy strategy.

Keywords: Biorefinery, Coffee cherry pulp, Optimization, Polyhydroxyalkanoate, Thermotolerant bacterium

Sustainable Pretreatment of Durian Rind for Cellulose Nanofiber Extraction as a Bio-Based Reinforcement Material

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ABSTRACT: Conventional physicochemical pretreatment processes of biomass rely on high temperatures (exceeding 150 °C), which lead to high operational costs and energy demand. In this study, sustainable pretreatment strategies comparing accessible, low-energy hydrothermal autoclaving (121 °C, 0.1 MPa, 30 min) with energy-intensive steam explosion (190 °C, 1.3 MPa, 15 min) were investigated to evaluate their effects on cellulose extraction from durian rind. The pretreatments were followed by bleaching using 5 wt% peracetic acid (PAA), autoclave-assisted 5 wt% oxalic acid hydrolysis, and mechanical hand homogenization (MHH) to isolate cellulose nanofibers (CNFs). Following pretreatment and PAA bleaching, cellulose content increased to 64.88% for autoclave-derived fibers and 78.14% for steam-exploded fibers, compared with 37.58% of raw, untreated durian rind. The fibers showed high thermal stability with T_{max} of 355 °C and 360 °C, respectively, while FTIR confirmed successful removal of non-cellulosic components. Oxalic acid hydrolysis followed by MHH yielded CNFs with high crystallinity indices of 70.58% and 71.44% for autoclave-derived and steam-exploded CNFs, respectively. Morphological analysis via transmission electron microscopy (TEM) showed average fiber diameters of 36.07 nm for autoclave-derived CNFs and 37.46 nm for steam-exploded CNFs. Furthermore, the suspensions exhibited strong colloidal stability, with zeta potential values of −38.70 mV for autoclave-derived and −36.10 mV for steam-exploded CNFs. Remarkably, hydrothermal autoclaving yielded cellulose fibers and CNFs with properties comparable to those produced via steam explosion, establishing a low-energy, cost-effective strategy aligned with circular economy principles for valorizing biomass waste into high-value functional materials for bio-based reinforced composites.

Keywords: Bio-based material, Cellulose nanofibers, Durian rind, Green defibrillation, Sustainable pretreatment

Plant-Microbe Interactions for Microplastic Removal Using Mangrove-Associated Herbaceous Plants in a Hydroponic System

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ABSTRACT: Microplastic (MP) pollution in mangrove ecosystems has become a growing environmental concern, yet effective and sustainable removal strategies remain limited. This study investigated the potential of mangrove-associated herbaceous plants and rhizobacteria to remove polypropylene (PP) MPs. Five herbaceous plant species, namely *Sesuvium portulacastrum*, *Wedelia biflora*, *Acanthus ebracteatus*, *Acrostichum aureum* L., and *Acanthus ilicifolius* L., collected from a mangrove forest in Samut Sakhon Province, Thailand, were screened for their capacity to adsorb MPs by their roots. *A. aureum*, *A. ilicifolius*, and *W. biflora*, which showed the highest efficiency in MPs removal, were selected for investigation of PP-MPs removal in a controlled laboratory experiment using a hydroponic system for 7 days at an initial concentration of 100 mg/L of PP. Although no significant difference was observed in PP-MPs removal between *A. ilicifolius* and *A. aureum*, *A. ilicifolius* showed a higher proportion of strongly adhered particles, whereas *W. biflora* showed significantly lower removal capacity. Three rhizobacterial strains were isolated from the mangrove soil, *Pseudomonas reactans*, *Pseudomonas gessardii*, and *Shewanella putrefaciens* were able to grow on PP-supplemented minimal medium. All strains could produce indole-3-acetic acid and exopolysaccharides. Based on these findings, *P. gessardii* was selected for inoculation with *A. ilicifolius* under PP-MP exposure in a hydroponic system to assess effects on root adsorption and plant growth. The results showed that inoculating *P. gessardii* with *A. ilicifolius* actively removed microplastics by 17%. Combining high MPs-removing mangrove herbaceous plants with beneficial rhizobacteria could provide a promising nature-based approach for remediating microplastic contamination in mangrove coastal environments.

Keywords: Mangrove ecosystem, Microplastics, Phytoremediation, Plant-microbe interactions, Rhizobacteria

1. INTRODUCTION

Plastic production has surged globally, leading to the widespread environmental accumulation of microplastics (MPs) [1]. Coastal environments, particularly mangrove ecosystems, are highly susceptible to MPs accumulation because their complex root networks act as highly effective sinks for suspended debris [1]. These ecosystems provide important support to coastal communities by protecting shorelines, storing carbon, and filtering pollutants [1]. The persistence of these pollutants poses severe ecological risks, including ingestion of plastics by local wildlife and subsequent transfer through the food web, underscoring the urgent need for sustainable remediation strategies [1]. Phytoremediation, which utilizes green plants to stabilize or remove contaminants, offers a promising, nature-based approach [2]. While herbaceous plants can accumulate and stabilize pollutants in their roots, it remains unclear whether herbaceous plants that naturally grow in mangrove environments have the potential to effectively remediate microplastics [11]. However, continuous exposure to microplastics can induce physiological stress in

plants, causing physical root blockage and triggering oxidative stress, which can ultimately hinder their remediation efficiency [3]. To overcome these limitations, integrating the physical adsorption capacity of native mangrove herbaceous plants with plant growth-promoting rhizobacteria (PGPR) provides a synergistic advantage [4]. PGPR colonize the root zone and enhance the plant's tolerance to environmental stress by producing hormones such as indole-3-acetic acid (IAA), which stimulate root expansion [4]. Furthermore, specific rhizobacteria secrete exopolysaccharides (EPS) that act as biological adhesives, facilitating the robust attachment of hydrophobic microplastics to plant roots while gradually initiating microbial degradation [5]. In Thailand, polypropylene and polyethylene were identified as the dominant polymer types, contributing to this widespread pollution in coastal sediment [3][4].

To date, plant-microbe interactions for microplastic removal in mangroves remain largely uncharacterized, this study investigated five native herbaceous species from Samut Sakhon Province (*Sesuvium portulacastrum*, *Wedelia biflora*, *Acanthus ebracteatus*, *Acrostichum aureum* L., and *Acanthus ilicifolius* L.) and native rhizobacteria from the mangrove soil for the removal of polypropylene (PP) microplastics. Microplastics adsorption capacity, Plant Growth-Promoting (PGP) traits including indole-3-acetic acid and exopolysaccharides production, and *in vitro*-degradation were investigated. Utilizing these interactions might provide a scientific basis for future remediation strategies.

2. MATERIALS AND METHODS

Native herbaceous mangrove plants including *Sesuvium portulacastrum*, *Wedelia biflora*, *Acanthus ebracteatus*, *Acrostichum aureum* L., and *Acanthus ilicifolius* L. and rhizosphere sediment were collected from the Mangrove Research and Development Center 2 in Samut Sakhon Province, Thailand. In this area, areas were contaminated with various plastic debris. Standard analytical chemicals for microbiological cultivation and biochemical assays were sourced commercially from Sigma-Aldrich, USA. To ensure controlled exposure, polypropylene (PP)-MPs were prepared from commercial products utilizing a modified matrix-assisted cryogenic grinding technique [3] to achieve a standardized target size fraction of 100-300 μm .

Acclimated plant species were subjected to a 7-day hydroponic screening to assess acute stress tolerance and MP removal capacity. The root systems were immersed in aerated vessels containing a 100 mg/L suspension of the PP-MPs in a half-strength Hoagland nutrient solution. Concurrently, MPs-degrading rhizobacteria were isolated from the collected sediment using carbon-limited Minimal Salt (MS) medium supplemented with 1% (w/v) PP microplastics as the sole carbon source. Purified bacterial isolates, identified as *Pseudomonas reactans*, *Pseudomonas gessardii*, and *Shewanella putrefaciens*, were subsequently screened for plant growth-promoting traits, including indole-3-acetic acid (IAA) and exopolysaccharide (EPS) production, and subjected to a 60-day *in vitro* gravimetric assay on MS agar to evaluate their polymer degradation capabilities. Finally, *A. ilicifolius* was inoculated with *P. gessardii* under 100 mg/L PP-MPs exposure for 7 days to assess the synergistic effects on root adsorption. Data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's Honestly Significant Difference (HSD) post-hoc test ($p < 0.05$). All experiments were performed at least in triplicate.

3. RESULTS AND DISCUSSION

The results showed that *A. aureum*, *A. ilicifolius*, and *W. biflora* had the highest microplastics removal by root adsorption among the mangrove herbaceous species (Figure 1). The 7-day hydroponic screening demonstrated significant differences in root-associated microplastic adherence among the tested mangrove plant species. *A. ilicifolius* adsorbed the highest amount of MP (approximately 100 item/g), successfully removing a significant proportion of suspended particles from the water column. Furthermore,

A. ilicifolius firmly anchored the MP directly to its root surface, demonstrating a high proportion of strongly adhered particles. Strong attachment is critical for phytoremediation applications, as it could prevent the trapped particles from re-entering the dynamic aquatic environment during tidal fluctuations [6].

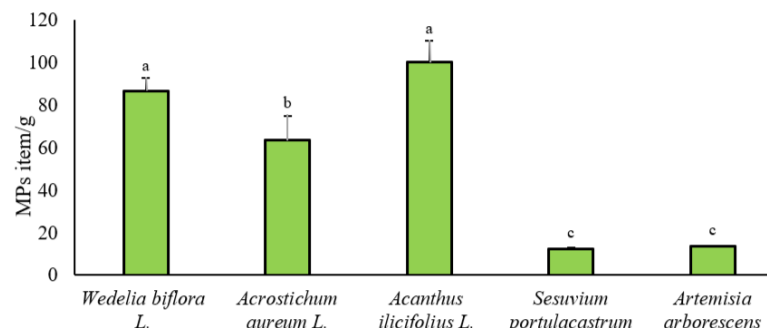


Figure 1. Microplastic removal by root adsorption of five native herbaceous mangrove plants. The removal efficiency is expressed as number of items per gram of fresh weight (items g⁻¹). “Items” is used to denote discrete particles counted microscopically. Bars with different letters indicate significant differences among samples ($p < 0.05$).

Plant Growth-Promoting (PGP) traits and 60-day PP film weight loss percentages of bacterial degradation capacity is shown in Table 1. Among the isolates, *Pseudomonas gessardii* demonstrated the most favorable profile for plant-microbe symbiosis. This strain produced high amounts of both exopolysaccharides (EPS) (636.12 mg/L) and indole-3-acetic acid (IAA) (127.95 $\mu\text{g mL}^{-1}$). EPS modifies the surface energy of hydrophobic plastics, acting as a biological adhesive that helps PP particles attach firmly to plant roots [5]. Simultaneously, IAA is an essential hormone that promotes lateral root development and enhances overall plant stress tolerance [4]. Furthermore, the *vitro* assay over 60 days, evaluated gravimetrically, revealed that *P. gessardii* initiated early-stage structural degradation of the PP polymer, resulting in a 0.19% weight loss, indicating an active metabolic response to the plastic substrate [8]. This highlights that this specific plant-microbe combination could physically stabilize microplastics and begin the biological breakdown process. Thus, to further evaluate the efficiency of the combined plant-microbe system, the mass of suspended PP microplastics remaining in the hydroponic solution was measured after 7 days (Figure 2.). It observed relatively greater reduction in suspended MPs in the treatment with bacteria (PBMPs) compared to the treatment without bacteria (PMPs). This indicates that the presence of *P. gessardii* actively facilitates the physical capture and stabilization of microplastics from the water column onto the root surface.

Table 1. Summary of Plant Growth-Promoting (PGP) traits and 60-day PP film weight loss percentages of bacterial degradation capacity.

Isolated rhizobacteria	IAA Production $\mu\text{g mL}^{-1}$	EPS Production (mg/L)	60 days film weight loss
<i>P. reactans</i>	133.60	604.00	0.00%
<i>P. gessardii</i>	127.95	636.12	0.19%
<i>S. putrefaciens</i>	11.78	501.00	0.68%

IAA: indole-3-acetic acid; EPS: exopolysaccharides

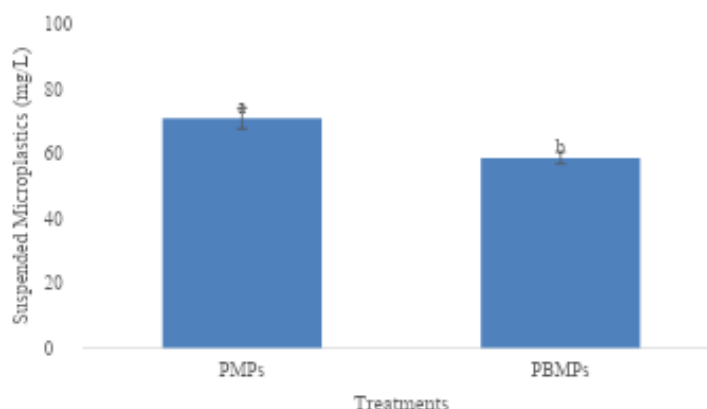


Figure 2. Mass of suspended PP microplastics remaining in the hydroponic solution after 7 days of exposure in the treatment without bacteria (PMPs) and treatment with bacteria (PBMPs).

4. CONCLUSIONS

This study confirms the potential of combining native mangrove flora with specialized rhizobacteria for microplastic phytoremediation. *Acanthus ilicifolius* effectively intercepts and physically stabilizes microplastics on its root system. Furthermore, *Pseudomonas gessardii* supports this process by producing EPS for particle attachment and IAA for plant stress tolerance, while initiating the degradation of the captured polymers. Thus, the symbiosis interaction may enhance microplastics adherence and initiate early-stage polymer degradation. This integrated, nature-based approach offers a viable, sustainable strategy to mitigate microplastic pollution in vulnerable coastal ecosystems.

5. ACKNOWLEDGEMENT

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Green Extraction and Isolation of Cellulose Nanofibrils from Kiam (*Cotylelobium melanoxyton*) Wood Waste

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ABSTRACT: *Cotylelobium melanoxyton*, commonly known as kiam wood, is a hardwood composed of major polymers, including cellulose, hemicellulose, and lignin, similar to other lignocellulosic biomass. This study investigates the extraction and characterization of cellulose and cellulose nanofibrils from *C. melanoxyton* waste, using extractive-free fibers obtained after Soxhlet extraction. Cellulose was subsequently extracted and purified through a delignification and bleaching process utilizing peracetic acid, resulting in a high alpha-cellulose yield of up to 70%. The resulting purified cellulose was then transformed into CNF via mechanical treatment using high-pressure homogenization. The morphological, structural, and chemical characteristics of the initial cellulose and the final CNF were comprehensively analyzed using FTIR, SEM, TEM, and XRD. These techniques confirmed the successful removal of lignin, significantly increasing the swelling and water-holding capacity of the fibers. Additionally, the isolated CNF exhibited a stable, well-dispersed suspension with a high zeta potential of up to -40 mV and a crystallinity of 65%. The findings demonstrate that *C. melanoxyton* is an effective, sustainable source for high-value CNF production, which can then be utilized to formulate effective edible coatings that extend the shelf life of perishable products. Furthermore, the application of these bio-based materials can decrease reliance on fossil-based synthetic polymers in the food packaging sector, aligning with current efforts to valorize lignocellulosic agricultural and forestry residues

Keywords: Biorefinery, Cellulose nanofibril, Green technology, Nanomaterials, Sustainable feedstock

Single-Site Polarity Modulation Improves the Catalytic Efficiency and Thermostability of D-allulose 3-epimerase through Flexibility–Rigidity Rebalancing

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ABSTRACT: The delicate balance between localized flexibility and global structural rigidity is often fundamental to enzymatic efficiency. Here, we demonstrate that a single point mutation (E35H) in *Arthrobacter psychrolactophilus* D-allulose 3-epimerase (ApDAEase) yields a 1.4-fold increase in catalytic efficiency compared to the wild-type, driven by a 1.3-fold decrease in K_m and a 1.1-fold increase in k_{cat} . Structural dynamics were elucidated using molecular dynamics simulations based on models generated via AlphaFold 3. The simulations revealed that the E35H substitution weakens the local hydrogen-bonding network surrounding residue 35, thereby relaxing structural constraints near the active site. Root mean square fluctuation (RMSF) analysis confirmed increased localized flexibility at residue 35, contrasted with a reduction in overall structural fluctuations, suggesting a more stabilized global scaffold. Additionally, a newly identified interaction between E152 and the substrate's O1 atom provides a supplementary anchoring effect. Circular dichroism (CD) spectroscopy and thermal unfolding assays confirmed a modest increase in thermostability, elevating the melting temperature from 92.6 °C in the wild-type to 94.3 °C. Ultimately, these results identify residue 35 as a key functional hotspot where polarity shifts successfully recalibrate the flexibility-rigidity balance, offering critical mechanistic insights into the catalytic enhancement of ApDAEase.

Keyword: D-allulose 3-epimerase, *Arthrobacter psychrolactophilus*, Rational mutation, Catalytic activity, Thermostability.

Development of Oil in Water Emulsion Formulations to Enhance the Efficiency of Vitamin D₃ Encapsulation

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ABSTRACT: Vitamin D₃ deficiency remains a global public health concern. Vitamin D₃ plays an essential role in calcium regulation and bone health. Beyond these roles, inadequate vitamin D₃ status has been associated with type 2 diabetes, cardiovascular disease, and mental health disorders. However, vitamin D₃ is sensitive to factors such as light, heat, and oxygen, which may cause degradation during processing and storage. Oil-in-water emulsions are used as delivery systems to improve the stability of lipophilic bioactive compounds. In this study, oil-in-water emulsions containing 0.025 g of vitamin D₃ dissolved in rice bran oil were prepared using emulsifier systems to investigate emulsion stability. The formulations contained a high level of lecithin (F1), a low level of lecithin (F2), wall materials (Arabic gum, Hi-Cap® 100, and maltodextrin) without an additional emulsifier (F3), Tween 80 (F4), and pea protein hydrolysate (F5). The wall-material concentration was fixed at 20% (w/w) in all formulations. The high-lecithin formulation exhibited phase separation after pH adjustment and was therefore excluded from processing. The remaining formulations were processed by high-pressure homogenization and spray drying to produce vitamin D₃ microcapsule powders. The emulsions were evaluated for visual appearance and viscosity, while the powders were evaluated for solubility and encapsulation efficiency. High-pressure homogenization increased the viscosity of F2, F3, and F5, whereas F4 showed only a small change. During the short-term visual stability test, F3 showed the smallest visible change. Powder solubility ranged from 67.28 to 80.52%, with F3 showing the highest value. All powders exhibited high encapsulation efficiency, ranging from 99.61 to 99.82%, with no significant differences among the formulations. F3 combined the highest numerical solubility with encapsulation efficiency comparable to the other formulations without requiring an additional emulsifier. These findings suggest that the wall materials provided an emulsifying effect and that F3 is a promising formulation for further development.

Keyword: Vitamin D₃, Spray Drying, Emulsifier, Encapsulation Efficiency

Application of Ohmic Heating Pretreatment to Accelerate the Aging Process and Modulate the Bioactive Profile of Black Shallots

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ABSTRACT: Shallot (*Allium ascalonicum* L.) is a widely consumed Allium vegetable containing bioactive compounds, particularly phenolic compounds and flavonoids, which contribute to its antioxidant properties. The production of black shallot through controlled aging induces physicochemical and biochemical transformations, including the formation of reducing sugars and Maillard reactions, which contribute to its characteristic color, flavor, aroma, and functional properties. However, conventional black shallot production requires approximately 15 days of aging, resulting in extended processing time, high energy consumption, and increased production costs. This study investigated the application of ohmic heating as a pretreatment to facilitate biochemical transformations and shorten the aging period. Fresh shallots were subjected to ohmic heating at 60, 70, and 80°C for 15, 30, and 60 min, with the applied voltage varying between 90 and 120 V to maintain the target temperature, before aging. Ohmic pretreatment significantly increased reducing sugar content, while no significant differences were observed in total phenolic content (TPC), total flavonoid content (TFC), or antioxidant activity compared with untreated shallots. Following aging, ohmic pretreated shallots produced black shallots within 9 days, representing a 40% reduction in aging time compared with the conventional 15 days process, while maintaining comparable TPC, TFC, and antioxidant activity. These findings suggest that ohmic pretreatment enhances the availability of reducing sugars, potentially facilitating subsequent biochemical transformations during aging and shortening the aging period while maintaining the chemical properties of conventionally produced black shallot.

Keyword: Antioxidant activity, Black shallot, Ohmic heatin, Reducing sugar

Evaluation of the Protective Effects of an Energy- and Electrolyte-Enhanced Mulberry Beverage Against Delayed Onset Muscle Soreness Through Redox

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ABSTRACT: Intense physical exercise promotes excessive generation of reactive oxygen species (ROS), leading to oxidative stress that damages muscle cells, triggers inflammation, this not only causes delayed onset muscle soreness but also impairs post-exercise recovery. This study aimed to evaluate the potential of an anthocyanin-rich mulberry beverage supplemented with energy sources and electrolytes to protect against DOMS by examining its antioxidant properties and redox-regulating mechanisms under oxidative stress conditions in C2C12 skeletal muscle cells. Mulberry aqueous extract was characterized for its antioxidant activity and phytochemical composition, focusing on free radical scavenging activity by the Ferric Reducing Antioxidant Power (FRAP), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, total phenolic content, total flavonoid content, total anthocyanin content, and total soluble carbohydrate content. The extract was then formulated into a sports beverage by incorporating energy substrates and electrolytes. The protective activity of the final product was subsequently evaluated using a hydrogen peroxide (H₂O₂)-induced oxidative stress model in C2C12 myoblasts. The result showed that mulberry extract contained total anthocyanin content of 678.25 mg C3G/L, ABTS VCE of 0.00621 mg/mg, FRAP VCE of 0.648 µg/mL, total phenolic content of 1.12 mg GAE/mL and total flavonoid content of 0.00230 mg CE/g. The formulated post-exercise recovery beverage retained anthocyanin at 645.41 mg C3G/L, ABTS VCE of 0.00605 mg/mg, FRAP VCE of 0.609 µg/mL, total phenolic content of 0.99 mg GAE/mL and total flavonoid content of 0.00218 mg CE/g. These findings demonstrate that both the mulberry extract and the formulated post-exercise recovery beverage retained substantial levels of anthocyanins and exhibited antioxidant activity, suggesting their potential to alleviate exercise-induced DOMS. However, further cellular studies are required to confirm their protective effects and underlying mechanisms.

Keyword: Anthocyanins, Delayed Onset Muscle Soreness, Mulberry, Redox Homeostasis, Sports Beverage

Physicochemical Characterization and 2D *In Vitro* Wound Healing Evaluation of a Novel Tripeptide

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ABSTRACT: Chronic and non-healing wounds impose a substantial healthcare burden. Therapeutic peptides have emerged as promising bioactive agents capable of regulating key regenerative processes; however, the clinical application of longer and structurally complex peptides is often limited by rapid degradation, short half-life and poor bioavailability. Increasing attention has been directed towards short therapeutic peptides with minimal yet strategically designed amino acid sequences that retain biological activity while offering improved stability, simplified design and cost-effectiveness. In this study, a novel synthetic Lysine-Arginine- Isoleucine (Lys-Arg-Ile; KRI) tripeptide, designed from a minimal bioactive motif of the human cathelicidin antimicrobial peptide LL-37, was evaluated for its regenerative potential using a two-dimensional (2D) *in vitro* wound healing model. The physicochemical characteristics of the tripeptide were determined using high-performance liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESI-MS), fourier-transform infrared spectroscopy (FTIR) and circular dichroism (CD) to assess the peptide purity, molecular weight, functional groups and secondary structure, respectively. The tripeptide exhibited high purity (>98%) by HPLC, an observed molecular weight of approximately 415 Da by ESI-MS, characteristic amide absorption bands by FTIR and a predominantly random coil secondary structure as determined by CD analysis. Dose-dependent evaluations were subsequently performed on human dermal fibroblasts (HDFs) using MTT, live/dead staining, and wound scratch assays to assess proliferative, cytocompatibility, and migratory effects, respectively. The tripeptide exhibited concentration-dependent activity, with 12.5-50 µg/ml promoting fibroblast viability and migration, while concentrations exceeding 100 µg/ml reduced cell viability. Live/Dead staining demonstrated 90-100% viable cells with minimal cell death at 12.5-50 µg/ml across 24 to 72 hours, whereas 100 µg/ml reduced viability to 74-89% with increased dead cells. Wound scratch assays further showed complete wound closure at 12.5-50 µg/ml after 24 hours, exceeding the untreated control (97.08%) and 100 µg/ml group (90.89%). Collectively, these findings support the KRI tripeptide as potential short peptide candidate for wound healing applications.

Keyword: Cytocompatibility; Physicochemical characterization; Short peptides; Wound healing

Effect of Peptide Sequence Length on the Physicochemical Properties and Biological Performance in Hyperglycaemic Wound Healing

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ABSTRACT: Hyperglycaemic wound healing, characterized by dysregulated tissue regeneration, persistent inflammation and elevated susceptibility to infection, remains a major healthcare challenge due to the limited therapeutic efficacy of current treatment approaches. Antimicrobial peptides (AMPs) represent promising biomolecular candidates for hyperglycaemic wound healing applications due to their multifunctional properties, including wound reepithelization and collagen synthesis activation, immunomodulation, antimicrobial activity and vascular regeneration. However, the relationship between peptide sequence length and biological functionality remains insufficiently characterized. This study evaluates the influence of peptide length on the physicochemical properties and biological performance of LL-37-derived peptides under hyperglycaemic wound conditions. The long (LL-37), medium (RI-10) and novel short peptides (KRI) were characterized with HPLC, LC-MS and FTIR to investigate sequence-dependent structural variation. All peptides recorded a high purity ($\geq 98\%$) with their molecular weight successfully identified (LL-37: 4493 Da; RI-10: 1273 Da; KRI: 415 Da). The functional groups of the peptides were successfully characterized. The hyperglycaemic condition was induced in human dermal fibroblasts (HDFs) using high-glucose DMEM for at least three passages. Hyperglycaemic induction in HDFs was confirmed through morphological observations and senescence assays, where HDFs displayed elongated morphology and increased SA- β -Gal staining, indicating cellular senescence. The MTT, Live/Dead cell viability and scratch assays were conducted to assess the proliferative, cytotoxic and migratory effects of different sequences of peptides on hyperglycaemic HDFs, respectively. The short peptide exhibited no cytotoxicity and enhanced cellular proliferation at concentrations up to 12.5 $\mu\text{g/mL}$, with 98% live cells retained at 100 $\mu\text{g/mL}$, whereas the longer peptides demonstrated cytotoxic effects at concentrations above 100 $\mu\text{g/mL}$. Following that, all peptides showed a concentration-dependent migratory effect from 12.5 to 100 $\mu\text{g/mL}$ on hyperglycaemia-induced HDFs. The findings demonstrate that peptide sequence length plays a critical role in determining the physicochemical properties of peptides. Ultimately, this research may contribute to the rational design and optimization of peptide-based biomaterials and therapeutic platforms for hyperglycaemic wound applications.

Keyword: Hyperglycaemic wounds, Antimicrobial peptides, Peptide sequence, Physicochemical characteristics, Biological properties

Cellulase-Assisted Enzymatic Remodeling of Bacterial Cellulose as a Scaffold for Tissue Engineering Applications

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ABSTRACT: Bacterial cellulose (BC) is a natural polymer synthesized by various bacteria. Notably, BC produced by *Komagataeibacter xylinus* has attracted considerable attention as a scaffold material for tissue engineering applications due to its outstanding physicochemical and biological properties, including high porosity, excellent water-holding capacity, biocompatibility, non-cytotoxicity, and a unique nanoscale fibrous structure. However, the direct use of native BC as a scaffold is limited by its densely entangled fiber structure, which restricts cell adhesion, migration, and proliferation. The objective of this study is to structurally modify BC using cellulase treatment in order to loosen the fiber network and increase surface area, thereby improving its suitability for cell attachment and migration. Surface morphology and structure was examined using scanning electron microscopy (SEM). Scaffold porosity was evaluated using a liquid displacement method. Crystallinity index was analyzed using wide angle x-ray scattering (WAXS). In addition, cytotoxicity and cell viability was assessed using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Results stated that BC modified with cellulase exhibited an increase in the porosity and results in 97.0103, 97.9861 and 98.7270 of BC control, BC3 and BC6 respectively. The swelling ratio of scaffold showed an increase of 2,532, 3,332 and 4,376 of BC control, BC3 and BC6, respectively. SEM analysis revealed a progressively loosened fiber structure with increasing cellulase concentration. The evaluation of cytotoxicity and cell proliferation indicated that the cell viability was above 70 and 80% in both BC control and cellulase-treated, demonstrating that the modified BC scaffolds provide a more favorable environment for cell growth. These modified BC scaffolds will serve as a prototype biomaterial with a loosened fibrous architecture, effectively overcoming the structural limitations of native BC. Ultimately, the findings of this study are anticipated to provide a fundamental basis for further development of bacterial cellulose-based scaffolds for tissue engineering applications.

Keyword: Bacterial cellulose, Cellulase, In-situ modification, Scaffold, Tissue engineering

Evolution of *Bacillus thuringiensis* Virulence During Passage through the Resistant and Susceptible Host

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ABSTRACT: *Bacillus thuringiensis* is one of the most common sources of biopesticide and gene modified crops used for pest management in the world. However, reduction or loss of virulence, morphological differentiation of *B. thuringiensis* when cultivated on an industrial scale, as well as the formation of pest populations resistant to biological insecticides, is a relevant area of research that requires the attention of scientists. Many cases of insect's resistance to *B. thuringiensis* were registered last decades. We experimentally selected a *Galleria mellonella* line over 40 generations for resistance against *B. thuringiensis*. The *B. thuringiensis* subsp. *galleriae* infection in susceptible and resistant populations of wax moth larvae was investigated to gain further insight into the "arms race" between *B. thuringiensis* virulence and insect defences. We found that a sub-population of highly virulent *B. thuringiensis* could survive in the resistant insects by disrupting the midgut microbiome and switching rapidly to a necrotrophic strategy, prior to sporulation in the cadaver. Arm race between virulence factors of *B. thuringiensis* and insect resistance is interesting example of coevolution in the field. Sequential passage, and isolation, of *B. thuringiensis* through resistant insects for developing highly virulent strains will be discussed in context of applied biotechnology for improvements of biological preparations.

Keyword: *Bacillus thuringiensis*, Passages, Virulence, Biological pest control

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Encapsulation of Anti-Dengue Virus NS3 Monoclonal Antibody in PEG-PLGA Nanoparticles for Intracellular Delivery

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ABSTRACT: Dengue fever, an infectious disease caused by dengue virus (DENV), remains a significant global health challenge with no specific antiviral treatment available. The non-structural protein 3 (NS3) of DENV plays an essential role in viral replication, making it an attractive therapeutic target. Among potential therapeutic approaches, antibodies could play a vital role in reducing disease severity by targeting and neutralizing specific viral components. We hypothesize that antibody-mediated neutralization of viral components within the intracellular environment could lead to a significant reduction in viral production. However, delivery of antibodies to intracellular targets is hindered by their inability to cross the plasma membrane and their susceptibility to intracellular degradation. In this study, anti-DENV NS3 monoclonal antibody was encapsulated within poly (ethylene glycol)-*block*-poly(lactide-*co*-glycolide) (PEG-PLGA) nanoparticles using a solid-in-oil-in-water (S/O/W) emulsion method. Prior to encapsulation, the anti-NS3 mAb demonstrated specific reactivity to DENV NS3 protein across all four serotypes by showing a single band at approximately 70 kDa by Western blot. The resulting nanoparticles exhibited a hydrodynamic diameter of 106.7 ± 1.6 nm with acceptable polydispersity index (PDI < 0.2), a negatively charged surface, and an encapsulation efficiency of 28.79%. Transmission electron microscopy confirmed spherical morphology. Cytotoxicity assessment in immortalized hepatocyte-like cells (imHC) by MTT assay showed that all antibody-loaded nanoparticles formulations maintained cell viability above 70%, although additional biological replicates are required for further validation. Cellular uptake analysis demonstrated efficient and concentration-dependent intracellular internalization of the antibody-loaded nanoparticles in imHC cells, indicating effective intracellular delivery of the encapsulated antibody. These preliminary results suggest that PEG-PLGA nanoparticles represent a promising carrier platform for intracellular antibody delivery targeting non-structural viral proteins in dengue-infected cells.

Keywords: Dengue Virus; Polymeric Nanoparticles; PEG-PLGA; Monoclonal Antibody; Intracellular Delivery

1. INTRODUCTION

Dengue fever is a significant and growing global health concern caused by dengue virus (DENV), a single-stranded positive-sense RNA virus belonging to the Flaviviridae family. The virus consists of four antigenically distinct serotypes (DENV-1 to DENV-4), each capable of causing diseases ranging from mild febrile illness to life-threatening severe dengue [1]. Over 14 million cases were reported globally in 2024, and more than 10,000 dengue-associated deaths were recorded, highlighting the urgent need for effective

therapeutic strategies [2]. To date, no specific antiviral drug is available, and clinical management remains largely supportive.

Among the seven non-structural proteins encoded by DENV, NS3 is a multifunctional ~70 kDa protein with critical roles in viral replication. It possesses both serine protease and RNA helicase activities required for viral polyprotein cleavage and genomic RNA unwinding [3]. Previous therapeutic strategies have mainly focused on structural proteins, particularly the envelope (E) protein, due to its role as a structure of viral particles that mediates both host cell attachment and membrane fusion [4]. However, target non-structural protein such as NS3 may provide an alternative therapeutic approach by inhibiting viral replication after the virus has entered host cells. Furthermore, NS3 high conservation across different serotypes makes NS3 a promising intracellular therapeutic target [3].

Monoclonal antibodies (mAbs) targeting viral proteins offer high specificity as therapeutic agents by mimicking the body's natural immune response [5]. However, delivering antibodies to intracellular sites of viral replication remains fundamentally challenging. Antibodies naturally cannot pass through the plasma membrane and are susceptible to degradation in the harsh endolysosomal environment [6]. Nanoencapsulation using poly (ethylene glycol)-*block*-poly(lactide-*co*-glycolide) (PEG-PLGA) nanoparticles offers a solution to this problem. PEG-PLGA is a well-established, FDA-approved polymeric carrier system with excellent biodegradability, biocompatibility, and PEGylation-mediated stealth properties that facilitate endocytic cellular uptake of proteins [7, 8].

The S/O/W emulsion method was developed to overcome limitations of conventional W/O/W techniques, including low encapsulation efficiency, burst release, and protein instability, by maintaining the active ingredient in solid state throughout the formulation process [9]. Building on an S/O/W formulation previously optimized in our laboratory [10], the present study aimed to encapsulate anti-DENV NS3 mAb (clone 1B11) within PEG-PLGA nanoparticles and systematically characterize the resulting nanoparticle system with respect to physicochemical properties, encapsulation efficiency, and biocompatibility in a dengue-relevant cell model.

2. MATERIALS AND METHODS

2.1 *Anti-DENV NS3 mAb characterization*

The reactivity and specificity of anti-DENV NS3 mAb (clone 1B11) were evaluated prior to encapsulation using Western blot assays. Briefly, C6/36 lysates from cells infected with all 4 dengue serotypes (DENV-1 strain Hawaii, DENV-2 strain 16681, DENV-3 strain H87 DENV-4 strain H241, all at MOI = 1) along with mock-infected controls were prepared under reducing and heat-denaturing conditions. Proteins were resolved by 10% SDS-PAGE, transferred to PVDF membrane using a semi-dry transfer system (PowerBlot™). After blocking, the membrane was probed with 1B11 mAbs at 20 µg/mL. Detection was performed with rabbit anti-mouse IgG/HRP (1:2000) and ECL substrate using an Amersham™ ImageQuant™ 800 imaging system.

2.2 *Preparation of anti-NS3 mAb-loaded PEG-PLGA nanoparticles*

1B11-loaded PEG-PLGA nanoparticles were prepared by S/O/W emulsion as previously described [9]. Briefly, 20 mg of PEG-PLGA (PEG avg. Mn 2,000; PLGA avg. Mn 11,500; Sigma-Aldrich) was dissolved in 1 mL acetone. 1B11 (160 µg, ≤100 µL) was added dropwise under continuous stirring for 1 minute to form a solid-in-oil (S/O) dispersion, which was subsequently added to 6 mL distilled water and stirred for 5 minutes to form the S/O/W emulsion. Acetone was removed by dialysis (MWCO 6,000–8,000 Da; Spectra/Por) against distilled water for 8 hours with water changes every 2 hours. Nanoparticles were purified using Sepharose 4B

gel permeation chromatography and concentrated using Amicon Ultra centrifugal filters (MWCO 30 kDa). Isotype control mAb (Myeloma)-loaded and empty PEG-PLGA NPs were prepared under identical conditions as controls.

2.3 Nanoparticle characterization

Hydrodynamic diameter, polydispersity index (PDI) to assess nanoparticle uniformity, and surface charge of nanoparticles formulations were measured by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Panalytical) at 25°C in triplicate. Nanoparticle morphology was assessed by transmission electron microscopy (TEM; Hitachi HT-7700) following negative staining with 2% (w/v) phosphotungstic acid (pH 7.0). Encapsulation efficiency (%EE) was determined by dissolving lyophilized nanoparticles in breaking solution (0.05 N NaOH, 1% SDS) and quantifying antibody content by micro-BCA protein assay (Pierce™, Thermo Fisher Scientific) with a BSA standard curve. The %EE was calculated according to the following equation:

$$\text{Encapsulation efficiency (\%EE)} = \frac{\text{Weight of mAbs in NPs}}{\text{Initial weight of mAbs}} \times 100.$$

2.4 Intracellular delivery evaluation

The intracellular delivery efficiency of anti-NS3 mAb-loaded PEG-PLGA nanoparticles were quantitatively assessed using flow cytometry. Immortalized hepatocyte-like cells (imHC; 2.5×10^5 cells/well, 12 well plate) were seeded and incubated until approximately 80% confluence was reached before treatment. The cells were treated with nanoparticles at antibody-equivalent concentrations of 5, 10, and 20 µg/mL for 6 hours, followed by incubation in complete medium until 24 hours. Cells were harvested, fixed with 3.7% formaldehyde, and permeabilized using 0.1% Triton X-100. After washing, cells were stained with Alexa Fluor® 488-conjugated goat anti-human IgG (1:500) for 30 min in the dark. Fluorescence was analyzed using a BD Biosciences BD FACSCalibur™ flow cytometer (10,000 events/sample).

2.5 Cytotoxicity assessment

Cell viability of imHC cells after nanoparticle treatment was evaluated by MTT assay. The imHC cells (3.0×10^4 cells/well, 96-well plate) were seeded and incubated until approximately 80% confluence was reached before treatment. The cells were then treated with five dilutions of empty PEG-PLGA NPs, Myeloma-loaded NPs, and 1B11-loaded NPs (corresponding to antibody equivalent concentrations according to Table 1.) for 24 hours.

Table 1. Final concentrations of mAb-loaded PEG-PLGA NPs used for cell viability analysis.

Concentration	Final concentration (µg/mL)	
	Myeloma-loaded NPs	1B11-loaded NPs
1X	16.11	27.64
0.75X	12.08	20.73
0.5X	8.06	13.82
0.25X	4.03	6.91
0.1X	1.61	2.76

Untreated cells and 10% (v/v) DMSO served as negative and positive controls, respectively. Absorbance was measured at 570 nm and 670 nm using an Agilent BioTek Epoch Microplate Spectrophotometer. The %cell viability was calculated according to the following equation:

$$\%Cell\ viability = \frac{A_{570}^{treated\ cell} - A_{670}^{treated\ cell}}{A_{570}^{control} - A_{670}^{control}} \times 100$$

3. RESULTS AND DISCUSSION

3.1 Anti-NS3 mAb (clone 1B11) specificity and reactivity

Prior to encapsulation, the specificity and cross-serotype reactivity of anti-NS3 mAb (clone 1B11) were confirmed by Western blot analyses (Figure 1). A distinct band at ~70 kDa was detected across all four DENV serotypes, with strongest signal observed in DENV-2, followed by DENV-3, DENV-4, and DENV-1, consistent with the reported size of DENV NS3 protein [3]. No signal was detected in the mock-infected control, confirming the high specificity of the antibody. These results collectively validate clone 1B11 as a specific anti-NS3 mAb suitable for use as a therapeutic cargo in nanoparticle formulation studies.

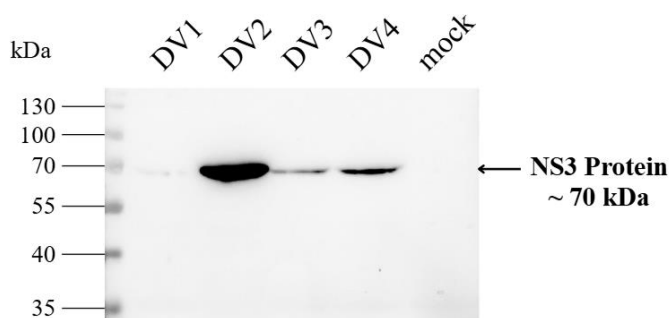


Figure 1. Western blot analysis confirms the specificity of anti-NS3 mAb (clone 1B11) against all four serotypes of DENV-infected C6/36 cell lysate.

3.2 Physicochemical characterization of PEG-PLGA nanoparticles

Anti-NS3 mAb (clone 1B11)-loaded PEG-PLGA nanoparticles were successfully prepared using the S/O/W emulsion method, and their physicochemical properties are shown in Table 2. DLS measurements revealed a hydrodynamic diameter of 106.7 ± 1.6 nm for 1B11-loaded NPs, which lies within the recommended 20–200 nm range for effective cellular uptake and prolonged circulation [11]. Empty PEG-PLGA NPs and isotype control (Myeloma)-loaded NPs measured 107.1 ± 1.0 nm and 113.4 ± 0.7 nm, respectively, indicating that antibody loading did not significantly alter nanoparticle size (Figure 2A). All nanoparticle formulation showed PDI values below 0.2, indicating acceptable size uniformity for polymer-based nanoparticle systems [12]. TEM imaging confirmed the spherical morphology of both empty and antibody-loaded nanoparticles (Figure 2B-E). The nanoparticle formulations showed surface charges ranging from –25 to –30 mV (Table 2), indicating excellent colloidal stability due to strong electrostatic repulsion and flocculation during storage. In the context of drug delivery, it is widely acknowledged that highly positive nanoparticles significantly enhance cellular uptake through strong electrostatic interactions with the negatively charged lipid bilayer of cell membranes [13]. However, these cationic formulations frequently induce severe non-specific cytotoxicity and cell membrane disruption. Conversely, the negative charge of our formulations minimizes these hazardous interactions, ensuring superior biocompatibility and lower cellular toxicity [14]. Interestingly, it has been reported that initial surface charges are normalized in biological media. Upon incubation with serum proteins, the zeta potential of nanoparticles consistently shifts to a range of –5 to –10 mV, regardless

of their original chemistry. Based on this, it is proposed that similar protein corona formation may mask highly charged interfaces that neutralize the inherent cytotoxicity of cationic particles while overcoming the initial electrostatic barrier of our negative formulations. This surface normalization ultimately ensures predictable cell membrane interactions and efficient receptor-mediated endocytosis [15], making our stable formulations highly favorable for drug delivery.

Table 2. Physicochemical properties of PEG-PLGA nanoparticle formulations.

Sample	Diameter size (nm.)	PDI	Zeta potential (mV)	%EE
Empty PEG-PLGA NPs	107.1 ± 1.0	0.1110 ± 0.0089	-29.94 ± 3.77	–
Myeloma-PEG-PLGA NPs	113.4 ± 0.7	0.1238 ± 0.0285	-29.25 ± 1.04	10.01
1B11-PEG-PLGA NPs	106.7 ± 1.6	0.1911 ± 0.0377	-25.77 ± 2.20	28.79

The encapsulation efficiency (%EE) of 1B11-loaded NPs was determined to be 28.79, which corresponds to a final antibody content of 138.19 µg. The isotype control (Myeloma)-loaded NPs demonstrated a lower %EE of 10.01. Notably, the %EE of 1B11-loaded NPs was lower than the 45.69 ± 6.62% reported for anti-E protein mAb-loaded PEG-PLGA NPs prepared using similar methods [9]. Subsequently, we proposed that the variation in encapsulation efficiency observed between 1B11-loaded NPs, Myeloma-loaded NPs, and other mAb-loaded NPs systems may be associated with variations in antibody physicochemical properties, including hydrophobic interactions and surface electrostatic patch distribution. As research established that antibodies cannot be characterized merely by their global net properties; rather, each antibody exhibits a highly unique, localized solvent-accessible electrostatic and hydrophobic patches within its variable (Fv) domain in the Fab region [16], these properties affect the interaction with the polymer. Molecular dynamics simulations of PEG-PLGA systems demonstrate that encapsulation is influenced by a localized hydrophobic and electrostatic surface match. During nanoparticle formation, the hydrophobic PLGA segment undergoes solvent-induced chain collapse, the interface shifts preferably to interact with hydrophobic protein patches to maximize the contact surface area [17]. Therefore, it is hypothesized that the 1B11 antibody exhibits a surface topology with greater hydrophobic accessibility than Myeloma antibody, allowing for stable non-covalent anchoring within the precipitating PLGA core. However, this remains a proposed mechanism that would require direct experimental validation. Overall, an encapsulation efficiency below 30% remains a limitation for translational applications. Several optimization strategies should be explored to improve encapsulation in future formulation work, such as adjusting the internal-to-external aqueous or organic-to-aqueous phase volume ratios, or modifying the polymer composition (PEG:PLGA ratio) and concentration, as these parameters have been reported to influence the encapsulation efficiency of PEG-PLGA nanoparticles [7].

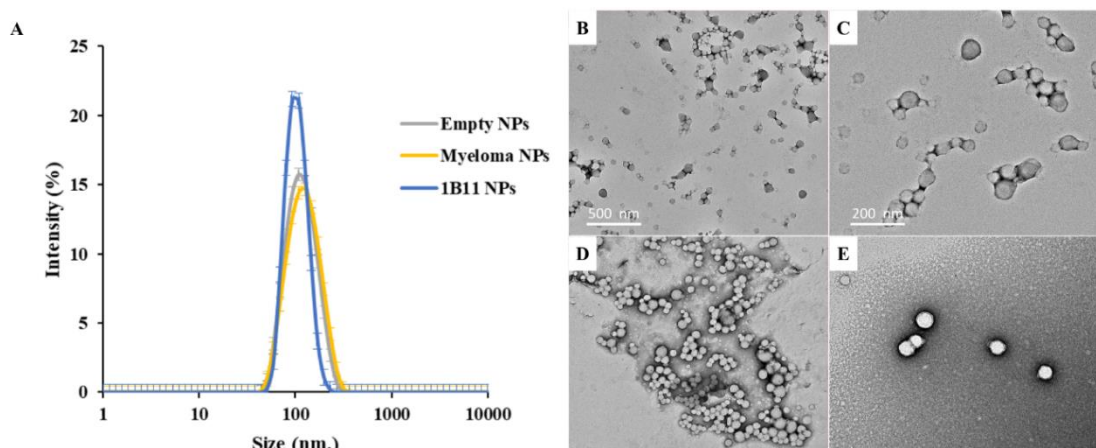


Figure 2. Characterization of PEG-PLGA NPs. (A) Size distribution profiles of empty PEG-PLGA NPs and mAb-loaded PEG-PLGA NPs measured by dynamic light scattering, showing average particle size versus intensity (%). (B-E) Transmission electron microscopy images of (B-C) empty PEG-PLGA NPs and (D-E) 1B11-loaded PEG-PLGA NPs.

3.3 Cytotoxicity of PEG-PLGA nanoparticles in imHC cells

Biocompatibility of nanoparticle formulations was assessed in imHC cells, a physiologically relevant hepatocyte-like model for dengue research [18]. As shown in Figure 3, the 10% DMSO positive control confirmed assay validity by reducing cell viability to $1.36 \pm 0.22\%$ relative to untreated controls. Both mAb-loaded NPs maintained cell viability above 70%, which all values were above non-cytotoxicity criterion defined by ISO 10993-5, where a material is classified as potential cytotoxic if cell viability falls below 70% of the untreated control [19]. Specifically, cell viability for Myeloma-loaded NPs ranging from $83.94 \pm 0.48\%$ to $108.75 \pm 6.79\%$, while 1B11-loaded NPs ranged from $81.89 \pm 3.89\%$ to $88.36 \pm 2.77\%$, corresponding to concentrations from highest to lowest. Values exceeding 100% at the lowest concentration in Myeloma-loaded NPs treated cells likely reflect inherent MTT assay variability and metabolic heterogeneity within cell populations rather than genuine proliferative effects [20].

Conversely, the empty nanoparticles exhibited concentration-dependent cytotoxicity, with cell viability decreasing from $80.46 \pm 1.89\%$ at the lowest concentration (0.1X) to $62.74 \pm 2.94\%$ at the highest concentration (1X), thereby falling below the ISO 10993-5 threshold. Notably, this pattern of greater cytotoxicity in empty NPs relative to antibody-loaded NPs contrasts with most reports in the polymeric nanoparticle literature, where blank or empty formulations are typically non-toxic or less toxic than their cargo-loaded counterparts [9]. We propose that this may be driven by the structural and chemical stabilizing effect of the encapsulated antibody within the PEG-PLGA matrix. In empty NPs, the absence of an internal protein cargo allows for autocatalytic hydrolysis of the core, leading to local pH drops in the micro-environment [21]. In contrast, the internally loaded antibody, in which exhibits unique charge, may help maintain the integrity of the polymer matrix to slow nanoparticle degradation, which reduces polymer-induced cytotoxicity. However, this mechanism was not directly assessed in this study and remains speculative.

It should be noted that the cytotoxicity data presented here are interpreted from a single biological replicate (with technical replicates within the assay). Therefore, these findings should be regarded as preliminary, and independent biological replicates are required to determine whether the observed differences between formulations are reproducible. Based on these trends, subsequent intracellular delivery and tracking experiments were restricted to fixed nanoparticle concentrations of 5, 10, 20 $\mu\text{g/mL}$. These specific doses were selected to optimize biocompatibility while ensuring sufficient carrier density for reliable downstream detection.

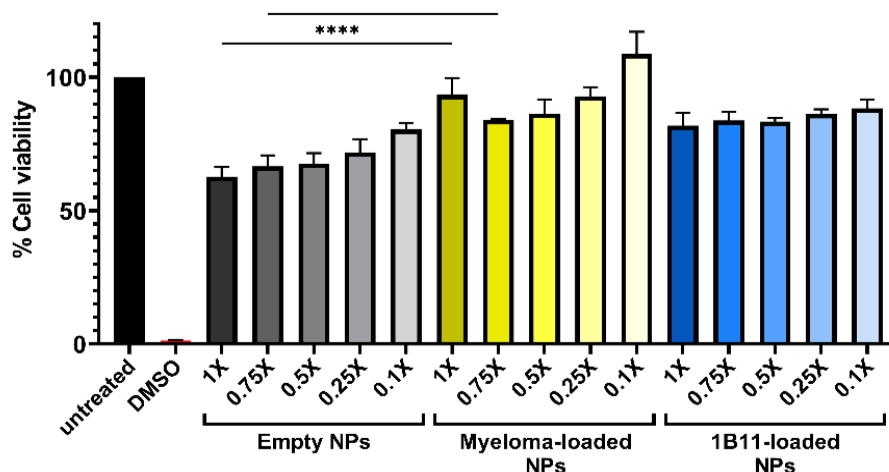


Figure 3. Cytotoxicity of PEG-PLGA nanoparticle in imHC cells assessed by MTT assay. Cell viability (%) is expressed relative to untreated controls. Data represents SD of three technical replications from one experiment.

3.4 Intracellular delivery of PEG-PLGA nanoparticles in imHC cells

To evaluate the intracellular delivery efficacy of NPs, cellular uptake of 1B11-loaded NPs was quantitatively assessed by flow cytometry (Figure 4). Unstained and mock-treated cells were used as controls to establish baseline background fluorescence (less than 1%). Following a 6-hour exposure to anti-1B11-loaded NPs at antibody-equivalent concentrations of 5, 10, and 20 $\mu\text{g/mL}$, a dose-dependent increase in cellular uptake was observed, yielding 23.03%, 30.83%, and 34.53% Alexa Fluor® 488-positive cells, respectively. This concentration-dependent profile demonstrates the capacity of imHC cells to progressively internalize the nanoparticles as the bioavailable particle concentration increases. Polymeric nanoparticles within the 100–200 nm size range have been reported to preferentially undergo cellular internalization through clathrin-mediated endocytosis [22], and the size of our nanoparticles falls within this range. However, this remains a proposed mechanism based on particle size alone, and direct experimental validation using approaches such as endocytosis inhibition assays will be required to confirm the specific internalization pathway involved.

In contrast, free antibody treatments resulted in minimal intracellular accumulation under identical conditions, confirming that nanoparticle encapsulation significantly enhances antibody internalization. Furthermore, empty NPs showed negligible background signal (less than 5%), indicated that the observed intracellular signals stem from the successful delivery of encapsulated antibodies rather than non-specific nanoparticle accumulation. Notably, Myeloma-loaded NPs showed a relatively higher cellular uptake compared to 1B11-loaded NPs across the equivalent concentrations (except the 10 $\mu\text{g/mL}$). This elevated uptake is likely attributed to the lower encapsulation efficiency (%EE) of the Myeloma formulation. To achieve an equivalent antibody payload, a greater total volume of nanoparticles had to be administered, thereby increasing nanoparticle exposure and subsequent cellular uptake.

Additionally, the cellular uptake results shown in Figure 4 do not indicate that the empty PEG-PLGA nanoparticles were poorly internalized by imHC cells, as the low percentage (<5%) of positive cells observed for the empty nanoparticles likely represents background signal resulting from nonspecific binding of the secondary antibody to the polymer. Importantly, this assay was designed to detect the encapsulated antibody using a fluorescently labeled secondary antibody and therefore cannot be used to quantify the cellular uptake of empty nanoparticles. It is possible that greater uptake of empty nanoparticles may contribute to the increased cytotoxicity observed in the MTT assay. However, this remains a hypothesis, and direct uptake studies using an appropriate nanoparticle-labeling strategy are required before any conclusions can be drawn.

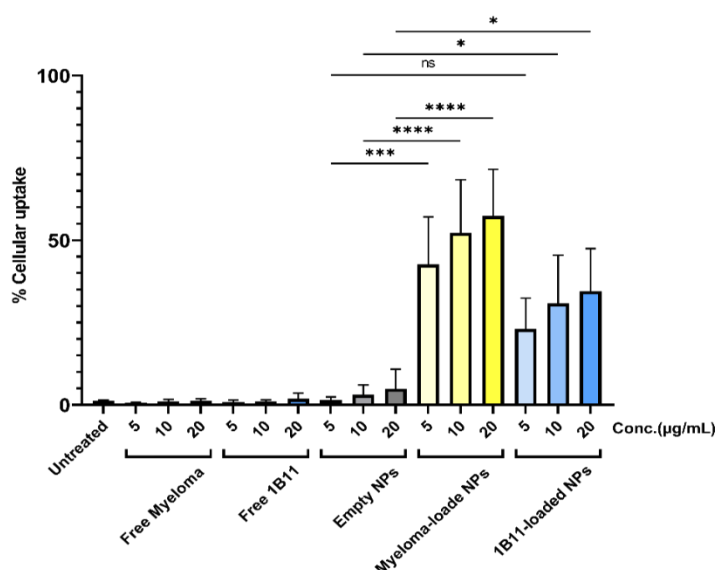


Figure 4. Cellular uptake (%) of PEG-PLGA nanoparticle in imHC cells assessed by using Flow cytometry. Data represents SD (n = 3) from three independent experiments analyzed by one-way ANOVA. ns: not significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

4. CONCLUSION

This study demonstrates the successful development of a PEG-PLGA polymeric nanoparticle platform using the S/O/W emulsion method for the intracellular delivery of the pan-serotype anti-DENV NS3 monoclonal antibody (clone 1B11). The formulated immunonanoparticles possessed favorable physicochemical properties, including a sub-200 nm spherical morphology and a stable negative surface charge, while preserving the broad-spectrum serotype specificity of the encapsulated antibody. Biological evaluations confirmed that the formulation is highly biocompatible and undergoes efficient, concentration-dependent intracellular internalization within hepatocyte-like cells, presumably through endocytic pathways. Although further biological replicates are warranted to comprehensively validate the cytotoxicity profile, these findings collectively establish a robust foundation for utilizing this polymeric carrier system as a promising intracellular antibody delivery strategy to target non-structural viral proteins in dengue-infected cells. Further studies evaluating antiviral efficacy are needed to fully demonstrate the therapeutic potential of this approach.

5. ACKNOWLEDGEMENT

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Development and Evaluation of a Pumpkin Seed-Enriched Functional Cream Cheese for Modulating Risk Factors of Polyendocrine Metabolic Ovarian Syndrome

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ABSTRACT: Polyendocrine Metabolic Ovarian Syndrome (PMOS) is a complex endocrine and metabolic disorder associated with insulin resistance, oxidative stress, chronic inflammation, and hormonal imbalance. Dietary approaches incorporating nutrient and bioactive-rich foods have garnered substantial attention for mitigating metabolic dysfunction. Pumpkin seeds (*Cucurbita pepo*) emerge as a promising functional matrix owing to their dense nutritional profile and naturally occurring bioactive compounds. However, their application in fermented plant-based dairy alternatives remains limited. This study aimed to develop a fermented pumpkin seed-based cream cheese via lactic acid bacteria inoculation and evaluate its physicochemical, textural, and sensory characteristics as an initial step toward functional food development. Fermentation with 0.1% (w/w) Lyofast SYAB 1 yielded a final product pH of 4.1 within 6 h. Texture profile analysis showed a firmness of 5.190 ± 0.966 N and adhesiveness of 9.629 ± 1.481 , indicating suitable spreadability. Just-About-Right evaluation (N=50) showed that while appearance (64%), aroma (62%), and texture (60%) reached satisfactory acceptance, flavor (38%) and cream cheese similarity (46%) fell below the benchmark. This indicates a critical need for formulation refinement to attenuate flavor intensity and enhance core cream cheese identity. These findings demonstrate the feasibility of developing a fermented pumpkin seed cream cheese with baseline properties. As this study did not quantify bioactives or evaluate PMOS-related biological activities in the final matrix, further research is required to characterize its precise bioactive profile and investigate its metabolic efficacy before its potential relevance to PMOS management can be established.

Keyword: Functional Food, Lactic Acid Bacteria, Plant-Based Cream Cheese, Polyendocrine Metabolic Ovarian Syndrome (PMOS), Pumpkin Seed

Fecal Microbiota Alterations Induced by Triphala Extract

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ABSTRACT: Diabetes mellitus is associated not only with impaired glucose metabolism but also with alterations in gut microbiota composition and microbial metabolic activity. Gut microbial imbalance may affect host metabolism through microbial metabolite production and nutrient utilization, suggesting that modulation of the gut microbiota could help support metabolic health in diabetic patients. This study investigated the potential of Triphala extract to modulate gut microbiota using a human gut model. Triphala extract, composed of *Terminalia chebula*, *Terminalia bellirica*, and *Phyllanthus emblica*, was tested in an *in vitro* fermentation of a human gut model inoculated with fecal microbiota from a diabetic female donor. Fermentation was conducted for 48 h with and without Triphala supplementation, followed by DNA extraction and shotgun metagenomic analysis to assess changes in microbial composition. At the phylum level, Triphala extract treatment was associated with a marked increase in Pseudomonadota from 2.89% to 51.04%, while Bacillota decreased from 49.01% to 22.99% compared with the control group. At the genus level, *Hungatella*, *Agathobaculum*, and *Bacteroides* showed higher relative abundance or better retention following Triphala treatment. These genera are known to contribute to beneficial gut metabolic activities, including complex carbohydrate degradation, short-chain fatty acid production, particularly butyrate, and maintenance of gut barrier function. In contrast, *Enterococcus* and *Clostridium* were reduced following Triphala extract treatment compared with the control. The reduction of *Enterococcus* and *Clostridium* may indicate decreased levels of potentially opportunistic bacteria and reduced risk of bacterial overgrowth associated with gut disturbance. Overall, these findings indicate that Triphala extract may contribute to restoring gut microbial balance and provide preliminary support for its potential use as a functional ingredient to modulate gut microbiota and metabolites in diabetic patients.

Keywords: Diabetes mellitus, Fecal microbiota, *In vitro* human gut model, Triphala extract.

In Vitro* Activity of a Co-Eluted Amphiphilic Crude Mixture Obtained from *Priestia aryabhattai* PTKU-123 Against *Phytophthora palmivora

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ABSTRACT: To address the need for alternatives to chemically synthesized fungicides, this study explored the extraction and enrichment of amphiphilic crude mixture from *Priestia aryabhattai* PTKU-123 to evaluate their anti-oomycete potential against the plant pathogen *Phytophthora palmivora*. The fermentation broth was precipitated and harvested to obtain the unrefined extract. Following with the enrichment process using preparative thin-layer chromatography and solid-phase extraction, an unresolved co-eluted fraction was recovered. Post-hydrolysis gas chromatography-mass spectrometry analysis identified this as an amphiphilic crude mixture, revealing a dominant β -hydroxy fatty acid-linked constituent at a 68.6% relative abundance alongside other co-migrating variants. When tested at a low concentration of 200 mg/L, this co-eluted crude mixture exhibited exceptional anti-oomycete activity, inhibiting mycelial growth by 95.55%, which demonstrated an increase in potency compared to the unrefined extract. Additionally, the fraction demonstrated thermal stability whilst retaining their potent anti-oomycete properties even after 28 days of continuous exposure to 50 °C. These active, naturally co-eluting compounds may represent as promising candidates for developing effective bio-pesticides to manage oomycete diseases in tropical agriculture.

Keyword: Anti-oomycete activity, Bio-pesticide, Co-elution, *Priestia aryabhattai*, *Phytophthora palmivora*

Co-Solvent-Driven Structural Engineering of Rice Bran Oil-Based Oleogels Through Multidimensional Characterization: Advancing Solid Fat Mimetics in Food Biotechnology

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ABSTRACT: The transition towards the use of trans-fat-free solid fats obtained from plants is one of the key objectives in the field of food biotechnology. Herein, the implementation of nutrient-rich agricultural products, such as rice bran oil (RBO), and designing them in the form of oleogels provides an effective strategy in the preparation of desirable lipids. In this study, polyethylene glycol (PEG 200) has been used as a crystal habit modifier (CHM) in stearic acid and RBO-based oleogels. The formulations with PEG 200 (0-10% w/w) have been prepared through a systematic process of heat-cool treatment. Subsequently, various analytical techniques such as microscopy, colorimetry, visible reflectance spectroscopy, and hyperspectral imaging (HSI) have been used to analyze the underlying structure–property relationships. Microstructural analysis has demonstrated that PEG 200 at a concentration of 1.25% w/w has induced a significant morphological change from one-dimensional (1D) needle-like structures to highly branched crystalline networks. As the concentration of the CHM was increased, it was observed that needle-like morphologies reappeared in the intermediate concentrations (2.5%-7.5% w/w) along with an increase in the density of the oleogel matrices. Specifically, the formulation P200-1.00 (5% w/w PEG) had shown an optimal structural balance wherein an increased scattering of short wavelengths (blue band) had occurred owing to the highly interconnected solid matrix. HSI resulted in a spatial analysis that confirmed this optical alteration with a distinct and concentrated cluster of high scattering domains within the 5% w/w PEG formulation observed at 450 nm. This strong local scattering created an effective ‘umbrella’ over the endogenous RBO carotenoid pigments, thereby imparting a large increase in the macroscopic whiteness needed to successfully mimic solid fats. Conversely, incrementing the PEG concentration to 10% w/w led to a structural breakdown of fat crystals into bulky agglomerates. Therefore, the formulation with 5% w/w PEG 200 showed an optimal balance between network density and the whiteness required for solid fat formulation, thus emphasizing the potential of co-solvent engineering of natural lipids as value-added food ingredients for the development of functional foods.

Keywords: Oleogels, Crystal habit modification, Hyperspectral imaging, Solid fat mimetics, Food biotechnology.

Multiscale Investigation of Pectin-Mediated Structural Reinforcement and Shelf-Life Enhancement in Whole Wheat Bread

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ABSTRACT: Development of functional whole wheat bread (WWB) with improved quality and extended shelf-life remains a significant challenge in the bakery industry. This study has focused on understanding the influence of incorporating pectin in WWB for the purpose of examining the changes in physicochemical, microstructural, textural, and shelf-life characteristics of the WWB formulations. Varying pectin concentrations have been employed to develop the formulations, which have been designated as SP0 (control), SP1 (1% w/w), SP2 (2% w/w), SP3 (3% w/w), and SP4 (4% w/w). Pectin incorporation has significantly enhanced the quality of bread in a concentration-dependent manner. Upon addition of pectin, loaf volume increased from 807.64 cm³ (SP0) to 1112.10 cm³ and 1194.22 cm³ in SP3 and SP4, respectively. However, SP3 and SP4 exhibited significant improvement in architecture and a finer and more uniform crumb. This change in architecture may be attributed to enhanced gas retention and increased dough stability caused by the incorporation of pectin. Further, the texture profile analysis has also shown a substantial crumb softening following pectin addition. Herein, the hardness of the crumb decreased from 1134.14 g in SP0 to 295.00 g and 272.62 g in SP3 and SP4 formulations, respectively. Moreover, stress-relaxation analysis also showed enhanced viscoelasticity and elastic recovery, indicating the development of a flexible network mediated by hydrocolloids. Additionally, sensory evaluation was conducted to validate the findings, and the results showed that SP4 got the highest texture and overall acceptability score (3.94 0.89). Based on the overall improvements in loaf volume, crumb structure, texture, sensory properties, and the efficiency of ingredient utilization, SP3 (3% w/w pectin) was selected as the optimum formula, achieving comparable results to SP4 at a lower concentration of pectin. Shelf-life studies have demonstrated that SP3 and SP4 formulations maintained freshness and were more structurally stable during storage. In this study, a clear link has been made between the microstructure induced by pectin, viscoelastic properties, sensorial characteristics, and storage properties. An attempt has been made to prove that pectin is a structure modifier that can improve bread quality parameters, including loaf volume, crumb texture, consumer acceptance, and storage properties, in a simultaneous way, while 3% (w/w) pectin was the optimum amount.

Keywords: Whole wheat bread, Pectin, Hydrocolloid, Sensory analysis, Shelf-life, Functional foods.

***In Silico* Evaluation of *Moringa oleifera* and *Solanum americanum*
Phytochemicals to Protect Red Blood Cell Membrane in Haemolytic
Anemia: A Network Pharmacology Approach**

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ABSTRACT: Disorders affecting red blood cell (RBC) membrane integrity, including sickle cell disease, stem from cytoskeletal destabilization and oxidative stress, resulting in haemolysis and reduced erythrocyte deformability. Although these conditions impact approximately 7–8 million individuals worldwide and significantly contribute to morbidity and mortality in endemic regions, effective multi-target therapeutic strategies remain scarce. In this study, we employed an integrative approach combining network pharmacology and molecular modeling to systematically evaluate the therapeutic potential of phytochemicals derived from *Moringa oleifera* (MO) and *Solanum americanum* (SA). From an initial set of 178 bioactive compounds, subsequent ADME profiling, QSAR filtering, and density functional theory (DFT) optimization led to the identification of nine promising lead candidates. Target prediction analyses revealed 853 drug–protein interactions, with 10 intersecting disease-associated proteins. Protein–protein interaction network topology and enrichment analyses highlighted three key hub regulators of membrane stability: SLC4A1 (4YZF), EPB42 (7UZS), and ALAS2 (5QQR). Molecular docking and molecular dynamics simulations demonstrated highly stable ligand–protein complexes, exhibiting binding affinities up to –9.5 kcal/mol for notable phytoconstituents, including gossypetin, apigenin, luteolin, and hesperetin. These results suggest that these four bioactive compounds may serve as potential therapeutic agents for sickle cell anemia and related hemolytic disorders. Further *In vitro* and *In vivo* validation is required for the therapeutic's applications of the screened molecules. This study is exclusively *In silico* in nature.

Keywords: *Moringa oleifera*, Network pharmacology, Molecular docking, Molecular dynamics, RBC Membrane integrity.

Characterization, Purification and Media optimization of *Saccharomyces cerevisiae* BHF5D35-2 Δ ade2 for Superoxide Dismutase Production

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ABSTRACT: Superoxide dismutase (SOD) is a potent antioxidant enzyme with growing demand as a bioactive ingredient in cosmetic and nutraceutical industries. As SOD in Thailand is predominantly imported, this study aimed to establish a domestic production platform by developing and characterizing a bioreactor-scale process for recombinant SOD using an engineered *Saccharomyces cerevisiae*. A chemically defined medium comprising yeast extract (15.0 g/L), ammonium nitrate (10.0 g/L), and glucose (10.0 g/L) was optimized for SOD production. Fed-batch fermentation in a 2 L bioreactor at 30°C, 200 rpm, and 1 vvm aeration over 24 h yielded a final cell density of OD₆₀₀ = 27.132, with stationary phase onset at 18 h. The maximum SOD activity of 813.14 U/mL was observed at 12 h during the exponential growth phase, declining to 443.50 U/mL by 24 h due to proteolytic degradation. Purification by immobilized metal affinity chromatography (IMAC) via fast protein liquid chromatography (FPLC) yielded a specific activity of 9,691.38 U/mg. SDS-PAGE confirmed a protein band at approximately 32 kDa, consistent with the predicted molecular weight of SOD. Biochemical characterization revealed optimal activity at pH 8.0 in Tris-HCl buffer (717.93 ± 12.93 U/mL) and 30°C (695.20 ± 14.66 U/mL). Stability assays demonstrated highest residual activity under alkaline conditions (pH 8–9; up to 753.10 ± 12.23 U/mL), while the enzyme was thermolabile above 45°C with complete inactivation at 50°C within 1 h. These findings establish a viable laboratory-scale platform for recombinant SOD production and purification with well-defined biochemical properties, providing a foundation for process scale-up and further formulation with delivery systems for cosmeceutical applications.

Keyword: Superoxide dismutase, Antioxidant enzyme, Recombinant, *Saccharomyces cerevisiae*

SPIDERS DETECH: An AI Innovation for Classifying Spider Species via a LINE Chatbot

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ABSTRACT: In our environment, arachnids are commonly found, and some species can be dangerous. However, many people are unable to identify different spider species or respond appropriately when encountering them. Therefore, we developed an AI-based system to promote awareness and understanding of the danger levels associated with spiders. Our project focuses on detecting and classifying five common spider species: Brown Recluse, Black Widow, Cellar, Jumping, and Huntsman spiders. The system not only identifies spider species, but also provides information about danger levels, safety responses, and educational knowledge regarding dangerous arachnids commonly found around homes. Several deep learning models were tested, including VGG16, Xception, EfficientNetB0, and ResNet50. The models were trained using a dataset of 532 images across five classes, consisting of 306 training images, 124 validation images, and 102 testing images. Among the tested models, ResNet50 achieved the highest F1-score of 0.96, followed by EfficientNetB0 with 0.95, Xception with 0.91, and VGG16 with 0.86. Each model was trained for 30 epochs to achieve optimal performance. The system was developed using Python on Google Colab, while the “LINE” application serves as the main platform for user interaction. Heatmaps are also used to display important features detected by the AI. Users can upload a spider image through the “LINE” app, and the AI automatically provides the species name, confidence score, danger level, heatmap, and cautions. In the future, we aim to expand the dataset, improve accuracy, and provide more detailed warnings and safety recommendations regarding dangerous spiders.

Keyword: Arachnid safety awareness, Deep learning, Image recognition, LINE Chatbot, Spider species classification

A Deep Learning-Based Smartphone Solution for Thai Jasmine Rice Quality Assessment

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ABSTRACT: Thai Jasmine rice (*Oryza sativa* L.) is globally renowned for its premium quality, long slender grains, and distinctive aroma, making it both a staple food and a major export commodity for Thailand. However, quality degradation from broken grains, chalky kernels, and discolored grains—whether from adulteration, improper storage, or poor processing—compromises national standards, market value, and consumer health. While precise quality assessment exists in large-scale laboratory facilities, everyday consumers, small restaurant owners, and local vendors lack accessible, affordable tools for real-time quality verification. This study addresses this gap by developing a smartphone-based rice quality assessment system integrated with a LINE Official Chatbot, enabling instant quality evaluation without specialized equipment. A custom dataset of 506 images was compiled from curated internet sources and categorized into four classes: Perfect (117 images), Broken (174 images), Brown/Yellow (112 images), and Chalky (103 images), aligned with internationally recognized defect criteria established by the International Rice Research Institute (IRRI) and ISO 7301 standards. Broken grains (fragments <75% of whole kernel length) reduce commercial value and cooking quality; chalky grains (kernels with $\geq 50\%$ opaque area) result from incomplete starch gelatinization, affecting texture and palatability; and yellow-brown discolored grains indicate heat damage or fungal contamination, historically linked to mycotoxin health risks. The dataset was split into training (306), validation (101), and testing (99) sets. To overcome the limited dataset size, extensive data augmentation techniques were applied, including rotation, horizontal and vertical flipping, brightness and contrast adjustments, and color space transformations to simulate diverse real-world smartphone capture conditions. Additionally, transfer learning was employed using four pre-trained deep learning architectures—ResNet-50, VGG, Xception, and EfficientNetB0—leveraging knowledge from ImageNet's millions of images to enable effective feature extraction with limited domain-specific data. Model performance was evaluated using F1 scores across the three defect categories. ResNet-50 achieved the highest performance for Broken grain detection (F1 = 0.9819), EfficientNetB0 excelled in Chalky grain identification (F1 = 1.0), and Xception performed best for Brown/ Yellow grain detection (F1 = 0.9555). These three specialized models were successfully embedded into a LINE Official Chatbot interface. Users simply upload a photograph of their rice sample, and the system evaluates it against the three defect criteria, generating an intuitive quality score: 3/3 (perfect quality), 2/3 (acceptable), 1/3 (fair), or 0/3 (poor quality), along with specific defect notifications and health warnings when applicable. This innovation democratizes rice quality assessment by transforming smartphones into portable quality assurance tools accessible to non-expert users. The system enables consumers to verify authenticity and safety before cooking, helps restaurant owners ensure premium grain quality, and allows small vendors to transparently demonstrate product quality to buyers. Future development will focus on enhancing contaminant detection capabilities, implementing automatic grain counting algorithms, and expanding the system to support diverse rice varieties beyond Thai Jasmine rice, contributing to improved food safety standards and agricultural quality control across the rice supply chain.

Keywords: Deep learning, Image classification, LINE Bot, Thai Jasmine rice, Quality assessment

Deep Learning-Based Skin Disease Classification Chatbot

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ABSTRACT: Skin diseases are common health problems in Thailand due to the hot climate, pollution, and genetic factors. Every year, many Thai people suffer from various skin conditions that affect not only physical health but also mental well-being and self-confidence. However, access to dermatologists remains difficult because of high medical costs, travel limitations, a shortage of specialists, and discomfort with hospital visits. To address these challenges, this project developed a LINE Chatbot embedded with a well-trained deep learning model for preliminary skin disease assessment and basic suggestions. The system allows users to upload skin images through mobile phones, making early screening more convenient and accessible. In its current stage, the system can detect Atopic Dermatitis, Chickenpox, Cellulitis, Ringworm, Urticaria, and Shingles, all of which are the skin diseases commonly found in Thailand. The project applies Deep Learning and Convolutional Neural Network (CNN) technology using Python on Google Colab. The classification utilizes four distinct pre-trained models, each trained using a standardized training, validation, and test method over 30 epochs. To facilitate this, a dataset of 892 images was divided into training (609 images), validation (164 images), and test (119 images) sets. Model performance was evaluated using Heat Maps, Confusion Matrices, and F1 Scores. Among the four models, ResNet50 achieved the highest F1 score at 91.19%, demonstrating the best overall performance. Xception ranked second with 84.40%, followed by EfficientNetB0 at 74.78%, while VGG16 showed the lowest F1 score at 54.85%. The trained model was integrated into the LINE Chatbot, enabling automatic image analysis and prediction of possible disease classes. This innovation is designed for people with early skin symptoms or those at risk of skin diseases, helping reduce medical expenses and travel difficulties while providing fast preliminary assessment. However, the current chatbot does not cover all skin diseases in Thailand. Future development will include more detectable diseases, normal skin data, and higher-quality datasets to further improve accuracy and system performance.

Keyword: Artificial Intelligence, Deep Learning, Image Classification, Line Chatbot, Skin Diseases

Evaluation of Antioxidant Activity, Total Phenolic Content, and Total Flavonoid Content of *Phyllanthus emblica* and *Garcinia mangostana* Extracts for Potential Biotechnological Applications in Herbal Oral Healthcare

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ABSTRACT: Medicinal plants are important sources of bioactive compounds with significant potential for biotechnological and healthcare applications. *Phyllanthus emblica* and *Garcinia mangostana* have been widely utilized in traditional medicine due to their antioxidant and antimicrobial properties. Natural antioxidants derived from medicinal plants have gained increasing attention in molecular biotechnology and nanobiotechnology for the development of sustainable healthcare products and bioactive formulations. This study aimed to evaluate and compare the antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC) of *P. emblica* and *G. mangostana* extracts for their potential application in herbal oral healthcare biotechnology. Dried plant materials were extracted using 90% ethanol as the extraction solvent. Antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays. TPC and TFC were analyzed using standard colorimetric methods and expressed as milligrams gallic acid equivalent per gram extract and milligrams quercetin equivalent per gram extract, respectively. *P. emblica* demonstrated stronger antioxidant activity than *G. mangostana*, with lower half maximal inhibitory concentration (IC₅₀) values for both the DPPH (29.29 ± 0.582 and 75.82 ± 0.614 µg/mL, respectively) and ABTS assays (15.16 ± 0.243 and 41.71 ± 0.302 µg/mL, respectively). Furthermore, *P. emblica* exhibited higher TPC (535.42 ± 3.752 mg gallic acid equivalent/g extract), while *G. mangostana* showed higher TFC (64.25 ± 2.682 mg quercetin equivalent/g extract). The findings suggest that phenolic compounds may contribute substantially to the antioxidant potential. These results support the potential utilization of medicinal plant extracts as sustainable bioactive materials for future biotechnological applications, including herbal oral healthcare formulations and natural antioxidant-based delivery systems.

Keywords: ABTS, Antioxidant activity, Biotechnology, DPPH, *Garcinia mangostana*, *Phyllanthus emblica*

Bid by Bit: A Micro: Bit-Based Wearable Device for Lower-Limb Motion Monitoring and Rehabilitation

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ABSTRACT: Falls are a major global health concern and the second leading cause of unintentional injury deaths worldwide, with older adults being especially vulnerable due to declines in lower-limb muscle strength, postural balance, and musculoskeletal function. While targeted physiotherapy can mitigate these fall risks and preserve independence, proper technique and long-term adherence during unsupervised home exercise remains a significant clinical challenge. To address this gap, we developed “Bid by Bit,” an affordable smart wearable device, designed to facilitate safe, accessible, and high-quality home-based physical therapy. Utilizing Micro: bit V2 technology and machine learning, the wearable system focuses on monitoring three core lower-body exercises crucial for mobility: straight-leg and lying-leg raises, which engage both the hip flexors and quadriceps; side-lying leg raises for hip stability; and lying knee bends for joint flexibility. Real-time motion data collected via an integrated accelerometer is processed by trained machine learning models to accurately recognize movement patterns, differentiate execution quality, and detect errors. Acting as an interactive “Personal Coach,” the device provides immediate audio-visual feedback and displays a live movement accuracy percentage on both the hardware interface and a synchronized Blynk Dashboard. In addition, the system actively prioritizes patient safety and tailored recovery; it prevents overexertion by automatically limiting daily sets and repetitions, while offering personalized configurations for pain-limit settings and individual calibration. Ultimately, “Bid by bit” successfully tracks long-term performance progress and securely stores motion data for future clinical analysis, offering a highly scalable, low-cost solution to enhance rehabilitation compliance and significantly reduce elderly fall risks.

Keywords: Elderly, Fall risks, Micro: bit technology, Rehabilitation, Wearable device

A Preliminary Study of Liquid Biopsy-Based Detection of Epidermal Growth Factor Receptor Mutations Using a Surface Acoustic Wave Biosensor

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ABSTRACT: Lung cancer remains one of the leading causes of cancer-related deaths worldwide, with non-small cell lung cancer accounting for more than 80% of cases. Mutations in the Epidermal Growth Factor Receptor (EGFR) gene are important biomarkers for targeted therapy selection. This study investigated the potential of a Surface Acoustic Wave biosensor for label-free detection of EGFR mutations in plasma samples. The biosensor operated through DNA hybridization between probes and complementary target DNA, which produced measurable phase shift responses due to mass loading on the sensing surface. Two clinically relevant EGFR mutations, Exon 18 and Exon 19 deletion, were evaluated. Experimental parameters, including incubation time, probe concentration, and target concentration, were systematically optimized to improve biosensor response. Results showed a detection of both EGFR mutations from plasma samples under label-free conditions with minimal reagent consumption. Phase shift differences between negative controls and mutation-positive samples confirmed the feasibility of mutation detection using the proposed platform. A detection cutoff derived from the mean plus three standard deviations of the negative control responses (8.92°) was applied to distinguish mutation-positive samples, and mutation-positive samples produced phase shift magnitudes approximately 2.8- to 3.0-fold higher than the negative control on average. Exon 19 samples showed more distinguishable responses than Exon 18 samples, which indicated better selectivity under the current experimental conditions. However, variations in phase shift responses among plasma samples showed no clear relationship with Ct values under the current experimental conditions. As this study represents a preliminary feasibility assessment using plasma samples rather than a synthetic target dilution series, analytical parameters such as the limit of detection, linear detection range, and analytical sensitivity could not be determined under the current experimental design. Therefore, additional studies with larger numbers of plasma samples are required to further evaluate biosensor sensitivity, selectivity, and quantitative detection capability.

Keywords: EGFR mutations, Exon 18 G719X, Exon 19 deletion, Non-small cell lung cancer (NSCLC), SH-SAW biosensor

A Low-Carbon Biochar Module for Nitrogen Reduction, Eutrophication Mitigation, and Sustainable Urban Water Resilience

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ABSTRACT: Bangkok's urban canals are affected by nitrogen and organic pollution, leading to eutrophication and ecological degradation. This study developed a low-carbon biochar module for nitrogen reduction using a 54-L solar-powered, biochar-based floating moving bed biofilm reactor (MBBR) as a decentralized wastewater treatment solution. The system used ring-shaped bamboo biochar as a microbial biocarrier and applied intermittent aeration powered entirely by an off-grid solar energy system without external electricity input. During startup, aeration schedules were adjusted to balance nitrate removal with solar energy availability; the 8 h aeration and 16 h non-aeration cycle showed the most favorable performance-energy balance. The module was evaluated using urban canal water during a 14-day startup period followed by a 7-day validation phase. Nitrate, biochemical oxygen demand (BOD), pH, dissolved oxygen, and oxidation-reduction potential (ORP) were monitored to assess performance and stability. Average nitrate removal reached 70–75%, with a maximum of 88%. Influent nitrate decreased from approximately 15 mg/L to 3–6 mg/L, while BOD decreased from about 30 mg/L to 3.8 mg/L. ORP shifted from positive values above +100 mV during aerobic periods to negative values below –50 mV during anoxic periods, indicating alternating aerobic-anoxic conditions that may support simultaneous nitrification-denitrification and alkalinity recovery. An indicative global warming potential (GWP) reduction estimate suggested 21.18–22.63 kg CO₂-eq module⁻¹ year⁻¹ from intermittent aeration, off-grid solar operation, and potential indirect methane and nitrous oxide reductions, excluding biochar. Including biochar carbon retention increased the unverified estimated benefit to 24.57–26.02 kg CO₂-eq per module. Longer-term operation, repeated trials, control comparisons, and additional nitrogen species data are recommended to confirm the mechanism and scalability. Overall, this study demonstrates the potential of solar-powered biochar-based MBBR technology as a decentralized, low-carbon approach for improving urban canal water quality.

Keywords: Biochar, Decentralized wastewater treatment (DWWT), Intermittent aeration, Nitrogen removal, Oxidation-Reduction Potential (ORP)

KneeHab Pro: A Wearable Motion Tracking System for Post Knee Surgery Rehabilitation

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ABSTRACT: Musculoskeletal knee injuries are a major source of long-term pain, disability, and healthcare expenditure worldwide. A critical limitation of current post-surgical recovery is that much of rehabilitation occurs unsupervised outside clinical facilities, contributing to poor exercise quality, low patient adherence, and an increased risk of reinjury. This study introduces KneeHab Pro, a low-cost wearable motion-tracking system designed to support home-based knee rehabilitation. The hardware comprises a Seeed Studio XIAO ESP32-C3 microcontroller, an Adafruit BNO085 nine-degree-of-freedom inertial measurement unit, a 3.7 V 500 mAh battery, and an Adafruit SPI MicroSD breakout. Synchronized sensors positioned on the thigh and shin continuously capture lower-limb orientation and movement data. The system supports two operating configurations: local data logging for long-duration assessment of recovery trends and real-time Bluetooth streaming to a patient mobile application and clinician web dashboard. The mobile application provides personalized rehabilitation exercises, guided range-of-motion measurements, and real-time movement feedback, while the clinician dashboard enables healthcare providers to monitor recovery data remotely, assign rehabilitation goals and exercise plans, and provide direct feedback. Raw inertial data are processed using a recursive low-pass filtering and kinematic analysis pipeline that calculates numerical metrics including knee joint angle, range of motion, angular velocity, movement smoothness, exercise repetitions, and exercise completion. These algorithmically calculated values form the basis of all displayed results and progress assessments. A Large Language Model is used only within the patient application to condense these precomputed medical metrics and clinician-provided information into clear, understandable progress summaries. It does not calculate measurements, diagnose conditions, or independently interpret raw sensor data. Patient movement data are protected using Fernet symmetric encryption to support the secure handling of sensitive rehabilitation information. KneeHab Pro therefore enables remote patient monitoring, real-time feedback, and at-home range-of-motion assessment beyond the limitations of periodic in-clinic measurements.

Keywords: Knee rehabilitation, Inertial measurement unit, Range of motion, Remote patient monitoring, Wearable sensor

AI-Engineered Nanoproteins: A De Novo Approach to Inhibit Hantavirus Transmission

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ABSTRACT: Andes virus (ANDV) is a highly pathogenic virus capable of causing severe Hantavirus Cardiopulmonary Syndrome (HCPS), presenting a critical threat to global public health. While monoclonal antibodies offer a traditional therapeutic pathway, their development and production are time-consuming, costly, and often limiting their accessibility. To overcome these limitations, this study proposes the development of *de novo* engineered nanoproteins binders targeted directly at the viral fusion loop of the ANDV Gc glycoprotein, a highly conserved region essential for host cell entry. By isolating the structural coordinates of the Gc glycoprotein, the fusion loop and its critical hotspots residues were mapped, RFDiffusion was used to design three-dimensional backbone structures that fit the specific epitope, the conditions used were iterations:200, num_designs:8, and hotspots included. Remarkably, the generated scaffolds achieved the best Root-Mean-Square Deviation (RMSD) of just 0.13 Å (PDB ID: 6Y5W) and 0.12 Å (PDB ID: 6Y5F), indicating highly stable and precise generation. ProteinMPNN was deployed to optimize the amino acid sequences to ensure stability and folding efficiency. Molecular Dynamic (MD) was applied to analyze movements of the molecules in real condition to verify binders' efficiency. For future downstream applications, these binders can be expressed using bacterial or yeast expression systems to validate their binding efficiency (EC₅₀ and KD) in laboratory assays, including Enzyme-Linked Immunosorbent Assay (ELISA) and Bio-Layer Interferometry (BLI). Ultimately, these stable nanoproteins hold immense potential for translation into innovative medical countermeasures, such as therapeutic nasal sprays, designed to effectively block viral-host cell attachment and neutralize infection at the primary site of entry.

Keywords: Andes virus, De novo protein design, Fusion loop binder, Gc glycoprotein

Gelatin/agar Film Combined with *Psidium cattleianum* Leaves Carbon Dots for Food Preservation

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ABSTRACT: This research created sustainable bioplastic films through integrating carbon dots (CDs) derived from strawberry guava leaves (*Psidium cattleianum*) into a gelatin/agar matrix. The CDs were made using an eco-friendly hydrothermal technique. UV-Vis spectroscopy and blue fluorescence upon UV irradiation confirmed that strawberry guava leaves underwent successful carbonization. Hydrothermal conditions, especially temperature and alkaline pH, influenced fluorescence behavior by promoting the synthesis of hydroxyl (OH) and amino (NH₂) functional groups linked to luminescence properties. Fourier-transform infrared spectroscopy (FTIR) proved the formation of GA/AG composite films with CDs. Characteristic functional groups, such as O-H, N-H, and C=O bonding, indicated the creation of carbonaceous structures and surface functionalization in CDs. The composite films showed a broad absorption band at 3200-3400 cm⁻¹ and a bathochromic shift of the amide band (from 3300 to 3280 cm⁻¹), confirming strong intermolecular hydrogen bonding and compatibility between gelatin and agar. Increasing the agar content greatly improved film performance, as increasing the GA/AG ratio from 6:1 to 6:5 increased film thickness from 0.087 ± 0.004 to 0.120 ± 0.003 mm, tensile strength from 0.77 ± 0.024 to 2.20 ± 0.347 MPa, and reduced water vapor transfer rate and permeability. Overall, agar reinforcement increased the film's mechanical strength and moisture barrier qualities. Combined with the functional properties of strawberry guava leaf-derived CDs, the produced films show remarkable potential for future active packaging applications.

Keywords: Agar, Bioplastic film, Carbon dots, Gelatin, Hydrothermal, *Psidium cattleianum* leaves

Population Dynamics of Ammonia-Oxidizing and Nitrite-Oxidizing Bacteria in Outdoor Shrimp Culture Ponds

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ABSTRACT: Nitrification is a key process governing water quality in shrimp aquaculture, sequentially converting toxic ammonia and nitrite into nitrate and thereby protecting animal health, growth, and survival. This two-step process depends on ammonia-oxidizing bacteria (AOB), such as *Nitrosomonas* spp., which convert ammonia to nitrite, and nitrite-oxidizing bacteria (NOB), such as *Nitrobacter* spp., which convert nitrite to nitrate. In outdoor earthen ponds, these communities are continuously exposed to fluctuating temperature, sunlight, salinity, and organic loading, yet their dynamics over a production cycle remain poorly characterized. This study quantified the abundance and tracked the dynamics of AOB and NOB in open-air outdoor ponds at Chaipattana Foundation demonstration shrimp farm (Chachoengsao, Thailand) managed without chemical pesticides or commercial probiotics. A SYBR Green real-time PCR assay was developed using two primer sets specific to AOB and NOB marker genes and applied to 69 pond-water samples collected across the culture period. Both bacterial groups were consistently detected, and their abundance and diversity varied among ponds and sampling times, revealing a dynamic nitrifying community that shifted with the changing nitrogen load of the ponds. The continuous presence and balance of AOB and NOB corresponded with effective on-farm nitrogen-waste management, stable water quality, and healthy cultured animals. These findings demonstrate that SYBR Green real-time PCR is a rapid and sensitive tool for monitoring temporal changes in nitrifier gene abundance in outdoor shrimp ponds, providing a practical molecular indicator that can support water-quality management and more sustainable shrimp production.

Keywords: Ammonia-oxidizing bacteria, Nitrite-oxidizing bacteria, Nitrification, Real-time PCR, Shrimp pond water quality

Development of a SYBR Green Real-Time PCR Assay Targeting the *amoA* and *nxrB* Genes for Monitoring Nitrifying Bacteria in a Recirculating Aquaculture System (RAS)

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ABSTRACT: Water quality is critical to shrimp health and productivity, and the accumulation of toxic ammonia and nitrite from excess feed and excretory waste is a major constraint in intensive culture. These nitrogenous compounds are removed by autotrophic nitrification, in which ammonia-oxidizing bacteria (AOB) convert ammonia to nitrite and nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate. Rapid, accurate monitoring of these functional groups, however, remains limited in aquaculture. This study developed and validated a SYBR Green real-time PCR assay targeting the *amoA* and *nxrB* genes, the functional marker genes of AOB and NOB, respectively, to monitor nitrifying bacterial populations in a Recirculating Aquaculture System (RAS) stocked with Pacific white shrimp (*Litopenaeus vannamei*) at Rajamangala University of Technology Tawan-ok (Chonburi, Thailand). Archived water samples from the culture cycle were filtered through a 0.22 µm membrane filter to collect the bacterial community, and genomic DNA was extracted. Seven newly designed short-amplicon primer pairs were screened against cloned *amoA* and *nxrB* plasmid standards, and one pair per gene was selected: *amoA* F1/R1 (180 bp) and *nxrB* F2/R1 (132 bp). Both pairs generated a single amplicon of the expected size on agarose gel electrophoresis and a single melt peak, with no specific amplification in no-template controls. Applied to the field-derived DNA, both genes were reliably detected, with Cq values of 22.4–27.7 for *amoA* and 22.7–23.4 for *nxrB*, and melt peaks (82.5–83.0 °C and 85.0–85.5 °C, respectively) identical to those of the corresponding plasmid controls, confirming target specificity against a complex environmental background. These findings demonstrate that *amoA*- and *nxrB*-targeted SYBR Green real-time PCR is a rapid and reliable tool for monitoring nitrification, supporting water-quality management and sustainable shrimp production in closed indoor systems. Absolute quantification against plasmid standard curves across the full culture cycle is in progress.

Keywords: *amoA* gene, Nitrifying bacteria, *nxrB* gene, Real-time PCR, Recirculating Aquaculture System (RAS)

Valorization of Sweet Sorghum Juice for Sustainable Protein Production Through Co-cultivation of Microalgae and Yeast Cells

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ABSTRACT: Microalgae are recognized as a highly promising feedstock due to their ability to synthesize a diverse array of high-value bioproducts, including lipids, proteins, carbohydrates, carotenoids, and other bioactive compounds. Especially, they represent an excellent alternative protein source, outperforming traditional crop and livestock systems in protein yield. This study aimed to evaluate the protein production of microalgae locally isolated from Si Than Lake (Khon Kaen University, Thailand). A single isolate, designated as *Chlorella* sp. SR-4, was previously recovered. In subsequent growth evaluation, mixotrophic cultivation of *Chlorella* sp. SR-4 outperformed heterotrophic cultivation a nearly five-fold increase, achieving a cell density of 7.83×10^6 cells/ml after 7 days of cultivation. Mixotrophic cultivation was selected to evaluate the growth and protein productivity of *Chlorella* sp. SR-4 using sweet sorghum juice as a carbon source. While a total sugar content of 5.0 g/l maximized biomass proliferation, a higher total sugar content of 15.0 g/l was required to enhance protein production, resulting in a high protein yield of 8.30 %(w/w). To further improve protein yield, a co-culture system pairing the isolated microalgae with the yeast *Saccharomyces cerevisiae* DBKKU-Y-53 at a 20:1 was detected. When grown in sweet sorghum juice at a total sugar content of 15.0 g/l, the co-culture achieved the higher protein yield of 32.60%(w/w), which was approximately four-fold more than yield of the monoculture. Based on these findings, this co-cultivation of microalgae and yeast strategy using sweet sorghum juice offers a highly viable approach to valorizing agricultural resources and producing high-value proteins.

Keywords: *Chlorella* sp., Co-cultivation, Protein production, *Saccharomyces cerevisiae*, Sweet sorghum juice

1. INTRODUCTION

Microalgae represent an attractive biological platform due to their unique nutritional characteristics, high photosynthetic efficiency, and capacity to thrive on marginal resources. Microalgae have garnered significant scientific and industrial interest due to their capacity to synthesize a wide range of valuable macromolecules, including lipids, carbohydrates, pigments, bioactive metabolites, and high-quality proteins [1,2]. Depending on the species and environmental conditions, microalgae can accumulate protein up to 50–70% of their dry cell weight, outperforming traditional protein crops like soybean (35–40%) and beef (20%) on a per-unit-area basis. Furthermore, microalgal proteins present an excellent, balanced essential amino acid profile that aligns closely with the joint standards of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) [3,4]. The microalgae market remains primarily restricted to high-value, low-volume niche products (e.g., astaxanthin, beta-carotene, and nutritional supplements) because the operating and capital expenditures of conventional autotrophic cultivation are relatively high. these physiological and economic limitations, researchers have explored alternative cultivation modes. Transitioning from photoautotrophic schemes to heterotrophic or mixotrophic regimes offers a promising avenue to dramatically boost cell densities and reduce overall harvesting costs. Mixotrophic cultivation

combines the advantages of both photoautotrophic and heterotrophic metabolisms. In a mixotrophic system, microalgal cells simultaneously utilize inorganic carbon via photosynthesis and organic carbon substrates (such as glucose, glycerol, or acetate) via aerobic respiration [5,6]. One highly promising agricultural resource is sweet sorghum (*Sorghum bicolor* (L.) Moench). Sweet sorghum is characterized by its rapid growth cycle, high drought tolerance, low fertilizer requirements, and adaptability to marginal lands. The stalks of sweet sorghum yield a sugar-rich juice contains high concentrations of fermentable sugars. This profile is supplemented by essential mineral ions, vitamins, and nitrogenous compounds, making sweet sorghum juice an ideal carbon substrate for low-cost, high-volume microalgal cultivation [7,8]. However, cultivating microalgae as a monoculture on raw agricultural juices poses technical challenges. Microalgal cells often lack the robust enzyme systems (such as extracellular invertases) required to efficiently hydrolyze complex sugars like sucrose into easily consumable monosaccharides [9,10]. This study aimed to evaluate the growth profiles and protein production of microalgae locally isolated from Si Than Lake (Khon Kaen University, Thailand) using sweet sorghum juice as a sole carbon source in heterotrophic and mixotrophic cultivation. Subsequently, co-cultivation of the microalgae and yeast cells was performed to examine the growth profiles and protein yield.

2. MATERIALS AND METHODS

2.1 Materials and microorganisms

Sweet sorghum juice, *Sorghum bicolor* (L.) Moench KKU-40, was obtained from the Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University (Khon Kaen, Thailand). To avoid storage issues, the juice was concentrated to total sugar content of 750 g/l and kept at -20 °C. Microalgal isolation from water samples collected from Si Than Lake (Khon Kaen University, Thailand) was conducted using Blue Green-11 (BG-11) medium supplemented with 2% (w/v) sucrose under incubation at 28 °C and a 16 h of light period with light intensity of 3,000 lux. Only one isolate, designated as *Chlorella* sp. SR-4, was previously obtained. The cells were then activated in 250 ml Erlenmeyer flasks containing 100 ml liquid BG-11 medium for 7 days. *S. cerevisiae* DB-KKU-Y-53 was kindly acquired from Associate Professor Pornthap Thanonkeo, Department of Biotechnology, Faculty of Technology, Khon Kaen University (Khon Kaen, Thailand). Yeast cells were cultured in yeast and malt extract medium. The cells were harvested by centrifugation in the late exponential phase of growth. The collected cells were washed in sterilized 0.9% (w/v) NaCl solution and resuspended in distilled water. The cell suspension was used in the subsequent experiments.

2.2 Heterotrophic and mixotrophic cultivation of the microalgae

Liquid BG-11 medium with 2% (w/v) sucrose was used in following experiments. Heterotrophic cultivation was incubated at 28 °C, at in an orbital shaker at 200 rpm under a dark environment. Mixotrophic cultivation was incubation at 28 °C, at in an orbital shaker at 200 rpm and a 16 h of light period with light intensity of 3,000 lux. The cultures were daily microscopic examined to monitor any contamination.

2.3 Mixotrophic cultivation of the microalgae using sweet sorghum juice

Sweet sorghum juice, with 5.0, 10.0 and 15.0 g/l of total sugar content, was utilized in experiment. Mixotrophic cultivation was incubation at 28 °C, at in an orbital shaker at 200 rpm and a 16 h of light period with light intensity of 3,000 lux. The cultures were daily microscopic examined to monitor any contamination.

2.4 Co-cultivation of the microalgae and yeast using sweet sorghum juice

Sweet sorghum juice, with 15.0 g/l of total sugar content, was utilized in experiment. Mixotrophic cultivation was incubation at 28 °C, at in an orbital shaker at 200 rpm and a 16 h of light period with light intensity of 3,000 lux. The initial cell counts were performed on pure cultures before co-cultivation. The initial ratios of microalgae and yeast set in co-culture experiments were 10:1, 20:1 and 30:1, respectively. The cultures were daily microscopic examined to monitor any contamination.

2.5 Analytical methods

During cultivation of the microalgae, cell growth was determined using a hemacytometer. Total sugar content of sweet sorghum juice was estimated utilizing the phenol-sulfuric acid method. Protein extraction of the samples was using alkaline solution, briefly, dried cells were grounded with mortar and incubated with 0.1 M NaOH solution at 90 °C for 1 h. Then, the mixture was centrifuged at 6000 rpm at 25 °C for 10 min. Protein content of the samples was examined using Lowry's method.

3. RESULTS AND DISCUSSION

3.1 Heterotrophic and mixotrophic cultivation of the microalgae

Chlorella sp. SR-4 was conducted heterotrophic and mixotrophic cultivation using BG-11 medium supplemented with 2% (w/v) sucrose. The results revealed that mixotrophic cultivation yielded a cell concentration of 7.83×10^6 cells/ml after 7 days of cultivation, which was substantially higher than that the achieved via heterotrophic cultivation (Figure 1). Mixotrophic cultivation combines the advantages of both autotrophic and heterotrophic metabolisms. In a mixotrophic system, microalgal cells simultaneously utilize carbon dioxide via photosynthesis and sucrose via aerobic respiration. This metabolic flexibility eliminates the light-limitation bottleneck typical of dense autotrophic cultures, as the cells can sustain growth using organic carbon even in poorly illuminated zones. Therefore, mixotrophic cultures routinely achieve biomass yields and growth rates that are significantly higher than those achieved under purely autotrophic or heterotrophic conditions [5,11].

3.2 Mixotrophic cultivation of the microalgae using sweet sorghum juice

Mixotrophic cultivation was selected to investigate the growth profile and protein production of *Chlorella* sp. SR-4 using sweet sorghum juice. The results showed that sweet sorghum juice with a total sugar concentration of 5.0 g/l was optimal for growth, resulting in a high cell concentration. However, for protein production, sweet sorghum juice with a total sugar concentration of 15.0 g/l demonstrated the higher protein content of 8.30 % (w/w) (Table 1). The higher cell concentration was achieved at a relatively low total sugar content of 5.0 g/l. This occurrence suggests that moderate organic carbon levels prevent the phenomena of substrate inhibition and osmotic stress, which are normally observed in mixotrophic cultivation of microalgae [5]. On the other hands, the higher protein content was obtained at a total sugar content of 15.0 g/l. This result can be attributed to the multi-nutrient profile of raw sweet sorghum juice. Rather than acting strictly as a carbon source, sweet sorghum juice naturally contains essential amino acids, vitamins, and minerals (including nitrogenous compounds, potassium, and magnesium) that scale proportionally with juice concentration. Direct dilution of the juice to low level of total sugar content concurrently dilutes these vital nitrogen sources and micronutrients, leading to nitrogen limitation [8,12].

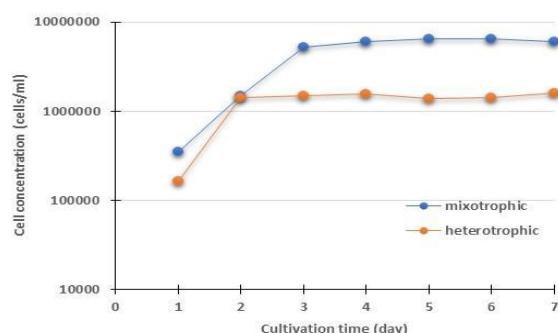


Figure 1. Growth profiles of *Chlorella* sp. SR-4 cells on BG-11 medium supplemented with 2% (w/v) sucrose through mixotrophic and heterotrophic cultivation

Table 1. Growth and protein production of *Chlorella* sp. SR-4 through mixotrophic cultivation using sweet sorghum juice

Initial total sugar content of sweet sorghum juice (g/l)	Cell concentration (cells/ml)	Specific growth rate (/day)	Yield of protein [% (w/w)]
5.0	2.83×10^6	0.87	2.05
10.0	2.03×10^6	0.82	7.77
15.0	1.81×10^6	0.58	8.30

3.3 Co-cultivation of the microalgae and yeast using sweet sorghum juice

The co-cultivation of *Chlorella* sp. SR-4 and *S. cerevisiae* DBKKU-Y-53 at initial ratios of 10:1, 20:1 and 30:1 using sweet sorghum juice with a total sugar content of 15.0 g/l was investigated. Initial ratios of the microalgae and the yeast cells at 20:1 exhibited the higher protein yield of 32.60 % (w/w), which was approximately four-fold more than yield of the monoculture (Figure 2). This dramatic enhancement in protein yield compared to the microalgal monoculture [8.30 % (w/w)] can be related to several cooperative mechanisms. The yeast cells naturally produce and secrete highly active extracellular invertase, which rapidly hydrolyze the sucrose in sweet sorghum juice into glucose and fructose monosaccharides. These easily metabolizable sugars are then readily assimilated by microalgae, facilitating enhanced amino acid biosynthetic pathways and resulting in a significantly elevated protein yield. In addition, the co-cultivation system establishes a mutually beneficial gas exchange. microalgal photosynthesis generates dissolved oxygen, which can accumulate to toxic levels and inhibit photosynthesis. During mixotrophic cultivation, yeast cells effectively consume the excess oxygen, mitigating oxidative stress on the microalgae. Concurrently, yeast respiration releases carbon dioxide, which is immediately captured and fixed by photosynthesis of the microalgae [13,14].

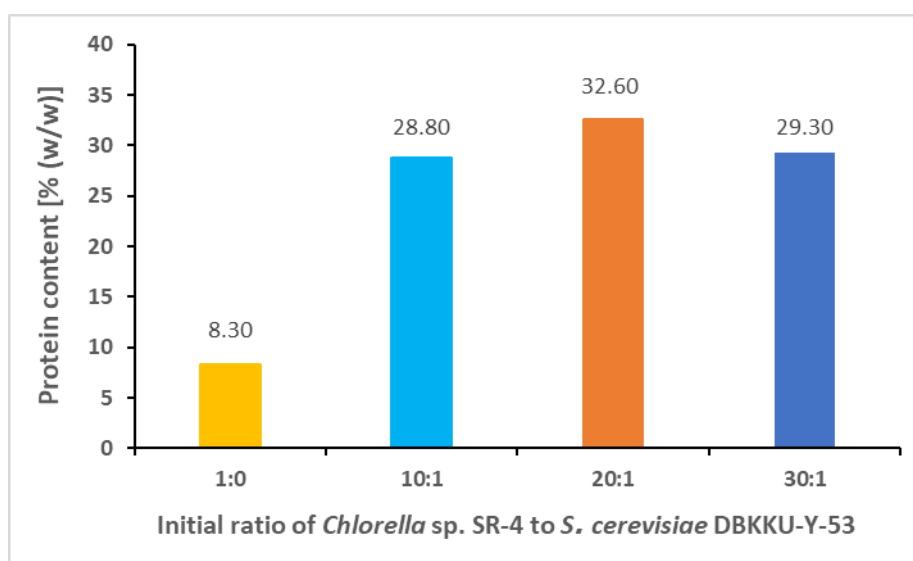


Figure 2. Yield of protein in cultivation and co-cultivation of *Chlorella* sp. SR-4 and *S. cerevisiae* DBKKU-Y-53 on sweet sorghum juice at a total sugar content of 15.0 g/l through mixotrophic cultivation

4. CONCLUSIONS

In this study, the co-cultivation of *Chlorella* sp. SR-4 with *S. cerevisiae* DBKKU-Y-53 at a 20:1 was detected as the optimal mutual benefit. When grown in sweet sorghum juice at a total sugar content of 15.0 g/l, the co-culture achieved the higher protein yield of 32.60%(w/w), which was almost four-fold more than yield of the monoculture. This co-cultivation of microalgae and yeast strategy offers a promising approach to valorizing agricultural resources and producing high-value bioproducts.

5. ACKNOWLEDGEMENT

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Effect of Maltose Concentration on the Production of Long-Chain Isomaltooligosaccharides by *Bacillus subtilis* strain AP-1

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ABSTRACT: This study investigated the effect of initial maltose concentration (50–200 g/L) as a carbon source on the production of a series of long-chain isomaltooligosaccharides (IMOs) without glucose and maltose contamination, using whole cells of *Bacillus subtilis* strain AP-1. Fermentations were conducted in Berg's mineral salt medium under aerobic conditions at 37 °C and 200 rpm. IMO profiles over time were monitored via thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), while final product yields were quantified gravimetrically. An evaluation of process efficiency revealed that complete depletion of residual glucose was achieved at lower initial maltose levels of 50 and 80 g/L within 36 h (1.5 days) and 54 h (2.25 days), yielding 36.33 and 58.60 g/L of IMOs, respectively. Although increasing the initial maltose loading from 80 to 200 g/L successfully achieved the highest IMOs concentration of 157.67 ± 0.08 g/L, the required cultivation time was substantially prolonged to 17 days. Consequently, the optimal productivity of 26.04 ± 0.04 g/L/day was achieved at 80 g/L of maltose, whereas higher maltose loadings (100–200 g/L) led to a marked decrease in productivity, ranging from 8.22 ± 0.01 to 13.95 ± 0.03 g/L/day. Under the selected operating condition, HPLC analysis confirmed no detectable residual glucose and maltose, with the oligomeric series spanning at least DP10. These findings demonstrate that an initial maltose concentration of 80 g/L represents an optimal operating condition, providing high productivity with a relatively short cultivation time and serving as a promising baseline for further process development and scale-up studies.

Keywords: *Bacillus subtilis*, Isomaltooligosaccharides, Maltose, Prebiotic

1. INTRODUCTION

Driven by rising consumer demand for therapeutic and preventative nutrition, food science research has increasingly focused on integrating natural functional ingredients into dietary products. Among these, naturally derived oligosaccharides-complex carbohydrates comprising 3 to 10 monosaccharide units have emerged as key targets for development due to their potential to mitigate chronic disease risk and support metabolic health [1]. These compounds serve as critical industrial food additives, functioning as low-calorie, low-glycemic-index sweeteners that enhance both the sensory attributes and physicochemical properties of diverse food matrices, including functional beverages, dairy products, and baked goods. Mechanistically, oligosaccharides exert prebiotic effects by resisting enzymatic hydrolysis in the upper gastrointestinal tract and reaching the colon intact. Within the large intestine, they undergo selective fermentation by saccharolytic microbiota, specifically stimulating the proliferation of beneficial taxa such as *Bifidobacterium*

and *Lactobacillus*. Consequently, this metabolic activity suppresses the colonization of enteric pathogens, including *Escherichia coli*, *Clostridium perfringens*, *Coliforms*, and *Salmonella spp.* [2]. Prominent types of these functional carbohydrates include galactooligosaccharides (GOS), fructooligosaccharides (FOS), mannanoligosaccharides (MOS), and isomaltoligosaccharides (IMOs) [3].

Isomaltoligosaccharides (IMOs) are a class of functional carbohydrates composed of D-glucose subunits linked primarily via α -(1-6)-glycosidic bonds. This structural group predominantly comprises isomaltose, isomaltotriose, panose, isomaltotetraose, and isomaltopentose. Due to their inherent resistance to endogenous enzymatic hydrolysis, IMOs have emerged as major targets for food industry applications [4]. Mechanistically, their non-digestible nature renders them low-calorie, low-glycemic sweeteners that do not elicit a marked postprandial insulinemic response. Consequently, these prebiotic sugars are ideally suited as functional ingredients in specialized dietary formulations designed for weight management and diabetic populations. In addition, IMOs promote the proliferation of health-promoting gut microbiota, thereby enhancing human well-being. They also possess desirable physicochemical properties, such as low viscosity and high stability under both acidic conditions [5] and the elevated temperatures typical of food processing. Consequently, they are widely utilized as functional ingredients in the formulation of health-promoting foods and beverages. Commercially available IMOs predominantly consist of short-chain oligomers with a degree of polymerization (DP) spanning 2 to 6 glucose units, such as isomaltose, isomaltotriose, panose, and isomaltotetraose. A critical limitation of these short-chain variants, however, is their susceptibility to partial enzymatic hydrolysis and subsequent absorption in the upper gastrointestinal tract, which severely attenuates their prebiotic efficacy downstream. Furthermore, commercial IMO preparations frequently retain substantial residual concentrations of glucose and other unintended mono- or disaccharides. These digestible impurities elevate postprandial blood glucose levels [5], thereby compromising the product's functional efficacy and market value. Conversely, long-chain IMOs exhibit superior resistance to upper gastrointestinal digestion, demonstrating enhanced efficacy in selectively promoting probiotic proliferation and imparting systemic health benefits. Conventionally, industrial IMO synthesis relies on a direct enzymatic strategy to biotransform maltose into branched oligomers. This process is driven by α -glucosidase (E.C. 3.2.1.20), which exhibits prominent transglucosylation activity [6]. Mechanistically, this glycoside hydrolase initiates the reaction by cleaving the terminal α -(1-4)-glucosidic bonds at the non-reducing ends of maltose and maltooligosaccharides, forming transient glucosyl-enzyme intermediates. Under elevated substrate concentrations, the enzyme shifts toward its transferase activity, catalyzing transglucosylation to synthesize IMOs via the 6-OH position. The structural elongation and chain length of the resulting oligosaccharides depend heavily on the selectivity and microbial source of the specific α -glucosidase. However, utilizing commercial enzymes is economically constrained at an industrial scale due to high market costs. To overcome this limitation, the current approach utilizes *in situ* enzymes produced during microbial growth to convert maltose into IMOs within a single cultivation stage. This whole-cell biocatalytic system drastically lowers production costs by bypassing separate enzyme generation, recovery, and purification steps, thereby improving both time efficiency and overall IMOs productivity.

In a previous report, *B. subtilis* strain AP-1, isolated from Thai fermented food demonstrated a unique capability to biotransform maltose into a series of long-chain IMOs ranging from DP 2 to 14 [7]. Under direct fermentation conditions, an IMOs yield of 36.33 g/L was achieved within 36 h of cultivation, representing a high bioconversion efficiency of 72.7%. Importantly, this optimized process yielded long-chain IMOs without residual contamination from simple glucose or maltose monomers [8]. Based on these findings, the current study investigates the effect of initial maltose concentration to further enhance long-chain IMOs productivity by *B. subtilis* strain AP-1, providing a technical baseline for scaling up this bioprocess toward industrial applications.

2. MATERIALS AND METHODS

2.1. Materials

Isomaltose and isomaltotriose were purchased from Megazyme Ltd. (Bray, Ireland). Yeast extract and peptone were obtained from HiMedia Laboratories (Mumbai, India). Maltose was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals, media, and media components were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Microorganisms, media, and culture conditions

B. subtilis strain AP-1 was isolated and identified using a previously described method [7]. The strain was grown at 37 °C under aerobic conditions on nutrient agar (NA) containing (per liter): 5 g peptone, 5 g NaCl, 1.5 g yeast extract, 1.5 g beef extract, and 20 g agar. To prepare the inoculum, a single colony was transferred into sterile nutrient broth (NB) and incubated at 37 °C with agitation at 200 rpm for 24 h. Cultivation was performed until the optical density at 600 nm (OD₆₀₀) reached approximately 0.6 (equivalent to 10⁸ CFU/mL), after which it was used in subsequent fermentation.

2.3 Investigation of the optimal maltose concentration for IMOs production by *B. subtilis* strain AP-1.

Fermentation was initiated by inoculating a 10% (v/v) culture of *B. subtilis* strain AP-1 into 100 mL of Berg's mineral salt (MS) medium [8] supplemented with maltose at varying concentrations (50, 80, 100, 120, 150, 180, and 200 g/L). Cultivation was maintained at 37 °C and 200 rpm until glucose was completely depleted from the system. Aliquots (0.5 mL) were sampled periodically and centrifuged (10,000 × g, 10 min) to obtain cell-free supernatants. Oligosaccharide profiles were monitored via thin-layer chromatography (TLC) [8] and high-performance liquid chromatography (HPLC) utilizing a Shimadzu RID-10A refractive index detector and an Aminex HPX-42A column (Bio-Rad, Hercules, CA, USA) using HPLC-grade water (Fisher Scientific, UK) as the mobile phase at 85 °C and a flow rate of 0.6 mL/min. For productivity calculations, the supernatant obtained under optimal conditions was harvested by centrifugation, frozen, and lyophilized using a laboratory freeze dryer (FD5-T-Series; SIM International Group Co. Ltd., Newark, DE, USA), and the resulting dried oligosaccharides were gravimetrically quantified.

3. RESULTS AND DISCUSSION

The effect of maltose concentration on IMOs production by *B. subtilis* strain AP-1 was monitored across a range of initial substrate concentrations (50–200 g/L) using thin-layer chromatography (TLC) (Figure 1), quantified gravimetrically via the lyophilized dried oligosaccharides (Table 1), and further characterized by HPLC (Figure 2). Results indicated that *B. subtilis* strain AP-1 successfully synthesized long-chain IMOs with a DP of up to 4 within 6 h of cultivation, across all maltose concentrations. This production was catalyzed by the extracellular α -glucosidase secreted by *B. subtilis* strain AP-1, which catalyzed the liberation of glucose from maltose and subsequently mediated transglucosylation by transferring a glucosyl moiety from the glucosyl-enzyme intermediate to the 6-OH position of available sugar acceptors, thereby forming α -1,6-linked disaccharides, trisaccharides, and higher oligosaccharides [8].

The initial maltose loading significantly affected both IMOs productivity and overall process efficiency. At lower initial maltose concentrations (50 and 80 g/L), rapid substrate utilization occurred,

leading to an increase in the length of synthesized oligosaccharides toward higher molecular weight chain structures. As visualized in the TLC chromatograms, glucose spots completely disappeared within 36 h (1.5 days) for 50 g/L of maltose and 54 h (2.25 days) for 80 g/L of maltose, respectively (Figure 1a, 1b). Conversely, increasing the initial maltose concentration from 100 to 200 g/L led to a substantial prolongation of the fermentation period. Specifically, the time required for complete glucose depletion extended significantly from 7 days (at 100 and 120 g/L), 9 days (at 150 g/L), and up to 17 days (at 180-200 g/L). These delayed glucose consumption dynamics were clearly captured by the persistent and dense glucose spots on the TLC plates at higher substrate loadings, which slowly faded over extended cultivation periods (Figure 1).

Although higher initial maltose concentrations successfully enhanced IMOs production, achieving a maximum concentration of 157.67 ± 0.08 g/L at 200 g/L of maltose, the extensively prolonged fermentation duration ultimately reduced overall bioprocess efficiency. Consequently, the productivity declined sharply at increased maltose loadings. For instance, increasing the maltose concentration from 80 to 100 g/L caused the productivity to drop drastically from 26.04 ± 0.04 g/L/day to 10.12 ± 0.01 g/L/day. The lowest productivity (8.22 ± 0.01 g/L/day) was recorded at 180 g/L of maltose due to the prolonged incubation period of 17 days. Thus, the higher final IMOs concentration obtained at 200 g/L was accompanied by a substantially longer cultivation time and considerably lower process productivity. These results demonstrate that maximizing product concentration alone may not represent the most efficient operating strategy for IMO production.

This inverse relationship between initial substrate concentration and productivity may be associated with slower glucose depletion and several possible biochemical and physicochemical limitations. One possible explanation is carbon catabolite repression (CCR), whereby glucose generated from maltose hydrolysis may influence carbohydrate metabolism and contribute to slower substrate utilization [9]. High substrate concentrations may also impose increased osmotic stress, potentially redirecting cellular resources toward maintenance rather than substrate utilization [10]. Moreover, elevated sugar and oligosaccharide concentrations could increase broth viscosity and potentially limit oxygen transfer, particularly during the later stages of cultivation, thereby affecting aerobic glucose consumption [11]. However, these mechanisms were not directly investigated in the present study and should therefore be regarded as possible explanations rather than confirmed mechanisms. Further investigation would be required to clarify their roles in substrate utilization and productivity.

Considering both product concentration and process productivity, an initial maltose concentration of 80 g/L was selected as the optimal operating condition. Although 200 g/L maltose produced the highest final IMOs concentration, the substantially longer cultivation time resulted in considerably lower productivity. In contrast, 80 g/L maltose provided a high productivity of 26.04 ± 0.04 g/L/day while requiring a much shorter cultivation period, thereby providing a more efficient operating condition for IMO production. This balance between product formation and cultivation time may be favorable for future industrial scale-up and economic evaluation. The resulting profile of synthesized IMOs and the absence of detectable residual glucose and maltose under this condition were further confirmed by HPLC analysis of dried oligosaccharides (Figure 2). The chromatogram showed no residual glucose and maltose peaks and revealed IMO species ranging from DP2 to at least DP10 (Figure 2a), corresponding well with the IMO standards (Figure 2b). Previous MALDI-TOF-MS analyses of these products further suggested the presence of long-chain IMOs extending up to DP14 [8], which may not be fully resolved by HPLC due to limitations in the separation and detection of highly polymerized oligosaccharides, particularly those present at low abundance or exhibiting overlapping retention profiles. Therefore, the synthesized IMOs mixture may contain higher-DP oligosaccharides up to DP14 that were not fully detectable by the current HPLC method.

The production performance of *B. subtilis* AP-1 can also be compared with previously reported microbial IMO production systems. A closely related study using *B. subtilis* SS-76 reported the production of the short-chain IMO panose (DP3) from 2% (w/v) maltose after 72 h of cultivation [12]. In comparison, the present *B. subtilis* AP-1 system produced an IMO profile ranging from DP2 to at least DP10 within 54 h using 8% (w/v) maltose. In addition, cell-bound α -glucosidase from *Microbacterium* sp. was used for IMO production, achieving 85 g/L after 24 h using a higher initial maltose concentration of 40% (w/v), with products ranging from DP3 to DP6, along with residual glucose and maltose in the final product [13]. Similarly, *Debaryomyces hansenii* SCY204 was used for continuous IMO synthesis from 30% (w/v) maltose, followed by selective fermentation with *Saccharomyces cerevisiae* to remove glucose and obtain IMOs with >91% purity [14]. Engineered *S. cerevisiae* expressing *Aspergillus niger* α -glucosidase has also been reported to produce mainly short-chain IMOs, with isomaltose becoming predominant after 24 h of incubation with maltose [15]. Compared with these reported microbial systems, the present *B. subtilis* AP-1 system achieved long-chain IMO production using a lower maltose concentration than the *Microbacterium* sp. and *D. hansenii* systems, while no detectable residual glucose and maltose were observed under the selected operating condition. Moreover, AP-1 achieved this product profile without requiring an additional selective fermentation step for residual sugar removal. These characteristics demonstrate the potential of AP-1 as a microbial platform for efficient long-chain IMO production and provide a basis for further process development and scale-up.

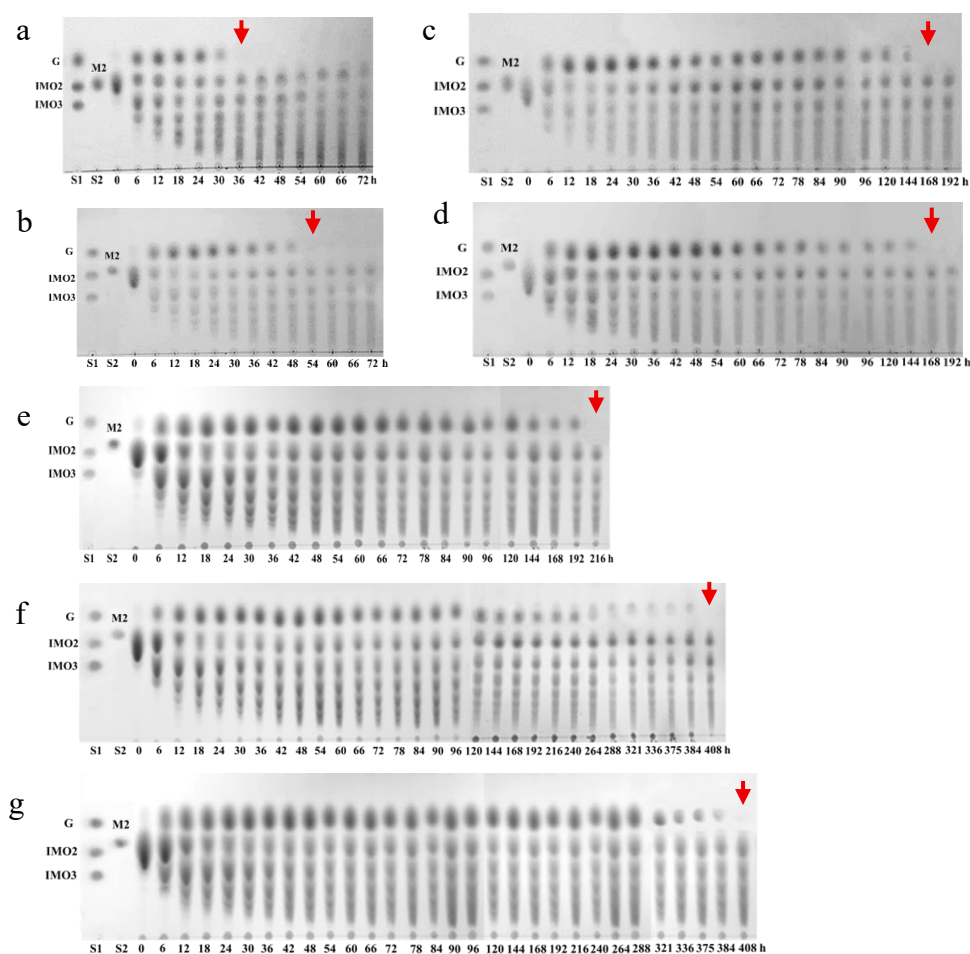


Figure 1. TLC chromatogram of IMOs production profiles over time using various initial maltose concentrations: (a) 50 g/L, (b) 80 g/L, (c) 100 g/L, (d) 120 g/L, (e) 150 g/L, (f) 180 g/L, and (g) 200 g/L. (Lane S1: glucose (G), isomaltose (IMO2) and isomaltotriose (IMO3) standard; lane S2: maltose (M2) standard).

Table 1. Effect of maltose concentration on the productivity of IMOs produced by *B. subtilis* AP-1.

Maltose concentration (g/L)	Production time (days)	IMOs concentration (g/L)	Productivity (g/L/day)
50	1.5	36.33 ± 0.09	24.24 ± 0.06
80	2.25	58.60 ± 0.08	26.04 ± 0.04
100	7	70.87 ± 0.09	10.12 ± 0.01
120	7	97.50 ± 0.05	13.95 ± 0.03
150	9	122.43 ± 0.05	13.60 ± 0.02
180	17	139.67 ± 0.05	8.22 ± 0.01
200	17	157.67 ± 0.08	9.27 ± 0.01

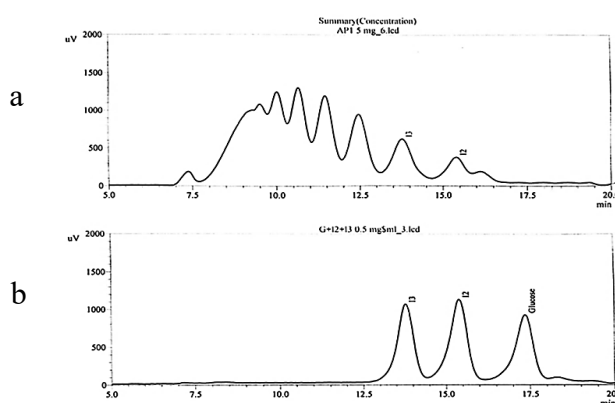


Figure 2. HPLC chromatograms of the synthesized IMOs: (a) profile at the optimal condition (80 g/L maltose), showing a distinct distribution of long-chain IMOs from DP2 to DP10 without residual glucose; (b) reference standards including glucose, isomaltose (IMO2), and isomaltotriose (IMO3).

4. CONCLUSIONS

In conclusion, this study demonstrates that the initial maltose concentration significantly affects both IMOs production and process efficiency during long-chain IMO synthesis by *B. subtilis* strain AP-1. While increasing the maltose concentration to 200 g/L resulted in the highest IMOs concentration of 157.67 g/L, the required cultivation time was substantially prolonged to 17 days, leading to considerably lower productivity. In contrast, 80 g/L maltose provided a favorable balance between IMOs concentration and cultivation time, achieving an IMOs concentration of 58.60 g/L and the highest productivity of 26.04 ± 0.04 g/L/day within only 2.25 days. HPLC analysis further confirmed the absence of detectable residual glucose and maltose, with the resulting IMO profile extending to at least DP10. Overall, 80 g/L maltose was identified as the optimal operating condition, providing an efficient process for the production of long-chain IMOs by *B. subtilis* AP-1 and demonstrating potential for further scale-up and process development.

5. ACKNOWLEDGEMENT

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Production of Mannan-oligosaccharides from Soybean Residues by Probiotic *Bacillus tequilensis* PLM1

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ABSTRACT: Soybean residue is an abundant and low-cost by-product generated from the soy sauce industry. It contains high amounts of proteins and carbohydrates, making it a promising substrate for the production of bioactive oligosaccharides. Mannan-oligosaccharides (MOSs) have attracted considerable attention due to their prebiotic properties and their applications in functional foods, dietary supplements, and pharmaceutical products. However, the complex structure of soybean residue limits its biodegradation by common microorganisms and results in heterogeneous oligosaccharide products. Therefore, this study aimed to investigate the potential of enzymes produced by *Bacillus tequilensis* strain PLM1 for the bioconversion of soybean residue into oligosaccharides. Crude enzyme extracts obtained from cultivation in mineral salt medium (MS) supplemented with soybean residue as a carbon source at 37°C exhibited the highest mannanase activity (568.72 ± 10.85 U/g protein), followed by pectinase, xylanase, and cellulase activities. High-performance liquid chromatography (HPLC) analysis revealed that increasing soybean residue concentrations to 5, 8, and 10 (w/v) enhanced MOSs with a degree of polymerization (DP) of 4–5 were detected. These results demonstrated the capability of *B. tequilensis* PLM1 enzymes to hydrolyze hemicellulose in soybean residue for oligosaccharide production. Furthermore, *B. tequilensis* PLM1 exhibited a high survival rate under simulated gastrointestinal conditions ($95.00 \pm 0.08\%$), indicating its probiotic potential. Owing to its efficient soybean residue-degrading capability, the strain effectively hydrolyzed cell wall polysaccharides and mannan, enabling the production of MOSs. Collectively, these results demonstrate the feasibility of using *B. tequilensis* PLM1 as a promising biocatalyst for the valorization of soybean residue and the co-production of probiotic bacteria and prebiotic MOSs. This study provides a sustainable strategy for converting soybean residue into value-added prebiotic oligosaccharides with potential applications in functional foods and health-related products. Further optimization of the hydrolysis process and biological validation of the produced MOSs are required to support their practical applications.

Keywords: *Bacillus tequilensis*, Mannan-oligosaccharides, Prebiotics, Soybean residue

1. INTRODUCTION

Mannan-oligosaccharides (MOSs) are bioactive oligosaccharides receiving increasing attention because of their prebiotic properties and health-promoting effects. Oligosaccharides are low-molecular-weight carbohydrates with degrees of polymerization (DP) ranging from 3 to 10 [8]. They can be produced from various biological sources, including bacteria, fungi, algae, and plants, and are widely used in functional foods, dietary supplements, and pharmaceutical applications. In addition to functioning as dietary fiber,

sweeteners, and humectants, oligosaccharides promote the growth of beneficial gut microbiota while inhibiting pathogenic bacteria. They also exhibit antioxidant activity, enhance mineral absorption, stimulate immune responses, prevent dental caries, and may help regulate blood glucose and lipid levels.

The soybean-processing industry generates large amounts of agro-industrial waste, particularly soybean cooking water and soybean residue, which are rich in proteins, cellulose, hemicellulose, and pectic substances. These by-products are therefore considered promising low-cost substrates for oligosaccharide production. Conventional production methods mainly rely on chemical hydrolysis, which requires harsh conditions and may generate toxic by-products and chemical waste. Consequently, enzymatic hydrolysis using microbial enzymes has emerged as a safer and more environmentally friendly alternative [2, 3, 6]. Hemicellulolytic enzymes, especially mannanases, can efficiently hydrolyze complex polysaccharides into bioactive short-chain oligosaccharides under mild conditions with high substrate specificity and minimal formation of undesirable compounds such as furfural and hydroxymethylfurfural. Thus, microbial enzymatic conversion represents a sustainable approach for producing functional oligosaccharides from agricultural biomass.

In Thailand, substantial quantities of soybean residue are generated annually from tofu and soybean-product industries. Valorization of these residues not only supports waste utilization and circular bioeconomy concepts but also provides an alternative substrate for MOSs production. However, the complex structure of soybean biomass limits efficient degradation, highlighting the need for effective microbial strains and enzymes.

In this study, *B. tequilensis* PLM1, isolated from Miang (pickled tea), was investigated for its potential to produce oligosaccharides from soybean residue. This Biosafety Level 1 strain produces hemicellulolytic enzymes, including xylanase, pectinase, and mannanase, which can serve as a biological pretreatment alternative to conventional chemical methods. By specifically targeting the structural polysaccharides of the substrate, these enzymes facilitate biomass deconstruction and simultaneously generate prebiotic MOSs through a single-step bioconversion process. Therefore, this study aimed to evaluate the potential of *B. tequilensis* PLM1 for the bioconversion of soybean residue into value-added MOSs for future biotechnological and industrial applications.

2. MATERIALS AND METHODS

2.1 Microorganisms and Culture

B. tequilensis strain PLM1 was cultivated on MS medium containing 1% (w/v) soybean residue incubated at pH 6.0 and 37°C for 24 h. Enzyme activities of cellulase, mannanase, xylanase, and pectinase were determined using corresponding substrates.

2.2 Production of the Cellulolytic and Hemicellulolytic Enzyme of *B. tequilensis* PLM1

The production of cellulolytic and hemicellulolytic enzymes was evaluated using modified methods described by [4]. The bacteria were grown in agar plates containing 5% w/v of different substrates such as galactomannan from locust bean gum for mannanase, pectin from orange peel for pectinase, xylan from corn core for xylanase, and carboxymethyl cellulose for cellulase, incubated at 37°C 48 h. After incubation, the plates were flooded with 0.5% (w/v) Congo red solution for staining and subsequently treated with 0.5% (v/v) sulfuric acid for color fixation and destaining. The formation of clear zones surrounding the colonies was observed and measured as an indication of polysaccharide-degrading enzyme activity.

2.3 Optimization of Soybean Residue Concentration for Oligosaccharide Production

The optimal concentration of soybean residue for oligosaccharide production was investigated using *B. tequilensis* PLM1 cultivated in liquid MS medium supplemented with soybean residue at concentrations of 1, 2, 5, 8, and 10% (w/v). The initial inoculum concentration of *B. tequilensis* PLM1 was adjusted to 10⁶ CFU/mL before inoculation into MS medium. The cultures were prepared at a working volume of 100 mL in 500 mL Erlenmeyer flasks and incubated at pH 6.0 and 37°C for 24 h. Control samples consisted of sterile MS medium supplemented with the same concentrations of soybean residue without bacterial inoculation. After incubation, the cultures were centrifuged at 10,000 ×g to separate the bacterial cells from the culture supernatants. The oligosaccharides in the supernatants were subsequently analyzed using HPLC. Separation was performed using an Aminex® HPX-87H column (300 × 7.8 mm), and detection was carried out using a flame ionization detector (FID) with nitrogen as the carrier gas.

2.4 Evaluation of Probiotic Survival under Simulated Gastrointestinal Conditions

The survival of *B. tequilensis* PLM1 in fermented soybean residue was evaluated using a modified static in vitro gastrointestinal digestion model based on the INFOGEST protocol of Minekus et al. (2014) [5]. Freshly fermented soybean residue containing viable *B. tequilensis* PLM1 and soybean-derived MOSs was subjected sequentially to simulated oral (pH 7.0, α-amylase, 37°C, 2 min), gastric (pH 3.0, pepsin, 37°C, 3 h), and intestinal (pH 8.0, pancreatin and bile salts, 37°C, 8 h) conditions. Viable cell counts were determined before and after digestion by serial dilution and spread plating on nutrient agar, followed by incubation at 37°C for 24 h. The survival rate was calculated as:

$$\text{Survival rate (\%)} = (N_t/N_0) \times 100$$

where N_0 and N_t represent the viable cell counts before and after simulated gastrointestinal digestion, respectively.

3. RESULTS AND DISCUSSION

3.1 Plant Cell Wall Degrading Enzyme Production by *B. tequilensis* PLM1

The ability of *B. tequilensis* PLM1 to produce cellulolytic and hemicellulolytic enzymes was evaluated using agar media supplemented with different polysaccharides as sole carbon sources. Carboxymethyl cellulose (CMC), galactomannan, pectin, and xylan (0.5%, w/v) were used to screen for cellulase, mannanase, pectinase, and xylanase production, respectively. After incubation at 37°C for 48 h, *B. tequilensis* PLM1 grew on all four polysaccharide-containing media. The results indicated that *B. tequilensis* PLM1 produced mannanase, pectinase, and cellulase. Although no clear zone indicative of xylanase activity was observed on xylan agar plates (Figure 1.), xylanase activity was detected during subsequent submerged fermentation using soybean substrate. This discrepancy may be attributed to the lower sensitivity of the agar plate screening method for detecting low levels of enzyme activity, as well as differences in enzyme induction under submerged fermentation conditions, where soybean substrate may provide more effective inducers for xylanase production. The largest clear zone was observed for pectinase (5.00 mm), followed by cellulase (1.26 mm) and mannanase (1.12 mm), respectively.

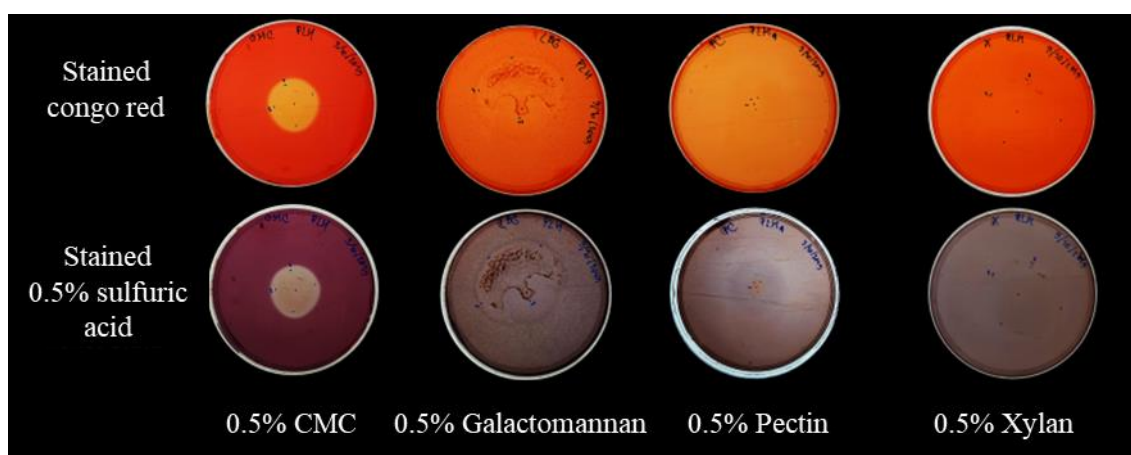


Figure 1. The production of mannanase, pectinase and cellulase of *B. tequilensis* PLM1 on a culture plate containing various types of hemicellulose after incubation at 37 °C for 48 h.

3.2 Specific Activities of Cellulolytic and Hemicellulolytic Enzymes Produced by *B. tequilensis* PLM1 Cultivated on Soybean Residue

The enzyme production by *B. tequilensis* PLM1 was evaluated in MS medium supplemented with 1% (w/v) soybean residue as the sole carbon source. The culture was incubated at pH 6.0 and 37°C for 24 h. Crude enzyme extracts were subsequently assayed for cellulase, mannanase, xylanase, and pectinase activities at 37°C using their respective substrates. As shown in Table 1, *B. tequilensis* PLM1 produced all four enzymes when cultivated on soybean residue. Among the enzymes tested, mannanase exhibited the highest specific activity (568.72 ± 10.85 U/g protein) when locust bean gum was used as the substrate. This was followed by pectinase (225.08 ± 8.95 U/g protein), xylanase (65.42 ± 4.27 U/g protein), and cellulase (47.14 ± 1.55 U/g protein), respectively.

The remarkably high mannanase activity may be attributed to the presence of mannan-containing polysaccharides in soybean residue, which can act as inducers for mannanase synthesis. Similar observations were reported by [1], who demonstrated that cultivation of *Bacillus* spp. in media containing mannan-rich substrates significantly enhanced mannanase production and enzyme activity.

Table 1. Specific enzyme activities of crude enzymes produced by *B. tequilensis* PLM1

Enzyme	Substrate	Specific activity (U/g protein)
Cellulase	Phosphoric acid swollen cellulose (PASC)	47.14 ± 1.55
Mannanase	Locust bean gum	568.72 ± 10.85
Xylanase	Beechwood xylan	65.42 ± 4.27
Pectinase	Citrus pectin	225.08 ± 8.95

3.3 Optimization of Soybean Residue Concentration for MOSs Production

The effect of soybean residue concentration on MOSs production by *B. tequilensis* PLM1 was investigated using MS medium supplemented with 1, 2, 5, 8 and 10% (w/v) soybean residue. After 24 h of cultivation at pH 6.0 and 37°C, the fermentation products were analyzed by HPLC. The oligosaccharide profile was strongly influenced by soybean residue concentration (Table 2.). At low substrate concentrations (1–2%, w/v), only mannotetraose (M4) was detected. Increasing the soybean residue concentration to 5–10% (w/v) resulted in the production of both mannotetraose (M4) and mannopentaose (M5), with M4 being the predominant product. The concentrations of M4 and M5 increased progressively with increasing soybean residue concentration and reached their highest levels at 10% (w/v).

In addition pectin-derived sugars as galacturonic acid (GalA), rhamnose, and arabinose were detected at soybean residue concentrations of 5–10% (w/v). These monosaccharides were likely released through the bacterial production of polysaccharide-degrading enzymes, including pectinases and xylanases. Their accumulation indicates the degradation of pectin and other hemicellulosic components, such as xylan and mannan, present in soybean residue. The monosaccharide concentrations increased markedly with increasing soybean residue concentration.

Among the tested conditions, 10% (w/v) soybean residue yielded the highest concentrations of both MOSs and pectin-derived sugars, indicating that this concentration was the most suitable for oligosaccharide production by *B. tequilensis* PLM1. The predominance of M4 and the absence of mannohexaose (M6) further suggest that the mannanase produced by *B. tequilensis* PLM1 preferentially generates medium-chain MOSs, particularly M4, through an endo-cleavage mechanism.

Table 2. Fermented products generated from soybean residue by *B. tequilensis* PLM1 cultivated in MS medium containing different concentrations of soybean residue and analyzed by HPLC.

Concentrations of soybean residue (%, W/V)	Oligosaccharide products (g/L)									Total products (g/L)	Yield (g/g substrate)	Yield (%)
	Rham nose	Arabi nose	GalA ^g	M6 ^a	M5 ^b	M4 ^c	M3 ^d	M2 ^e	M1 ^f			
1.0	0.259	-	-	-	-	0.253	-	0.020	-	0.532	0.0532	5.32
2.0	0.718	0.801	-	-	-	0.502	-	-	-	2.021	0.1011	10.11
5.0	1.712	1.825	13.533	-	0.147	1.049	-	-	-	18.266	0.3653	36.53
8.0	2.794	2.94	25.277	-	0.208	1.765	-	-	-	31.219	0.3902	39.02
10.0	3.661	3.833	33.153	-	0.239	2.197	-	-	-	42.883	0.4288	42.88

^aM6 = mannohexaose, ^bM5 = mannopentaose, ^cM4 = mannotetraose, ^dM3 = mannotriose, ^eM2 = mannobiose, ^fM1 = mannose,
^gGalA = Galacturonic acid

The results showed that increasing the soybean residue concentration from 1% to 10% (w/v) significantly enhanced MOSs production by *B. tequilensis* PLM1, with total MOSs levels increasing from 0.3 to 2.4 g/L (Figure 2.). M4 was the predominant MOSs produced, whereas M5 was detected only at substrate concentrations of 5% (w/v) and above. In addition, GalA, rhamnose, and arabinose were detected at soybean residue concentrations of 5–10% (w/v), indicating the concurrent hydrolysis of pectin and the formation of pectin-derived sugars during fermentation. These findings suggest that higher soybean residue concentrations provide more accessible polysaccharides for enzymatic hydrolysis, thereby

enhancing the production of both MOSs and pectin-derived sugars. Furthermore, the conversion yield, calculated as the total concentration of HPLC-detected products per gram of soybean residue supplied, increased progressively from 5.32% at 1% soybean residue to 42.88% at 10% soybean residue (Table 2.). No reduction in conversion yield was observed at the highest substrate concentration tested, suggesting that substrate inhibition or mass transfer limitations were not significant under the experimental conditions.

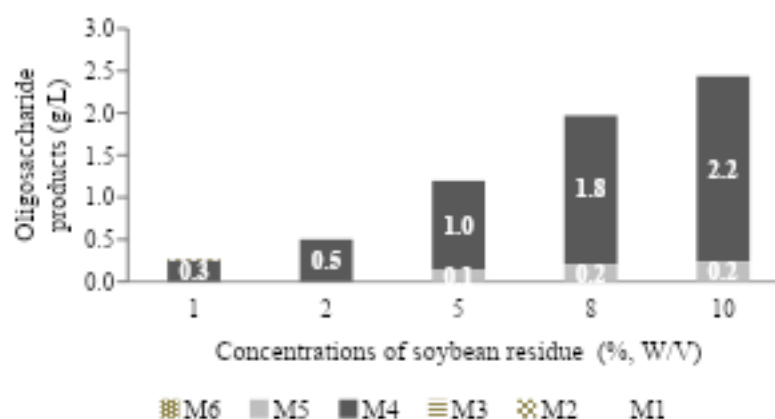


Figure 2. Effect of soybean residue concentration on MOSs production by *B. tequilensis* PLM1.

3.4 Evaluation of Probiotic Survival under Simulated Gastrointestinal Conditions

In addition to promoting MOSs production, fermented soybean residue supported the survival of the probiotic bacterium *B. tequilensis* PLM1 during gastrointestinal digestion. Probiotic survival was evaluated using an in vitro digestion model comprising sequential oral, gastric, and intestinal phases. As shown in Figure 3., *B. tequilensis* PLM1 retained $95.0 \pm 0.08\%$ of its initial viable cell population after sequential exposure to all digestion stages.

The high survival rate under acidic gastric conditions (pH 3.0) and subsequent intestinal digestion may be attributed to the ability of *B. tequilensis* PLM1 to form endospores, which confer resistance to acidic environments, digestive enzymes, and bile salts. These findings demonstrate that *B. tequilensis* PLM1 possesses excellent gastrointestinal tolerance and therefore has potential as a probiotic candidate capable of surviving gastrointestinal transit.

Because *B. tequilensis* PLM1 can simultaneously produce soybean-derived MOSs during fermentation, the fermented product may provide both probiotic bacteria and prebiotic oligosaccharides, suggesting its potential as a synbiotic product. Nevertheless, further investigations using more representative gastrointestinal models and in vivo studies are required to confirm its probiotic functionality and the health benefits of the synbiotic formulation.

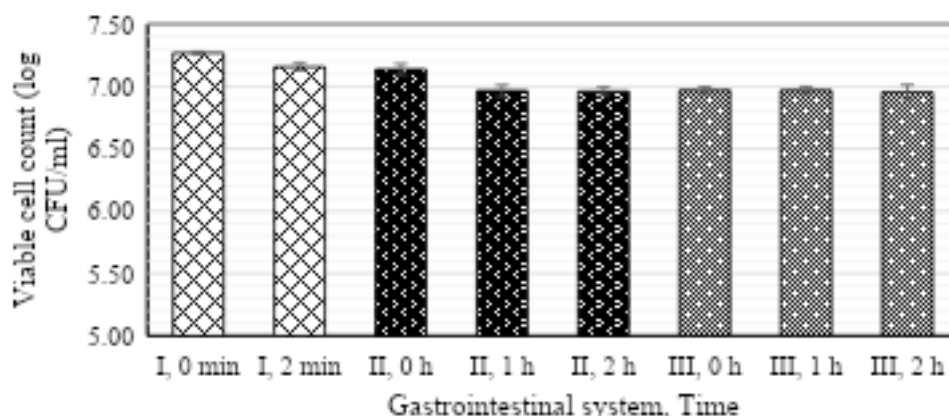


Figure 3. Survival of *B. tequilensis* PLM1 under simulated gastrointestinal conditions, including oral (I, 2 min), gastric (II, 2 h), and intestinal (III, 2 h) phases.

4. CONCLUSIONS

B. tequilensis PLM1 demonstrated the ability to produce cellulolytic and hemicellulolytic enzymes, particularly mannanase, when cultivated on soybean residue. The strain efficiently converted soybean residue into MOSs and pectin-derived sugars, with 10% (w/v) soybean residue yielding the highest total oligosaccharide production and conversion yield. M4 was the predominant MOSs produced, indicating the selective hydrolysis of soybean mannan by bacterial mannanase. Furthermore, *B. tequilensis* PLM1 exhibited excellent survival under simulated gastrointestinal conditions, retaining 95.0% of its initial viable cell population. These findings demonstrate the feasibility of producing a fermented soybean product that combines the probiotic *B. tequilensis* PLM1 with soybean-derived prebiotic MOSs in a single fermentation process, highlighting its potential as a synbiotic product that may provide synergistic benefits for gut health and host well-being.

5. ACKNOWLEDGEMENT

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Genomic Resequencing Reveals Genetic Diversity and Population Structure of Durian in Uttaradit Province, Thailand

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ABSTRACT: Durian (*Durio zibethinus* L.) is an important economic fruit in Southeast Asia, particularly in Thailand, the world's largest durian exporter. Currently, only a few durian varieties are commercially cultivated, resulting in a decrease in the diversity of other durian varieties. However, in Uttaradit province, durians were propagated from seeds, which might lead to higher genetic diversity than in typical commercial growing areas. For this reason, the objective of this research was to assess the population structure and genetic diversity of thirty durian varieties in the Uttaradit province. The results revealed 26,793,798 SNPs across the genome, providing genome-wide markers for characterizing the genetic diversity of durian in the study area. Population structure analysis using IBS, phylogenetic tree, and PCA revealed three genetic clusters: group 1 (ID1-ID5, ID7, ID9-ID19, ID22, and ID25-ID30); group 2 (ID6 and ID8); and group 3 (ID20, ID21, ID23, and ID24). Furthermore, identified two ancestral sub-populations ($K = 2$). However, despite the clear classification of these population groups, the overall genetic differentiation of the 30 durian varieties was generally low to moderate. This study enhances our understanding of the population structure and genetic diversity of durian in Uttaradit province and can serve as a basis for future genetic resource conservation, germplasm management, and durian breeding programs.

Keyword: Durian, Uttaradit province, Population structure, Genome analysis, Single nucleotide polymorphisms (SNPs)

1. INTRODUCTION

Durian (*Durio zibethinus* L.) is one of the most economically important tropical fruit crops in Southeast Asia, particularly in Vietnam, Malaysia, and Thailand. Thailand is the world's biggest exporter of durian. In 2024, the country exported US\$4,404.9 million (about 157,506 million baht) worth of durian [1]. Approximately 641 durian varieties are surveyed, collected and founded in Thailand. However, only five durian cultivars are commercially planted, 'Mon Thong', 'Cha nee', 'Kradum Thong', 'Kanyao', and 'Phuang Manee' [2].

Currently, most durian propagation uses asexual methods such as grafting and budding. These techniques help preserve the characteristics of the parent plant and maintain consistent fruit quality [3, 4]. However, relying heavily on only a few commercial cultivars may reduce genetic diversity within the cultivated durian population, which can increase susceptibility to diseases, pests, and environmental changes, poses a significant threat to the biodiversity of local durian varieties and may also limit future breeding potential. Therefore, studying the genetic diversity and population structure of durian is important for both conservation and crop improvement [4].

Molecular markers and genomic technologies have recently emerged as important tools in durian research. The first durian draft genome was reported for the Musang King cultivar, with a genome size of about 738 Mb [5]. Subsequently, genome sequencing and chromosome-scale assemblies were extended for several Thai cultivars, including Monthong, Kradumthong, and Puangmanee [6]. In addition, the haplotype-resolved genomes of Thai Kanyao cultivar are available as Hap1 and Hap2, which provide sequence information for each haplotype [7]. These genome studies provided a better understanding of the durian genome structure and genetic variation. Several molecular markers, including RAPD, ISSR, SSR, DAF, HAT-RAPD, AFLP, and single nucleotide polymorphisms (SNPs), have been used to investigate genetic diversity and relationships among durian varieties. Among these markers, SNPs are particularly useful because they are widely distributed throughout the genome and provide high-resolution genetic information [3, 4, 8-10]. The usefulness of SNP markers in durian population studies has been shown by previous studies. For instance, a genome-wide SNP analysis of Malaysian durian varieties showed distinct genetic differences among varieties including D101, Black Thorn, Musang King, and D13. Because of clonal propagation through grafting and budding, individuals from the same variety showed significant genetic similarity, and admixture analysis also divided the samples into five primary genetic clusters [11]. Similarly, a study of 114 durian accessions conducted in China identified over 40 million high-quality SNPs, divided the samples into three distinct genetic groups, and found indications of population admixture [4]. These results show that SNP markers are effective tools for analyzing durian population structure and genetic variation.

Uttaradit province is one of the important durian-producing areas in northern Thailand. This region is an interesting source of genetic variation because durians were propagated from seeds on high, sloping mountains [12]. However, information regarding the genetic diversity and population structure of durian in this area is still limited. Therefore, this study aims to investigate the genetic diversity and population structure of durian in Uttaradit province using SNP markers. The results of this research provide useful information for genetic resource conservation, germplasm management, and future durian breeding programs.

2. MATERIALS AND METHODS

2.1 Sample collection and preparations

A total of 30 durian accessions were purchased at the fully ripe stage from local markets in Uttaradit province, northern Thailand (Figure1). After peeling, the flesh was collected without a seed, immediately frozen in liquid nitrogen, then ground into a fine powder, and stored at -80 °C for further use.

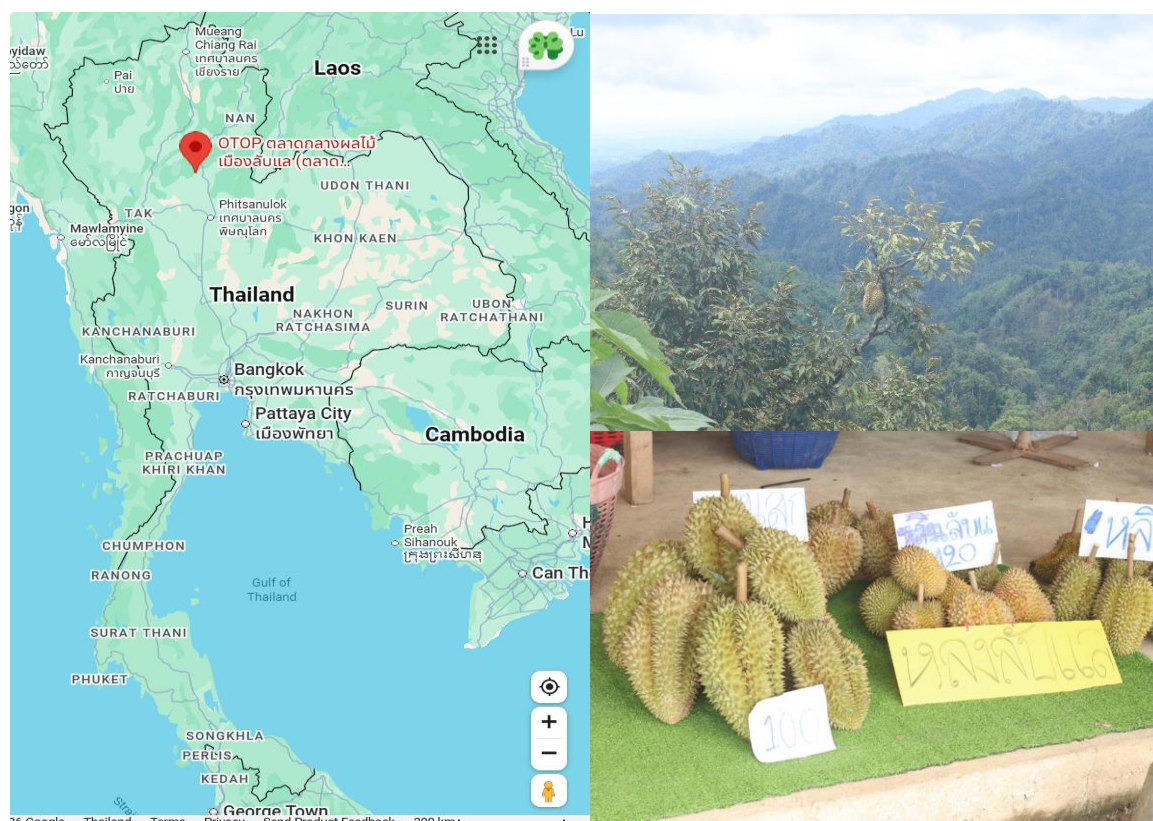


Figure 1. Sampling locations of durian accessions in Uttaradit Province. (A) Location of Hua Dong Market in Uttaradit Province, Thailand. (B) Mountain-grown durian orchards in Uttaradit Province. (C) Durian fruit at Hua Dong Market.

2. Genomic DNA (gDNA) Extraction, Library Preparation and Sequencing

Crushed thorn samples from 30 durian accessions were used to extract genomic DNA, employing a modified CTAB protocol based on the method outlined by Porebski [13]. DNA quality was assessed using 1 % agarose gel electrophoresis and the concentration was determined with a Biospectrophotometer (Eppendorf). The DNA samples were submitted to Biomarker Technologies (BMK) GmbH, where whole-genome resequencing (WGRS) library preparation and sequencing were performed using the Illumina platform, producing 150 bp pair-end reads.

3. Reference Mapping and Variant calling

Raw sequencing reads (150 bp paired end) were filtered using the FASTQ quality check package of the Omixbox program (v.3.5.3) with default parameters. Then, the clean reads were aligned to the reference genome of durian cv. Kan Yao [7] using BWA-MEM with default parameters in the Omixbox program (v.3.5.3). The resulting alignments were converted to BAM format, sorted and indexed using SAMtools (v1.17) and subsequently marked with PCR duplicates using Picard (v1.9). Variant calling was performed for each sample using GATK HaplotypeCaller (v4.2.0.0) in GVCF mode for each sample. All per-sample GVCFs were then combined using GATK's GenotypeGVCFs to jointly genotype across all 30 accessions, producing a multi-sample VCF. SNPs and indels were separated using GATK SelectVariants. Hard filtering was performed using GATK VariantFiltration with the following criteria: for SNPs, QD < 2.0, FS > 60.0, MQ < 40.0, SOR > 3.0, MQRankSum < -12.5, and ReadPosRankSum < -8.0; for indels, QD < 2.0, FS > 200.0, and SOR > 10.0. The variants were further filtered to exclude

those with >20% missing data across samples or a minor allele frequency (MAF) below 0.05 to obtain informative polymorphisms.

4. Population Structure and Genetic Relatedness Analyses

A total of 26,793,798 high-quality SNP variants were subjected to linkage disequilibrium (LD) pruning using PLINK (v.1.9). Population structure and identity by state (IBS) were computed using PLINK (v.1.9). The IBS matrices obtained were visualized as heatmaps with the Pheatmap package in R (v.4.4.1). The phylogenetic tree was constructed using the neighbor-joining (NJ) method in R (v.4.4.1). In addition, principal component analysis (PCA) based on the pruned SNPs was performed using the R package SNPRelate. The optimum number of K was estimated with K = 2–10 using Omixbox program (v.3.5.3) to obtain the cross-validation (CV) error curve and admixture plot. Furthermore, genetic differentiation among populations was evaluated by calculating FST using PLINK v1.9.

3. RESULTS AND DISCUSSION

3.1 Summary of Sequencing and Variants discovery

In this study, thirty durian accessions were assessed. Sequencing analysis generated between 168.5 million and 185.8 million total read alignments per sample providing a comprehensive and reliable dataset for further analysis. The Kanyao reference genome's mapping rates ranged from 80.5% to 86.6%, indicating the reference genome's usefulness as a sample reference. However, due to the durian genome's complexity and high number of repetitive regions, approximately between 13.36% to 16.75% of the reads remained unmapped. This work employed different sequencing methods and an alternative reference genome compared with a previous resequencing study of Thai durian cultivars, which had reported a higher mapping rate of 91.3–93.5% [6]. Additionally, the mean coverage depth across all samples ranged from 26× to 30×, which was greater than the generally recommended threshold of 20× [14]. The observed duplication rates were low (18.50% - 22.35%), reflecting high molecular diversity and minimal bias from PCR amplification, making them suitable for downstream analysis (Table 1.).

3.2 Genetic Distance of the Conserved Population of the 30 Durian accessions

After quality filtering and variant calling, a total of 26,793,798 high-quality SNPs were identified across the 30 durian accessions. To reduce redundancy caused by linkage disequilibrium (LD) and to minimize computational bias in population genetic analyses, the SNP dataset was pruned, resulting in 539,821 LD-pruned SNPs. These LD-pruned SNP datasets were utilized to construct an IBS distance matrix, which was used to estimate pairwise genetic distances among samples based on genome-wide SNP data. The result shows pairwise genetic distances in the range of 0–0.25, demonstrating that the studied Uttaradit durian collection comprises both highly related and accessions with moderate genetic divergence. From the analysis, the 30 durian genotypes were divided into three distinct groups. Group 1 comprised 24 accessions (ID1–ID5, ID7, ID9–ID19, ID22, and ID25–ID30), Group 2 included ID6 and ID8, and Group 3 consisted of ID20, ID21, ID23, and ID24 (Figure 2). Accessions within Groups 2 and 3 showed lower pairwise IBS distances than those in Group 1, indicating closer genetic relationships within these groups. This study identified a broader distribution of genetic distances than a previous study on 32 durians cultivated in Hainan Province, China, where a high frequency of near-zero IBS distances, indicating identical or closely related lineages.

Table 1. Summary of sequencing quality and alignment statistics of the 30 durian varieties

Sample	Total Alignment	Mapped (%)	Unmapped (%)	Duplication Rate	Coverage
ID1	185,899,768	86.572	13.428	22.35	30.994×
ID2	174,366,081	86.638	13.362	18.50	29.177×
ID3	168,597,981	83.247	16.753	18.99	26.785×
ID4	171,939,575	85.526	14.474	19.76	28.181×
ID5	236,636,596	89.252	10.748	26.88	40.718×
ID6	171,641,387	87.230	12.770	18.43	28.864×
ID7	169,079,923	86.638	12.066	19.81	28.976×
ID8	176,177,704	88.177	11.823	19.37	29.898×
ID9	164,567,128	90.784	9.216	18.48	29.196×
ID10	166,822,284	84.744	15.256	18.54	27.352×
ID11	179,696,044	91.500	8.500	22.37	32.048×
ID12	170,522,342	87.890	12.110	18.43	29.114×
ID13	169,537,898	80.545	19.455	19.07	25.818×
ID14	242,810,909	85.298	14.702	39.06	40.359×
ID15	164,484,215	88.588	11.412	18.27	28.364×
ID16	175,918,184	87.486	12.514	18.58	29.784×
ID17	187,040,949	89.745	10.255	22.65	32.667×
ID18	165,789,741	86.540	13.460	17.91	27.681×
ID19	176,500,596	88.543	11.457	22.23	30.166×
ID20	187,567,707	88.781	11.219	20.11	32.136×
ID21	166,988,626	84.399	15.601	18.32	26.713×
ID22	177,242,454	87.052	12.948	18.79	29.761×
ID23	179,868,207	88.565	11.435	21.41	30.813×
ID24	196,698,754	88.174	11.826	20.55	33.201×
ID25	179,649,962	86.894	13.106	22.36	30.160×
ID26	192,179,570	86.413	13.587	27.39	32.374×
ID27	194,771,387	84.321	15.679	24.13	31.807×
ID28	215,792,003	86.172	13.828	31.59	36.243×
ID29	166,013,477	82.195	17.805	18.51	26.425×
ID30	179,413,412	84.957	15.043	22.40	29.581×
Mean	181,807,162	86.762	13.195	21.508	30.512×

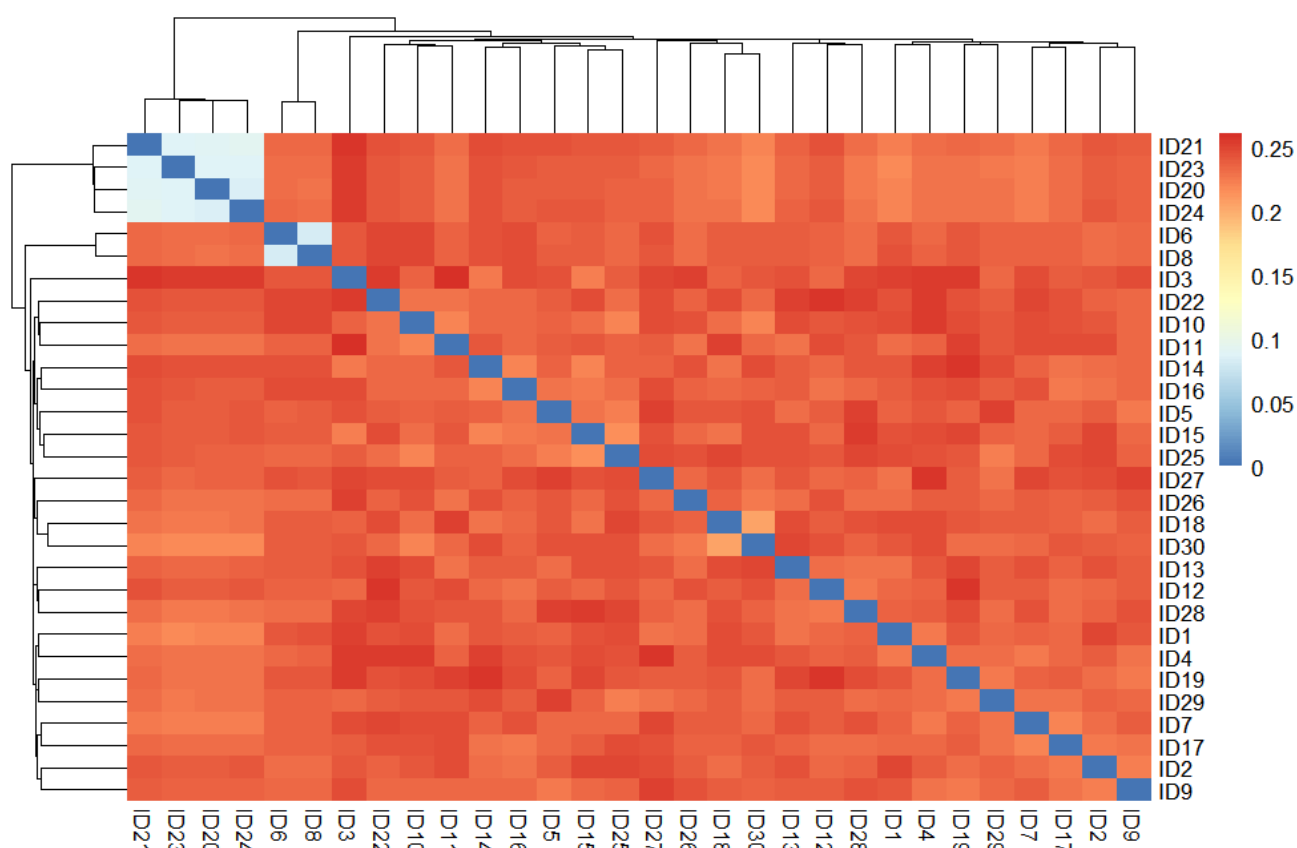


Figure 2. Heatmap of identity by state (IBS) distance matrix of the conserved population of 30 durian genotypes based on pruned SNPs, ranging from 0 to 0.25. Blue indicates low genetic distance, whereas red indicates high genetic distance.

3.3 Phylogenetic tree

NJ phylogenetic tree was constructed from the pruned SNP dataset to estimate genetic relationship among the 30 durian genotypes. The 30 genotypes of durian were divided into three clades (Figure 3). The majority of accessions were distributed across the major clades (Cluster1) without forming distinct subclusters, reflecting high genetic diversity, which serves as a valuable resource for genetic conservation and selective breeding. Meanwhile, Cluster 2 contains only ID6 and ID8, which were clustered together early in the clustering process, demonstrating a higher level of genetic similarity compared to the remaining samples. This pattern may suggest that the two accessions represent the same variety. Conversely, Samples ID20, ID21, ID23, and ID24 were clustered together within Cluster3, reflecting genetic closeness or common origin.

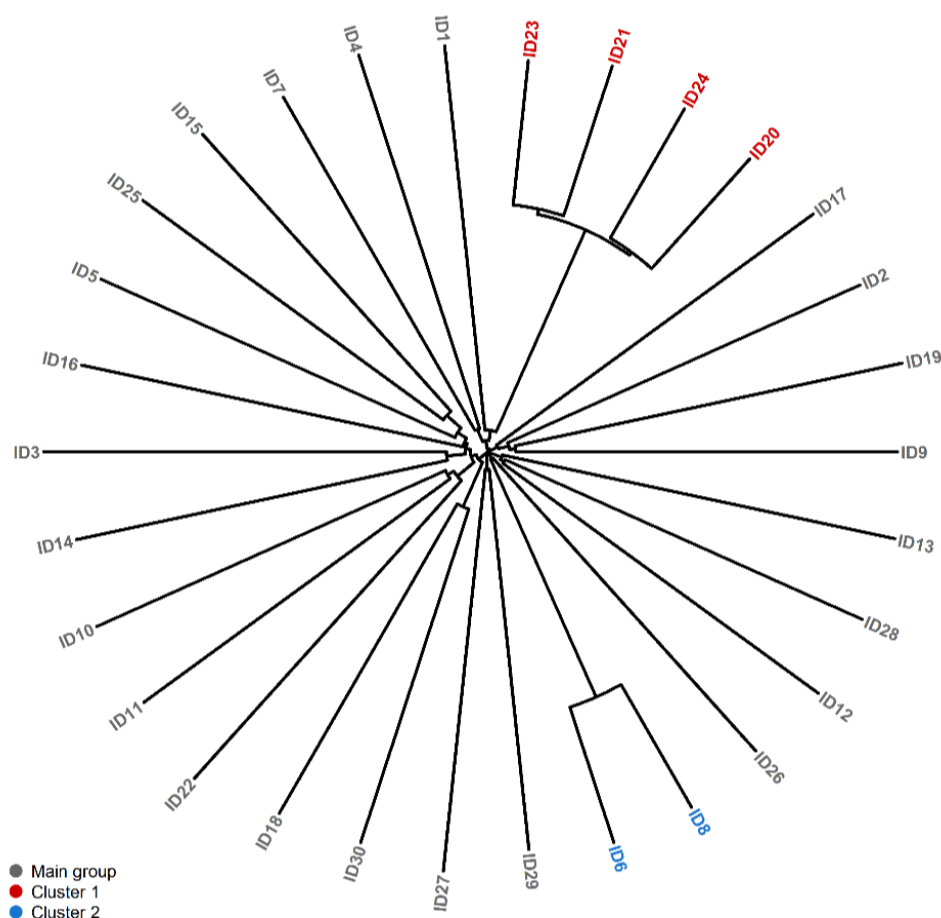


Figure 3. Neighbor-joining (NJ) phylogenetic tree showing genetic distance among 30 durian genotypes. The label with black, blue, and red represent main group (cluster 1), cluster 2, and cluster 3, respectively.

3.4 Principal Component analysis

Principal component analysis (PCA) of the pruned SNP dataset revealed the sample distribution along the principal component axes (PC1–PC4), where PC1 explains 10.4% of the variance, PC2 6.94%, PC3 5.66%, and PC4 5.65% (Figure 4). Although each component does not explain clearly genetic differentiation among the cultivars, this implies a relatively homogenous genetic background across the collection and may suggest that no durian varieties from other locations have been imported. However, PC1 clearly separates samples ID 20, 21, 23, and 24 from the rest of the samples across the PC1–PC2, PC1–PC3, and PC1–PC4 planes. Similarly, PC2 is crucial in separating samples ID06 and ID08 from the remaining accessions when examined across the PC1–PC2, PC2–PC3, and PC2–PC4 planes.

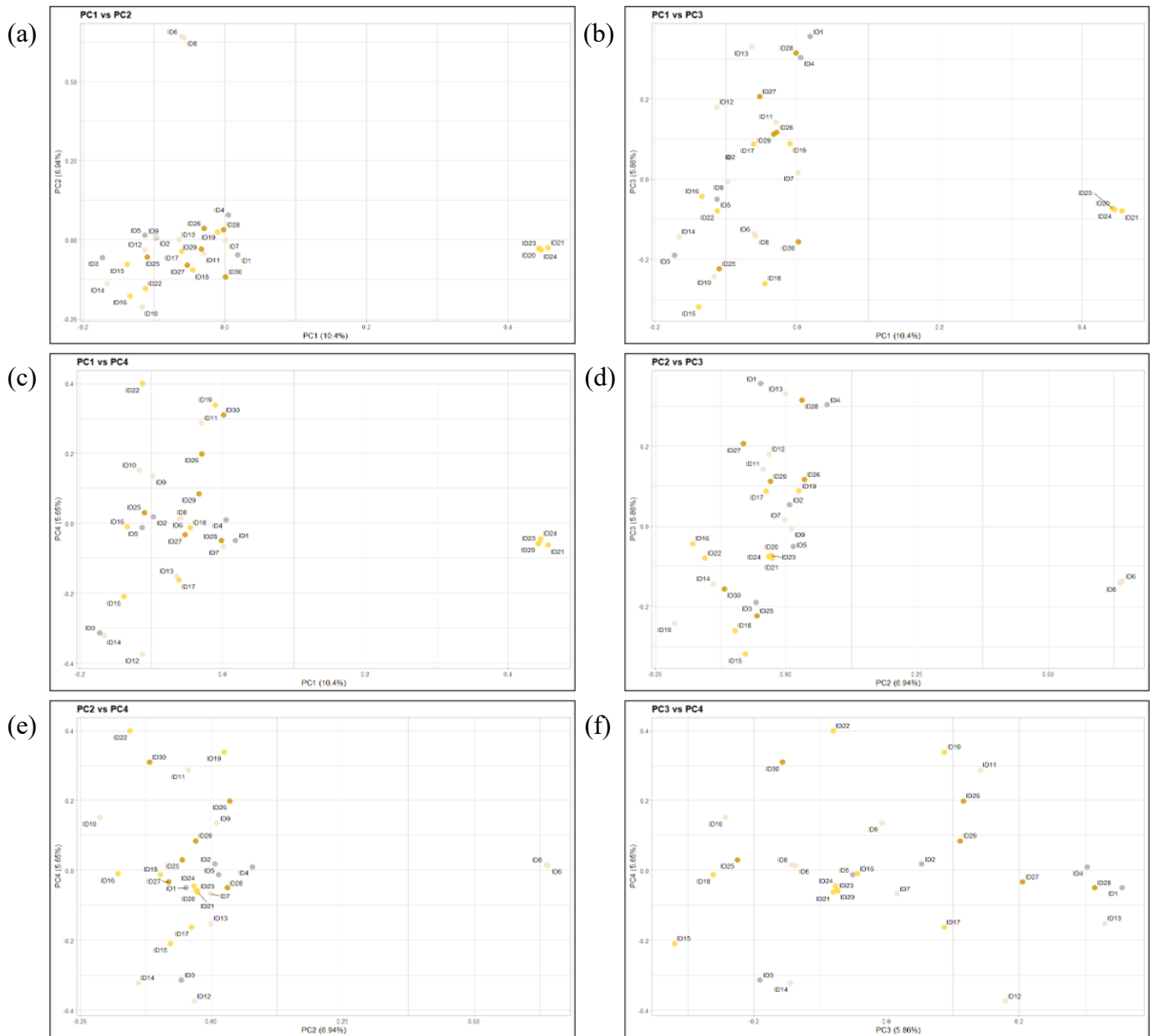


Figure 4. 2D PCA plots showing different pairwise combinations of the first four principal components (PC1–PC4) derived from pruned SNP data. (a) PC1 vs PC2, (b) PC1 vs PC3, (c) PC1 vs PC4, (d) PC2 vs PC3, (e) PC2 vs PC4, and (f) PC3 vs PC4

3.5 Model-Based Population Structure

The model-based population structure analysis was performed with $K = 2$ –10 runs. The 30 durian genotypes were divided into two ancestral population size (Figure 5(b)) based on the cross-validation (CV) error estimates ($K=2$, 0.55575) (Figure 5(a)). The genotypes ID1, ID2, ID3, ID4, ID5, ID6, ID7, ID8, ID9, ID10, ID11, ID12, ID13, ID14, ID15, ID16, ID17, ID18, ID19, ID22, ID25, ID26, ID27, ID28, ID29, and ID30 from Sub-population I (red color bars) share a possible ancestry source with the genotypes in Sub- population II, comprising ID20, ID21, ID23, and ID24 (green color bars). However, these results stand in contrast to the patterns observed in the IBS heatmap (Figure 2.), the phylogenetic tree (Figure 3.) and the PCA (Figure 4), where three distinct groups were partitioned among 30 durian cultivars. Specifically,

Cluster 1 and Cluster 2, derived from the IBS analysis, were classified as belonging to a common ancestral population by ADMIXTURE, while Group 3 was classified as belonging to a different ancestral population. This indicates that Group 1 and Group 2 share a common ancestral background but are genetically distinct enough to be separated by model-free methods such as IBS, PCA, and phylogenetic tree analysis, while Group 3 is genetically distinct from the other two groups.

In genetic studies with poor population structure, the differences between ADMIXTURE (K=2) and the model-free methods (PCA, IBS, and phylogenetic tree, which revealed three clusters) are clearly demonstrated. Lawson et al. [15] explain that when allele frequency differences are minor, model-based clustering algorithms such as ADMIXTURE may not be able to identify small-scale substructures, frequently preferring a lower, more reasonable K value to prevent overfitting. Nevertheless, Patterson et al. [16] revealed that Principal Component Analysis (PCA) is highly sensitive to variance and can recognize limited kinship relationships or minor genetic variations that model-based methods ignore. As a result, the three clusters discovered by PCA, IBS, and phylogenetic analyses most likely represent ancient genetic divergence within a shared ancestral gene pool or a real, fine-scale population substructure.

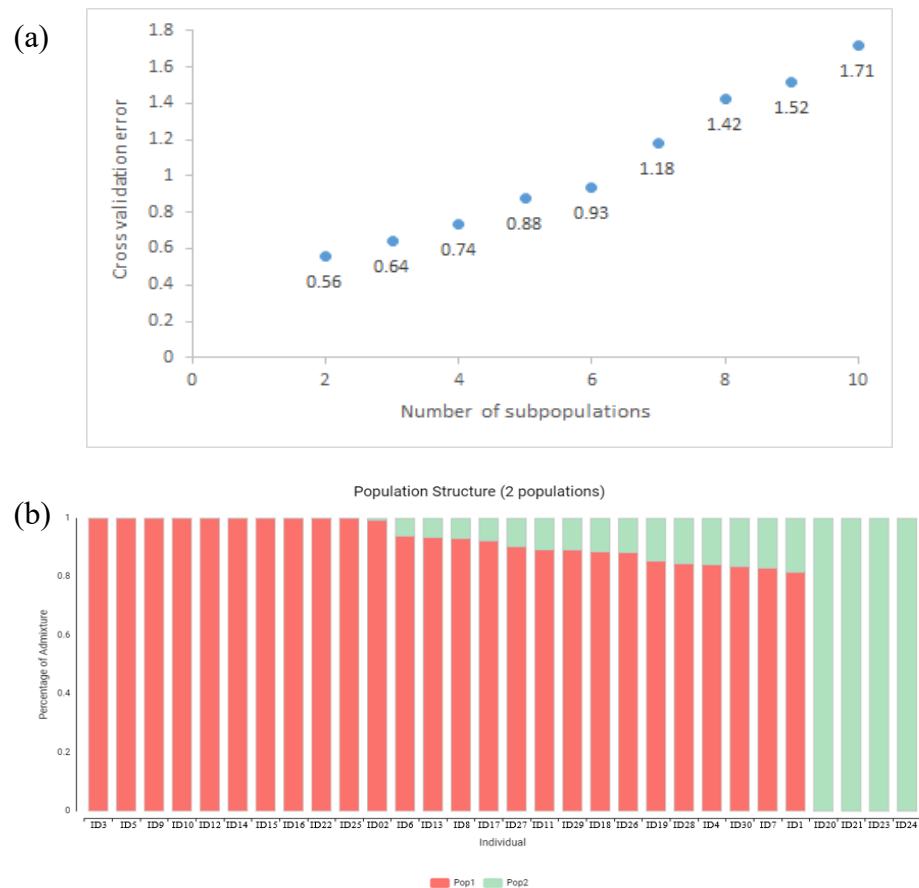


Figure 5. Admixture analysis reveals two subpopulations among 30 durian samples (a) The cross-validation (CV) error curve based on ADMIXTURE output reveals that the CV error value of 2 was the smallest at K = 2. (b) The subgroup assignment by ADMIXTURE when K = 2.

3.6 FST analysis

Table 2. Pairwise Fst comparisons among genetic clusters and sub-populations

Pairwise comparison	Mean Fst	Weighted Fst	Interpretation*
Cluster 1 vs Cluster 2	0.0191107	0.0736555	Moderate differentiation
Cluster 1 vs Cluster 3	0.0558021	0.0997064	Moderate differentiation
Cluster 2 vs Cluster 3	0.1966130	0.2728440	Very strong differentiation
Sub-Population 1 vs Sub-Population 2	0.0556646	0.0987000	Moderate differentiation

* Weighted Fst values: (1) 0–0.05, negligible differentiation; (2) 0.05–0.15, moderate differentiation; (3) 0.15–0.25, significant differentiation; and (4) >0.25, very strong differentiation. [17]

Pairwise FST analysis among the three genetic clusters supported the clustering patterns inferred from PCA, IBS distance, and phylogenetic analyses. Moderate genetic differentiation was observed between Cluster 1 and Cluster 2 (weighted FST = 0.0737) and between Cluster 1 and Cluster 3 (weighted FST = 0.0997). In contrast, Cluster 2 and Cluster 3 exhibited very strong genetic differentiation (weighted FST = 0.2728), indicating that these two clusters are the most genetically distinct. Furthermore, pairwise FST was also calculated between the two ancestral sub-populations identified by ADMIXTURE. A moderate level of genetic differentiation was detected between Sub-population 1 and Sub-population 2 (weighted FST = 0.0987). This result is consistent with the ADMIXTURE analysis, which separated the 30 accessions into two ancestral populations while indicating that several accessions possessed admixed ancestry.

Additionally, the relatively moderate genetic differentiation among durian accessions in Uttaradit province suggests a shared ancestral origin. According to historical records, durian seeds were introduced and planted in Uttaradit approximately 200 years ago, marking the beginning of durian cultivation in the province. Over time, natural cross-pollination among those founding populations likely facilitated the emergence of several local varieties. However, resulting genetic variation may have remained very limited because these cultivars most likely originated from a small founding population. Furthermore, sustained genetic relationship among accessions may result from the continuous reproduction of selected cultivars through grafting and budding [18].

4. CONCLUSIONS

In summary, this study identified 26,793,798 high-quality single nucleotide polymorphisms (SNPs) across 30 durian accessions collected from the Uttaradit region. Identity-by-state (IBS) analysis, phylogenetic tree construction, and principal component analysis (PCA) were performed utilizing the 539,821 LD-pruned SNPs. Despite the relatively narrow pairwise genetic distances across the 30 durian accessions, population structure analyses resolved three distinct genetic clusters. Notably, Group I, comprising 24 accessions (ID1–ID5, ID7, ID9–ID19, ID22, and ID25–ID30), represented the largest genetic cluster. Group II consisted of two genetically distinct accessions (ID6 and ID8), whereas Group III comprised four accessions (ID20, ID21, ID23, and ID24) that formed a separate cluster. Furthermore, ADMIXTURE analysis identified K = 2 as the optimal number of ancestral populations, revealing admixture between the two inferred ancestral populations. Taken together, these findings significantly increase our understanding

of the genetic diversity and population structure of durian in Uttaradit province and provide invaluable insights for genetic resource conservation, germplasm management, and future durian breeding programs.

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Postbiotic Potential of Cell-Free Supernatants from Khao-mak Microbial Isolates: Antimicrobial Activity and Stability

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ABSTRACT: Growing demand for natural antimicrobial agents has increased interest in postbiotics derived from fermented foods. Khao-mak, a traditional Thai fermented rice dessert, serves as a rich source of diverse microbial communities with potential bioactive properties. This study evaluated the antimicrobial activity, thermal and pH stability, and minimum inhibitory concentrations (MIC) of cell-free supernatants (CFS) from Khao-mak microbial isolates against *Escherichia coli* K12 and *Bacillus subtilis* CU1065. Ten bacterial and ten yeast isolates were isolated and screened using the agar well diffusion method. Yeast isolates exhibited no inhibitory effects, while several bacterial isolates demonstrated significant antibacterial activity: L02, L05, L07, and L08 inhibited *E. coli*, and L02, L07, and L08 were active against *B. subtilis*. CFS from the promising isolates were freeze-dried and reconstituted for further characterization. Thermal stability testing at 60°C, 80°C, and 100°C showed that L02 and L07 maintained complete stability against *E. coli* ($p \geq 0.05$), whereas L08 exhibited a significant decrease above 60°C ($p < 0.05$). Against *B. subtilis*, L02 and L08 retained full heat tolerance up to 100°C, while L07 showed a slight reduction at higher temperatures ($p < 0.05$). CFS demonstrated excellent stability across pH 2.0–9.0; L08 achieved maximum inhibition against *E. coli* at pH 7.0 (1.70 ± 0.10 cm), and L02 peaked against *B. subtilis* at pH 2.0 (1.83 ± 0.06 cm). MIC determination indicated that L07 had the strongest potency against *E. coli* K12 (MIC = 0.07 g/mL), while all three isolates exhibited an identical MIC of 0.13 g/mL against *B. subtilis* CU1065. These findings highlight the postbiotic potential of CFS from Khao-mak microbial isolates, demonstrating heat- and pH-stable antimicrobial properties with efficacy dependent on the isolate and pathogen.

Keywords: Antimicrobial activity, Cell-free supernatant, Heat stability, Khao-mak, pH stability

1. INTRODUCTION

Khao-mak is a traditional Thai fermented dessert made from sweetened glutinous rice and consumed for generations. The fermentation process is initiated by Look-pang, a starter containing a diverse community of molds, yeasts, and bacteria that convert starches into sugars, organic acids, alcohol, and aromatic compounds [1–3]. Recent studies have identified beneficial microorganisms in Khao-mak, particularly the lactic acid bacterium *Pediococcus pentosaceus* and the yeast *Saccharomyces cerevisiae*, both widely recognized for their probiotic properties [1,2].

In recent years, scientific interest has increasingly focused on postbiotics, which are formally defined as “preparations of inanimate microorganisms and/or their components that confer health or functional benefits on the host” [4]. Unlike live probiotics, postbiotics offer various industrial advantages, including

longer shelf life, ease of standardization, and resistance to high-temperature processing [5–7]. This has prompted growing research toward identifying novel microbial strains capable of producing stable bioactive metabolites for applications in food safety and functional food development [6–9].

Despite the recognized microbial richness of Khao-mak, its promise as a source of antimicrobial postbiotics remains largely unexplored. Here, this study aimed to isolate yeast and bacterial strains from Khao-mak and evaluate the cell-free supernatants (CFS) of these strains for postbiotic potential. Isolates were screened for antimicrobial efficacy against *Escherichia coli* and *Bacillus subtilis*, and the most promising CFS preparations were further characterized for minimum inhibitory concentrations (MICs) and stability under thermal and pH stress conditions.

2. MATERIALS AND METHODS

2.1 Bacterial and Yeast Isolation, and Chemical Preparation

Khao-Mak was purchased from a local vendor at the Wednesday Market, Silpakorn University, Nakhon Pathom Province, Thailand. After that a 10 g Khao-mak sample was homogenized in saline peptone water (SPO water) and serially diluted. The diluted samples were then spread onto de Man, Rogosa and Sharpe (MRS) agar containing cycloheximide (0.1 mg/mL) and incubated at 37°C for 2 to 3 days for bacterial isolation. Yeasts were isolated from the same diluted samples by using Malt extract Glucose Yeast extract Peptone (MYGP) agar supplemented with chloramphenicol (0.1 mg/mL) and chlortetracycline (0.05 mg/mL), followed by incubation at 30°C for 2 to 3 days. After incubation, ten colonies were randomly selected from each medium and purified by repeated streaking on the corresponding medium without antibiotics. The resulting pure isolates were subjected to preliminary characterization based on Gram staining and cell morphology to facilitate selection for subsequent antimicrobial screening. The purified microorganisms were then freshly cultured overnight on the respective medium prior to experimentation.

E. coli K12 and *B. subtilis* CU1065 were used as indicator microorganisms and maintained on Mueller–Hinton (MH) agar. Prior to use, the cultures were propagated in MH broth and incubated at 37°C for 18–24 h.

All culture media were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Chemicals and reagents were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL, Maharashtra, India), unless otherwise specified.

2.2 Screening for Antimicrobial Activity

Ten bacterial isolates (L01–L10) and ten yeast isolates (Y01–Y10) obtained from Khao-Mak were screened for antimicrobial activity against *Escherichia coli* K12 and *Bacillus subtilis* CU1065 using the agar well diffusion method. Cell-free supernatants (CFS) from each isolate were used as test samples. Chloramphenicol served as the positive control, while uninoculated MRS broth and MYGP broth served as the negative controls for bacterial and yeast isolates, respectively. After incubation at 37°C for 24 h, antimicrobial activity was evaluated by measuring the diameter of the inhibition zones (cm).

2.3 Cell-free Supernatant Powder Preparation

Microbial isolates exhibiting antimicrobial activity against both targeted microorganisms were centrifuged at 9,000 rpm and 4°C for 10 minutes. The CFS were collected, filtered and freeze-dried using a ScanVac CoolSafe freeze-dryer (UK) (condenser temperature: –110 °C connected to 4×10^{-4} mbar vacuum pump). The freeze-dried CFS was subsequently used as the postbiotic preparation in this study, following the method described by Moradi *et al.* [10].

2.4 Thermal and pH Stability

The stability of postbiotic preparations under various temperature and pH conditions was evaluated. Freeze-dried postbiotic powder was dissolved in deionized water (100 mg/mL) and subjected to heat treatments at 60, 80, and 100°C for 15 minutes. For pH stability assessment, postbiotic preparations were dissolved in buffer solutions adjusted to pH 2–9 (50 mg/mL).

The antimicrobial activities of treated samples against *E. coli* K12 and *B. subtilis* CU1065 were determined using the agar well diffusion assay. Chloramphenicol and the corresponding buffer solutions served as positive and negative controls, respectively. After incubation at 37°C for 24 hours, inhibition zone diameters were measured. The pH stability assay was performed triplicately according to the method of Moradi *et al.* [10]. The inhibition zone of each sample was corrected by subtracting the inhibition zone produced by the corresponding buffer control, where applicable.

2.5 Minimal Inhibitory Concentration Assay

The MIC of the CFS preparations against *E. coli* K12 and *B. subtilis* CU1065 was determined using the broth microdilution method, as described by Kowalska-Krochmal, B. and Dudek-Wicher, R. with modifications [11]. Briefly, bacterial suspensions were adjusted to approximately 10⁶ CFU/mL and inoculated into 96-well microplates. CFS-solution preparations were serially diluted in culture medium to obtain final concentrations ranging from 0.01 to 0.50 mg/mL (w/v). After incubation at 37°C, the MIC was defined as the lowest concentration that completely inhibited visible bacterial growth. All assays were performed in triplicate. Sterile culture medium and inoculated medium without postbiotic treatment served as negative and growth controls, respectively.

2.6 Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. Differences were considered statistically significant at $p < 0.05$. Data analysis and graph preparation were performed using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Activity Screening

An initial screening of ten bacterial isolates (L01–L10) and ten yeast isolates (Y01–Y10) from Khao-mak. The CFS from these active isolates were then evaluated using an agar well diffusion assay (Figure 1.).

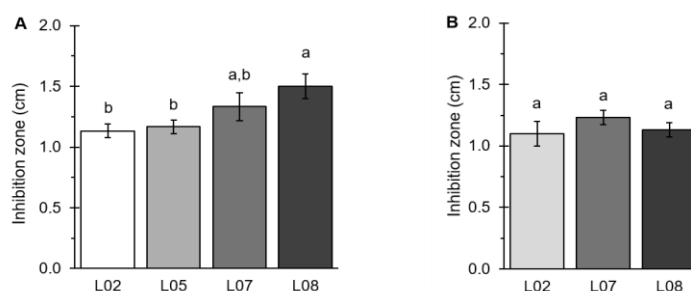


Figure 1. Inhibitory activity of cell-free supernatants produced by isolates L02, L05, L07, and L08 against (A) *E. coli* K12 and (B) *B. subtilis* CU1065. Results are expressed as inhibition zone diameter (cm) and presented as mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences among isolates (Tukey's HSD, $p < 0.05$).

Inhibition zones against *E. coli* K12 revealed that only four bacterial isolates (L02, L05, L07, and L08) exhibited antimicrobial activity against the strain (Figure 1A). The inhibition zones were ranged from approximately 1.1 to 1.5 cm. L08 demonstrated the highest potency (1.50 ± 0.10 cm), which was significantly greater than that of L02 (1.13 ± 0.06 cm) and L05 (1.17 ± 0.06 cm), while L07 exhibited intermediate activity (1.33 ± 0.12 cm). For *B. subtilis* CU1065 (Figure 1B), L02, L07, and L08 produced comparable inhibition zones (1.10–1.23 cm) without statistically significant differences. No inhibition zones were observed against either indicator bacterium following treatment with the yeast cell-free supernatants (CFS).

These findings indicate that these Khao-mak isolates secrete bioactive metabolites, such as organic acids and bacteriocins, that are active against both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria [3]. L08 was the most effective against *E. coli* K12, whereas *B. subtilis* CU1065 exhibited similar susceptibility to all active treatments. Although the inhibition zones were modest (1.1–1.5 cm), they are consistent with reported values for unconcentrated CFS from fermented food isolates. Consequently, isolate L02, L07, and L08 were selected for further characterization due to their inhibitory activity against both target pathogens. L05 was excluded from subsequent experiments because it did not inhibit *B. subtilis* CU1065.

3.2 Thermal Stability of Antimicrobial Activity

The thermal stability of reconstituted CFS (100 mg/mL) from isolate L02, L07, and L08 was assessed following exposure to 60, 80, and 100°C for 15 minutes (Figure 2.). Freeze-drying, reconstitution, and thermal treatment did not reduce efficacy, as inhibition zones (1.17–1.47 cm) remained comparable to those of fresh, untreated CFS (Figure 1). These results demonstrate that the bioactive metabolites are resilient to lyophilization and exhibit high heat tolerance.

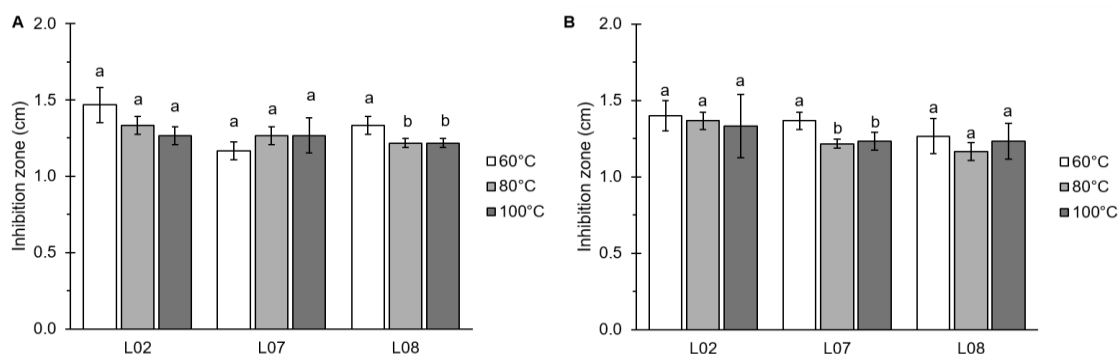


Figure 2. Effect of heat treatment on the inhibitory activity of CFS produced by isolates L02, L07, and L08 against (A) *E. coli* K12 and (B) *B. subtilis* CU1065. CFS were exposed to 60, 80, and 100°C prior to analysis. Inhibitory activity was expressed as inhibition zone diameter (cm). Data are presented as mean $\pm$ SD (n = 3). Different lowercase letters above bars within the same isolate indicate significant differences among temperature treatments according to Tukey's HSD test following one-way ANOVA ($p < 0.05$).

Briefly, all reconstituted CFS inhibited *E. coli* K12 (Figure 2A). The inhibitory activity of L02 and L07 was unaffected by temperature, whereas L08 exhibited a minor reduction in inhibition zones at 80°C and 100°C ($p < 0.05$). Meanwhile, L02 and L08 maintained stable inhibition ($p > 0.05$), while L07 demonstrated a significant decrease in activity at 80°C and 100°C ($p < 0.05$) against *B. subtilis* CU1065 (Figure 2B.).

Therefore, these findings suggest that the bioactive agents released by the bacterial isolates are heat-stable compounds, such as organic acids or low-molecular-weight bacteriocins, which are commonly produced by bacteria in fermented foods [8,9,12]. The high thermal tolerance of these CFS preparations supports their potential application in thermal food processing.

3.3 pH Stability of Antimicrobial Activity

The effect of pH on the antimicrobial activity of CFS from isolates L02, L07, and L08 was evaluated against *E. coli* K12 and *B. subtilis* CU1065 across a pH range of 2 to 9 (Table 1). Overall, all three isolates retained measurable antimicrobial activity across the entire pH range tested, indicating that the bioactive compounds present in the CFS preparations are broadly stable under both acidic and mildly alkaline conditions.

Table 1. Inhibitory activity of cell-free supernatants produced by isolate L02, L07, and L08 at different pH values against *E. coli* and *B. subtilis*.

pH	Inhibition zone (cm)					
	<i>E. coli</i> K12			<i>B. subtilis</i> CU1065		
	L02	L07	L08	L02	L07	L08
2	1.37 $\pm$ 0.06 ^{ab}	1.37 $\pm$ 0.12 ^{ab}	1.47 $\pm$ 0.06 ^{ab}	1.83 $\pm$ 0.06 ^a	1.43 $\pm$ 0.15 ^a	1.23 $\pm$ 0.06 ^b
3	1.43 $\pm$ 0.06 ^{ab}	1.43 $\pm$ 0.15 ^{ab}	1.47 $\pm$ 0.06 ^{ab}	1.43 $\pm$ 0.06 ^b	1.33 $\pm$ 0.06 ^a	1.40 $\pm$ 0.10 ^{ab}
4	1.50 $\pm$ 0.00 ^a	1.23 $\pm$ 0.06 ^b	1.43 $\pm$ 0.06 ^b	1.43 $\pm$ 0.06 ^b	1.07 $\pm$ 0.06 ^b	1.50 $\pm$ 0.10 ^a
5	1.47 $\pm$ 0.06 ^{ab}	1.30 $\pm$ 0.00 ^b	1.50 $\pm$ 0.10 ^{ab}	1.43 $\pm$ 0.06 ^b	1.40 $\pm$ 0.10 ^a	1.40 $\pm$ 0.00 ^{ab}
6	1.37 $\pm$ 0.06 ^{ab}	1.30 $\pm$ 0.00 ^b	1.63 $\pm$ 0.15 ^a	1.27 $\pm$ 0.12 ^b	1.27 $\pm$ 0.06 ^{ab}	1.47 $\pm$ 0.06 ^a
7	1.40 $\pm$ 0.10 ^{ab}	1.30 $\pm$ 0.00 ^b	1.70 $\pm$ 0.10 ^a	1.30 $\pm$ 0.10 ^b	1.40 $\pm$ 0.00 ^a	1.40 $\pm$ 0.00 ^{ab}
8	1.50 $\pm$ 0.00 ^a	1.33 $\pm$ 0.06 ^{ab}	1.40 $\pm$ 0.10 ^b	1.43 $\pm$ 0.06 ^b	1.37 $\pm$ 0.06 ^a	1.27 $\pm$ 0.12 ^b
9	1.30 $\pm$ 0.10 ^b	1.53 $\pm$ 0.06 ^a	1.37 $\pm$ 0.06 ^b	1.53 $\pm$ 0.06 ^b	1.17 $\pm$ 0.06 ^b	1.23 $\pm$ 0.06 ^b

¹Values are presented as mean $\pm$ SD (n = 3). Different superscript letters within the same column indicate significant differences among pH treatments within each isolate according to Tukey's HSD test ($p < 0.05$).

For *E. coli* K12, the isolate L02 maintained consistent inhibition zones across most pH values

(1.30–1.50 cm), with a significant reduction only at pH 9 (1.30 ± 0.10 cm, $p < 0.05$), indicating that its antimicrobial components are stable across a wide pH range but may be slightly affected under alkaline conditions. Isolate L07 exhibited its lowest activity at pH 4–7 (1.23 – 1.30 cm) compared to pH 9 (1.53 ± 0.06 cm), where a significant increase was observed ($p < 0.05$), an unexpected result that warrants further investigation. Isolate L08 showed notably higher inhibition at pH 6 and 7 (1.63 ± 0.15 cm and 1.70 ± 0.10 cm, respectively), which were significantly greater than those at other pH values ($p < 0.05$), suggesting that its antimicrobial compounds may be most active or stable under near-neutral conditions.

For *B. subtilis* CU1065, the isolate L02 showed the highest inhibition at pH 2 (1.83 ± 0.06 cm), significantly greater than at most other pH values ($p < 0.05$), indicating that its antimicrobial activity is particularly pronounced under strongly acidic conditions. Isolate L07 displayed variable activity, with the lowest inhibition at pH 4 (1.07 ± 0.06 cm) and pH 9 (1.17 ± 0.06 cm), both significantly lower than at other pH values ($p < 0.05$). Isolate L08 exhibited its highest inhibition at pH 4 (1.50 ± 0.10 cm), with a significant decline at pH 2 (1.23 ± 0.06 cm) and pH 9 (1.23 ± 0.06 cm) ($p < 0.05$).

This broad pH-tolerance range suggests the presence of stable bioactive peptides or bacteriocins alongside organic acids, which typically deprotonate and lose efficacy at near-neutral pH [8,13]. The strain- and target-dependent variations likely reflect differences in the structural charge of these bioactive peptides and the cell wall characteristics of the target pathogens [12,13]. Although some of these pH-induced variations are statistically significant, the relatively small numerical differences indicate that the CFS preparations remain effective across a wide pH range, suggesting their suitability to apply for diverse food formulations.

3.4 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the CFS preparations was evaluated against *E. coli* K12 and *B. subtilis* CU1065 to quantify antimicrobial potency. Isolate L07 demonstrated the highest efficacy against *E. coli*, yielding the lowest MIC of 0.07 mg/mL. For the Gram-positive *B. subtilis*, all three isolates exhibited an identical MIC of 0.13 mg/mL, suggesting a similar mechanism of action against this species.

These quantitative findings are consistent with the zone-of-inhibition trends in Figure 1, confirming the strong antimicrobial activity of these Khao-mak-derived cell-free supernatant preparations. The strain- and target-dependent MIC variations likely result from differences in outer membrane permeability between Gram-positive and Gram-negative bacteria, as well as the unique profiles of bioactive metabolites secreted by each isolate [13]. These low MIC values underscore the significant postbiotic potential of these preparations, offering safe, viable-cell-free, and highly stable alternatives to live probiotics for targeted food preservation and biomedical applications.

4. CONCLUSIONS

The antimicrobial potential of CFS derived from bacterial isolates of Khao-mak, a traditional Thai fermented rice dessert, was investigated in this study. Although the isolated yeasts showed no antimicrobial activity, three bacterial isolates (L02, L07, and L08) produced CFS that exhibited significant antibacterial activity against both *E. coli* K12 and *B. subtilis* CU1065. These CFS preparations maintained stability under high-temperature treatments (up to 100°C) and across a broad pH range (2–9). Isolate L07 exhibited the greatest potency against *E. coli* (MIC = 0.07 g/mL), while all three isolates were equally effective against *B. subtilis* (MIC = 0.13 g/mL). The observed stability of these supernatants under thermal and pH stress reveals their potential application as natural bio-preservatives in the food industry, particularly for pasteurized or acidic food products. The following research will address the taxonomic identification of

these bacterial isolates using 16S rRNA gene sequencing and the purification of their specific active antimicrobial compounds.

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Nested Recombinase Polymerase Amplification Coupled with a CRISPR-Cas12a Assay for Sensitive and Specific Detection of *Streptococcus iniae* in Fish

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ABSTRACT: *Streptococcus iniae* is one of the major pathogens in fish, causing Streptococcosis that results in massive fish mortalities and economic losses. Rapid diagnostic methods are crucial for early detection and prevention of this disease, but conventional molecular approaches often require laboratory settings and trained personnel, making field implementation challenging. In this study, we developed an integrated CRISPR-Cas12a platform for rapid, sensitive *S. iniae* detection that can be completed at 37°C within one hour. Specifically, this assay uses one-tube nested RPA to amplify target DNA, followed by the CRISPR-Cas12a assay to detect the target sequence, generating strong fluorescence that can be observed by the naked eye. While the conventional RPA-Cas12a protocol failed to attain the expected sensitivity, using the one-tube RPA allowed the CRISPR detection assay to achieve high analytical sensitivity, detecting as few as 10² copies of target DNA, without cross-reactivity observed in other *Streptococcus* species or fish-related pathogens. Furthermore, the clinical validation using both field and artificially spiked samples exhibited 100% diagnostic accuracy. Collectively, the results confirm that our assay is a rapid, highly sensitive, and specific field-deployable diagnostic tool for *S. iniae* detection, providing early protection and prevention of Streptococcosis in aquaculture.

Keywords: CRISPR-Cas12a, Diagnosis, RPA, *Streptococcus iniae*, Tilapia

Rapid CRISPR-Based Detection Platform for Species-Specific Identification of *Edwardsiella* Bacteria

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ABSTRACT: Sudden outbreaks of bacterial pathogens in aquaculture represent a persistent threat to productivity and economic sustainability. Among the most pressing concerns are *Edwardsiella tarda* and *Edwardsiella ictaluri*, two pathogens capable of infecting both freshwater and saltwater fish species and, in rare cases, causing disease in humans. However, existing detection methods often lack the speed and accessibility required for routine, onsite surveillance. In this study, we developed a DNA detection platform based on a CRISPR-Cas12a assay combined with Recombinase Polymerase Amplification (RPA), referred to as the RPA-Cas12a assay, for the detection of both *E. tarda* and *E. ictaluri*. This assay detects as few as 100 copies of target DNA from both bacteria. No cross-reactivity was observed with closely related *Edwardsiella* species, nor with a panel of other fish-associated bacterial and viral pathogens. Validation using clinical samples confirmed a 100% diagnostic accuracy of the developed RPA-Cas12a assays. Importantly, the assay operates isothermally at 37°C and generates visual readouts within 50 minutes, removing the need for specialized equipment. Overall, this RPA-Cas12a assay provides a rapid, sensitive, and highly specific point-of-care tool for maintaining proactive, biosecure aquaculture systems.

Keywords: CRISPR-Cas12a, *Edwardsiella tarda*, *Edwardsiella ictaluri*, Fish, RPA

Whole Genome Characterization of *Streptomyces angustmyceticus* as a Potential Biological Control Agent Against Southern Blight Disease Caused by *Agroathelia rolfsii*

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ABSTRACT: Southern blight caused by *Agroathelia rolfsii* is an economically important soil-borne disease of many crops worldwide causing severe yield losses and decreased agricultural productivity. The widespread use of chemical fungicides for disease control has raised concerns about environmental pollution, pathogen resistance, and adverse effects on soil health and beneficial microorganisms. Thus, biological control using antagonistic microbes has become a sustainable approach, especially those belonging to the genus *Streptomyces*. *Streptomyces* spp. are known to be potent biocontrol agents due to their ability to produce several bioactive secondary metabolites that have significant antifungal activity against many phytopathogenic fungi. In this study, genetic and biological investigations were performed on *Streptomyces angustmyceticus* isolated from agricultural soil in southern Thailand to examine its antifungal potential against *A. rolfsii*. For genetic characterization, the isolate was subjected to whole-genome sequencing using the Illumina platform. The draft genome was 8.22 Mb with a GC content of 72.21% and showed high completeness (98.3% BUSCO). Genome analysis identified 32 putative biosynthetic gene clusters (BGCs) associated with the production of antimicrobial and antifungal secondary metabolites, including polyketides, non-ribosomal peptides, terpenes, and siderophores. In a biological investigation, the antifungal activity of *S. angustmyceticus* against *A. rolfsii* was examined by the toxic-medium method using different concentrations of culture filtrate (1%, 2%, 5%, 10%, and 20%). The results demonstrated a concentration-dependent inhibition of *A. rolfsii* mycelial growth, with higher concentrations showing significantly greater antifungal activity. These findings suggest that extracellular metabolites produced by *S. angustmyceticus* contribute substantially to fungal suppression. The integration of genomic characterization and antifungal assays highlights the potential of *S. angustmyceticus* as a promising biocontrol agent for sustainable management of southern blight disease. This study also provides genomic insights supporting the future development of environmentally friendly strategies for crop protection and sustainable agriculture.

Keywords: *Agroathelia rolfsii*, Biological control, Biosynthetic gene clusters, *Streptomyces angustmyceticus*, Whole-genome sequencing

Substantial Catalytic Enhancement of D-allulose 3-epimerase from *Arthrobacter psychrolactophilus*: A Sustainable Chitosan Immobilization Strategy for Production of Low-Calorie Sweetener

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ABSTRACT: D-allulose, a low-calorie rare sugar, has gained significant industrial interest; however, cost-effective enzymatic production remains a bottleneck. Enzyme immobilization offers a promising approach to enhance the overall catalytic efficiency and operational feasibility of bioprocesses. While D-allulose 3-epimerase is conventionally immobilized on expensive synthetic resins or inorganic supports, there is a growing demand for sustainable alternatives. Chitosan—an abundant, inexpensive, and biocompatible natural biopolymer—emerges as an ideal support matrix. Therefore, this study aims to optimize the immobilization of recombinant *Arthrobacter psychrolactophilus* D-allulose 3-epimerase (ApDAEase) onto chitosan beads to maximize its catalytic performance. The immobilization was conducted using a glutaraldehyde cross-linking method. Specifically, the chitosan beads were formulated using a syringe dropping technique with a 2% chitosan concentration dissolved in 1% acetic acid. The resulting matrix was subsequently activated using 0.25% glutaraldehyde for 3 hours, followed by an enzyme loading phase optimized for 4 hours. Direct comparative analysis between the free and immobilized enzyme systems demonstrated that the optimized microenvironment within the porous chitosan matrix successfully preserved the functional conformation of the active site. Under these established optimum conditions, the chitosan-immobilized ApDAEase exhibited a remarkable nearly 19-fold increase in catalytic activity compared to its free counterpart. In conclusion, this optimized immobilization strategy provides a highly efficient, cost-effective, and robust biocatalytic platform, demonstrating strong potential for the scalable production of D-allulose.

Keywords: Chitosan beads, D-allulose 3-epimerase, D-allulose, Enzyme immobilization, Glutaraldehyde cross-linking

Preparation and Characterization of Mealworm-Contained Rice Starch Soft Foods for Older Adults with Dysphagia

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ABSTRACT: Sustainable protein sources have attracted considerable interest in the development of functional foods designed to meet both nutritional and textural requirements for aging populations. In this study, rice starch-based soft foods containing mealworm powder were developed to investigate the effects of mealworm powder content (0.0–5.0 wt%) on the physicochemical and rheological properties of dysphagia-friendly formulations. A gelatinized rice matrix was prepared under controlled thermal conditions, followed by mealworm incorporation during the cooling stage. The resulting formulations were characterized by microstructural, thermal, and rheological analyses. The effects of mealworm incorporation on the structural characteristics and flow behavior of the food formulations were investigated using rheological analyses across a range of shear rates (1–1000 s⁻¹) relevant to oral processing and chewing. The results demonstrated that increasing mealworm content altered the intermolecular interactions of the rice starch matrix, resulting in changes in thermal stability, viscosity, and viscoelastic properties. The formulations exhibited rheological characteristics consistent with the texture requirements for pureed foods defined by the International Dysphagia Diet Standardization Initiative (IDDSI), suggesting their suitability for safe swallowing. These findings highlight the potential of mealworm-enriched rice starch systems as sustainable, protein-fortified soft foods for elderly individuals with dysphagia.

Keywords Age-friendly soft food, Dysphagia, rheological property, Mealworm powder, Viscoelasticity

Isolation and Characterization of α -1, 3-Glucanase from Alkaliphilic Bacterium, *Alkalihalobacillus* sp.

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ABSTRACT: Various kinds of oligo saccharides have been recognized to be useful for physiologically functional food ingredients. The authors have been interested in the application of α -1,3-glucan oligosaccharides to food. To develop efficient enzymatic production of α -1,3-glucan oligo saccharides, α -1,3-glucanases capable of hydrolyzing α -1,3-glucan under alkali condition where α -1,3-glucan can be retained in solubilized condition were examined in the various microbial samples. As a result of extensive screening, several bacteria were grown on the agar medium containing α -1,3-glucan as a carbon source at pH 10. Among these isolates, the strain RTS232 exhibited highest activity at pH 10 where α -1,3-glucan was partially solubilized. Based on 16SrRNA sequence analysis, it was classified in *Alkalihalobacillus pseudoalcalophilus*. It was designated to be *A. pseudoalcalophilus* RTS232 and used for further study. The enzyme was extracellularly produced in the culture medium containing α -1,3-glucan as a main carbon source and homogenously purified through Butyl-cellufine and HiTrap Q HP column chromatography. The purified enzyme showed a specific activity of 4.08 U/mg with 57.8-fold purification. The enzyme exhibited maximum activity at pH 9.0 and an optimal temperature of 50°C. It was stable in a pH range of 7.0–9.5 (retaining greater than 95% activity) and retained 90% residual activity after heat treatment up to 45°C. The main hydrolysis product from α -1,3-glucan was a tetra-oligosaccharide. It showed the highest activity toward α -1,3-glucan, with slight activity toward α -1,6-glucan. Further study will clarify the production efficiency of tetra-oligosaccharide from α -1,3-glucan using the enzyme. This is the first report regarding α -1,3-glucanase from alkaliphilic *A. pseudoalcalophilus*.

Keywords: α -1,3-glucan, α -1,3-glucanase, Alkaliphilic, *Alkalihalobacillus*, Oligosaccharide

Optimal Condition for Mycelial Growth of Ectomycorrhizal Mushroom Het Ra-ngok Lueang (*Amanita javanica*)

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ABSTRACT: *Amanita javanica*, commonly known as Het Ra-ngok Lueang, is a highly popular and delicious edible ectomycorrhizal mushroom that commands high market prices. It forms mutualistic symbioses with dipterocarp trees and plays a critical role in forest restoration under challenging environmental conditions. However, laboratory cultivation remains challenging due to fastidious growth requirements. The objective of this study was to optimize the culture media, nutritional components, and initial pH levels to enhance the mycelial growth of *A. javanica*. To identify the most suitable basal medium, *A. javanica* strain KK was screened on eight solid media, including MMN, ½MMN, PDA, ½PDA, MEA, ½MEA, Heli medium, and ammonium chloride glucose agar. Among these, ½MMN agar at pH 5.3 was the most effective medium for supporting mycelial growth. Subsequently, nutrient optimization using a Response Surface Methodology (RSM) based on a Central Composite Design (CCD) revealed that the interactive effects of glucose and malt extract followed a significant quadratic model ($p = 0.0109$, $R^2 = 0.9135$). The RSM analysis identified a ½ MMN medium supplemented with 0.05% glucose and 0.10% malt extract as the optimal nutrient formulation. Following this nutrient optimization, evaluation of initial pH conditions identified pH 5.0 as the most effective level for supporting optimal growth, yielding a maximum colony diameter of 4.50–4.95 cm after 35 days of cultivation. In conclusion, these findings provide practical knowledge for producing high-quality mycelial inoculum to colonize host plant roots, facilitating successful dipterocarp reforestation and ultimately yielding edible wild mushrooms for sustainable local consumption.

Keywords: Central Composite Design; Dipterocarpaceae; Fungal biotechnology; Inoculum production, Reforestation.

1. INTRODUCTION

Amanita javanica (Corner & Bas) T. Oda, C. Tanaka & Tsuda, commonly known as Het Ra-ngok Lueang, is an ectomycorrhizal fungus belonging to the family Amanitaceae. It is highly regarded as an edible mushroom due to its unique flavor, nutritional benefits, and potential immunomodulatory properties [1]. As an ectomycorrhizal fungus, *A. javanica* forms a mutualistic association with plant roots, in which the fungus receives photosynthetically derived carbon from the host plant, while enhancing plant nutrient and water uptake, improving drought tolerance, and protecting roots from phytopathogenic microorganisms [2]. Ectomycorrhizal networks may also facilitate resource transfer and signaling among neighboring plants, thereby contributing to forest resilience and ecosystem stability [3–4]. In Thailand, *A. javanica* is primarily associated with trees in the family Dipterocarpaceae, including *Dipterocarpus alatus*, *Hopea odorata*, *Dipterocarpus obtusifolius*, *Shorea obtusa*, and *Anthoshorea roxburghii* [5]. Dipterocarp forests are ecologically and economically important in Southeast Asia, serving as vital resources for hardwood timber and supporting forest biodiversity, including wild edible mushrooms [6]. However, forest degradation caused by agricultural expansion, climate variability, drought, and forest fires has reduced dipterocarp forest cover and hindered

forest regeneration [7]. In addition, intensive harvesting of wild mushrooms, including the *A. javanica*, may increase pressure on forest ecosystems [8]. Since the establishment and survival of dipterocarp seedlings are strongly influenced by ectomycorrhizal associations, *A. javanica* holds significant potential for inoculation in dipterocarp reforestation programs to improve seedling establishment and ecosystem restoration [9]. Nevertheless, successful ectomycorrhizal inoculation relies on effective fungal strain selection and inoculum production, which remain challenging because *Amanita* species are notoriously difficult to isolate and maintain in pure culture [10–11]. To date, limited studies have investigated the cultivation and inoculum development of *Amanita* species, including *A. javanica* [12]. Therefore, the present study aimed to evaluate a high-performance strain of *A. javanica* and determine suitable culture media and growth conditions for mycelial development. The findings are expected to contribute to the production of effective mycelial inoculum for dipterocarp reforestation and to support sustainable forest restoration, with the potential to generate edible fruiting bodies as an additional economic resource for local communities.

2. MATERIALS AND METHODS

2.1 Strain selection of *A. javanica*

Three strains of *A. javanica* were obtained from the Mycology Laboratory, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The strains, namely KK, CM, and LP, represent origins from Khon Kaen, Chiang Mai, and Lamphun provinces, respectively. Mycelium was maintained on half-strength Modified Melin-Norkrans ($\frac{1}{2}$ MMN) agar medium [13] at pH 5.3 and subcultured every 4 weeks. The inoculum was prepared by culturing the selected strain for 20 days. To determine the appropriate strain, a mycelium plug (9 mm in diameter) was cut and placed in the center of petri dish containing 25 ml of $\frac{1}{2}$ MMN agar medium, pH 5.3, and incubated in the dark at room temperature ($30 \pm 2^\circ\text{C}$) for 35 days. The experiment was conducted using a completely randomized design (CRD). The diameters of the grown colonies were measured every 7 days and compared among the three strains with three replications. The fastest-growing strain was selected for further studies.

2.2 Optimization of culture conditions for mycelial growth of the selected *A. javanica* strain

2.2.1 Effect of culture media on mycelial growth of *A. javanica*

The effect of culture media on the mycelial growth of *A. javanica* was evaluated by comparing eight solid media, namely MMN, $\frac{1}{2}$ MMN, PDA [14], $\frac{1}{2}$ PDA, MEA, $\frac{1}{2}$ MEA [15], Heli medium, and ammonium chloride glucose agar [16] (Table 1). The experiment was conducted as previously described in 2.1. The medium that supported the fastest mycelial growth was selected for subsequent experiments.

Table 1. Composition of the growth media used in this study.

Growth medium	Composition (per Liter)
Modified Melin-Norkrans agar (MMN)	Glucose (2 g), Malt extract (3 g), KH ₂ PO ₄ (0.5 g), (NH ₄) ₂ HPO ₄ (0.25g), MgSO ₄ ·7H ₂ O (0.15 g), CaCl ₂ (0.05 g), NaCl (0.025 g), FeCl ₃ (1 g), Thiamine HCl (10 mg), agar (15 g)
Potato dextrose agar (PDA)	Glucose (20 g), potato (200 g), agar (15 g)
Malt extract agar (MEA)	Maltose (12.75 g), glucose (2.75 g), peptone (0.78 g), agar (15 g)
Heli medium	Glucose (0.5 g), KH ₂ PO ₄ (1g), CaCl ₂ (0.1 g), MgCl ₂ (0.3 g), NH ₄ Cl (0.5 g), FeCl ₃ (0.01 g), NaCl (0.1 g), agar (20 g)
Ammonium chloride glucose agar	Glucose (10 g), NH ₄ Cl (1 g), KH ₂ PO ₄ (1 g), MgSO ₄ ·7H ₂ O (0.5 g), agar (15 g)

2.2.2 Optimization of glucose and malt extract concentrations

The concentrations of glucose (a carbon source) and malt extract (a nitrogen source) were varied to investigate their effects on the mycelial growth of *A. javanica*. Response surface methodology (RSM) was applied using a Central Composite Design (CCD) with two independent variables of glucose concentrations (0.05%–0.2%w/v) and malt extract concentrations (0.1%–0.3%w/v) (Table 2). Mycelial growth was evaluated at 7-day intervals over a total of 35 days, with three replicates per treatment. The treatment that produced the highest mycelial growth diameter was chosen for further studies.

Table 2. The experimental design for optimizing the concentrations of glucose and malt extract to enhance the mycelial growth of *A. javanica* based on the Central Composite Design (CCD).

Treatment	Factor 1 A: Glucose (%)	Factor 2 B: Malt extract (%)	Treatment	Factor 1 A: Glucose (%)	Factor 2 B: Malt extract (%)
T1	0.020	0.200	T7	0.125	0.200
T2	0.050	0.100	T8	0.125	0.340
T3	0.050	0.300	T9	0.200	0.100
T4	0.125	0.060	T10	0.230	0.300
T5	0.125	0.200	T11	0.230	0.200
T6	0.125	0.200			

2.2.3 Optimization of initial pH

To determine the optimal initial pH for *A. javanica* mycelial growth, the optimized medium (containing the optimal concentration of glucose and malt extract) was prepared at four pH levels (5.0, 5.5, 6.0, and 6.5), adjusted using 1N HCl or 1N NaOH. A completely randomized design (CRD) was utilized to execute the experiment. Mycelial growth was measured at 7-day intervals for 35 days with three replicates per pH treatment. Colony diameters were measured and compared among the pH treatment to identify the optimal initial pH for mycelial growth.

2.3 Statistical analysis

Data obtained from experiments 2.1, 2.2.1, and 2.2.3 were analyzed using one-way analysis of variance (ANOVA). Mean comparisons were performed using Duncan's Multiple Range Test (DMRT) at $P < 0.05$ using IBM SPSS Statistics 22.

3. RESULTS AND DISCUSSION

3.1 Selection of the optimal *A. javanica* strain

Strain selection of *A. javanica* was performed by evaluating the mycelial growth after 35 days of incubation. The results revealed significant differences in mycelial growth among the three *A. javanica* strains, as shown in Figure 1. The average of colony diameters ranged from 2.43 to 4.70 cm. All strains produced white mycelia with smooth margins and uniform mycelial density (Figure 1). Among the tested strains, the KK strain demonstrated the highest growth performance on the $\frac{1}{2}$ MMN medium and was therefore selected for use in further experiments. All strains demonstrated notable intraspecific variation in mycelial growth. This phenotypic divergence is consistent with observations reported for *Amanita caesareoides*, in which the vegetative growth and mycorrhization efficiency vary markedly among individual isolates [12]. This type of variation is prevalent in ectomycorrhizal fungi, as evidenced by the study of *Astraeus odoratus* [11].

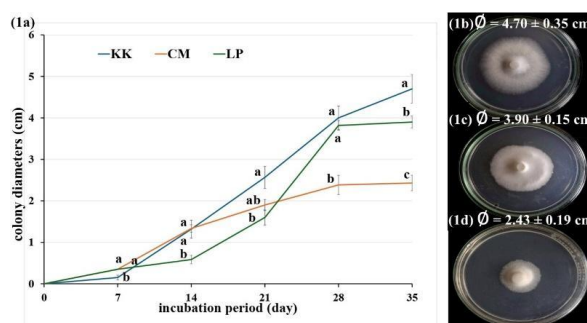


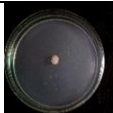
Figure 1. Mycelial growth of the three *A. javanica* strains on half-strength Modified Melin-Norkrans (MMN) agar medium at pH 5.3: (1a) Colony diameter growth curves (different letters at the same incubation time indicate statistically significant differences at $P < 0.05$); and colony morphology on solid medium of (1b) strain KK, (1c) strain CM, and (1d) strain LP at day 35, showing colony diameter (mean $\pm$ SD) (scale bar = 1 cm).

3.2 Optimal conditions for mycelial growth of the selected *A. javanica* strain

3.2.1 Effect of culture media on mycelial growth of *A. javanica*

The mycelial growth of *A. javanica* strain KK was evaluated on eight different culture media, as shown in Table 3. Among these media, the $\frac{1}{2}$ MMN medium exhibited the largest colony diameter and subsequently was selected for further experiments. These results support that fungi in the *Amanita* genus grow efficiently on MMN medium, in contrast to high carbon standard media that inhibit mycelial growth. The optimal conditions for successful *Amanita* mycelial development are provided by the balanced blend of inorganic salts, trace elements, and low glucose with malt extract in MMN [12]. In this study, *A. javanica* strain KK grew most effectively on $\frac{1}{2}$ MMN, while vegetative growth was inhibited by excessive nutrient concentrations in full-strength media. This phenomenon is consistent with the reported inhibitory effects of elevated carbon and nitrogen levels [17].

Table 3. Colony diameters of *A. javanica* strain KK cultured on eight different media for 35 days

culture medium	colony diameters (cm)	colony	culture medium	colony diameters (cm)	colony
½ MMN	4.38 ± 0.06 ^a		½ MEA	0.00±0.00 ^c	
MMN	1.32 ± 0.03 ^b		MEA	0.00±0.00 ^c	
½ PDA	0.04 ± 0.06 ^b		Heli medium	0.00±0.00 ^c	
PDA	0.00 ± 0.00 ^c		Ammonium chloride glucose agar	0.00±0.00 ^c	

*Means with different superscript letters differ significantly ($P < 0.005$) based on Duncan's multiple range test (scale bars = 1 cm)

3.2.2 Determination of glucose and malt extract concentrations

The effects of glucose and malt extract concentrations on the mycelial growth of *A. javanica* strain KK showed that the quadratic model was statistically significant ($p = 0.0109$), with an R^2 value of 0.9135. and a coefficient of variation (CV%) of 6.15. The optimum conditions for *A. javanica* mycelial growth were determined to be a glucose concentration of 0.05% and a malt extract concentration of 0.10%. This balanced nutritional profile is further supported by the choice of specific carbon and nitrogen sources. In this study, glucose was selected as an easily metabolizable energy source, while malt extract served as an organic nitrogen and vitamin source [18]. The finding that the optimal growth occurred at relatively low nutrient concentrations is in accordance with the observation that fungi of the genus *Amanita* perform best at moderate nutrient levels, whereas higher levels may suppress or inhibit mycelial development [14]. Similar trends have been observed in other ectomycorrhizal species, such as *Tricholoma matsutake*, where excessive glucose suppresses vegetative growth due to catabolite repression [19].

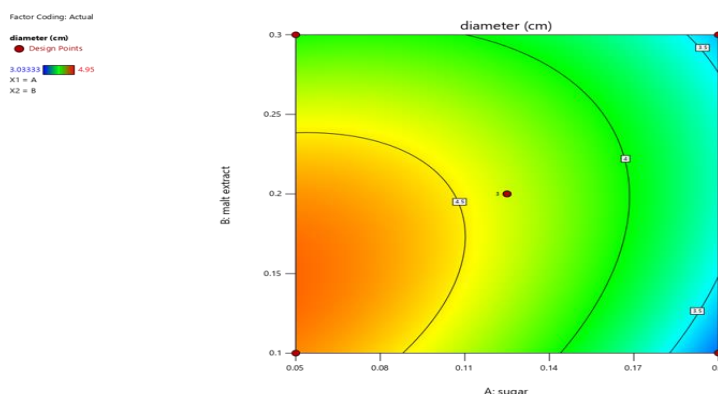


Figure 2. Response surface contour plot of colony diameter of *A. javanica* strain KK as a function of glucose and malt extract concentrations.

3.2.3 Optimization of initial pH

The effect of initial pH on the mycelial growth of *A. javanica* strain KK was demonstrated in Figure 3. The most significant mycelial growth was observed on the selected medium at pH 5.0, with a colony diameter of 4.60 cm after 35 days of incubation. This is consistent with previous studies that fungi of the genus *Amanita* grow best at pH values of 5.0–5.5 [20].

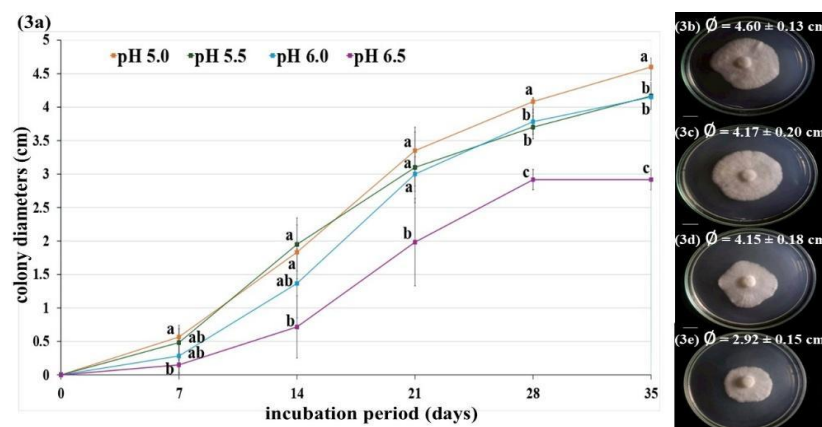


Figure 3. Effect of initial pH values on the mycelial growth of *A. javanica* strain KK over 35 days: (3a) Colony diameter growth curves (different letters within the same incubation day indicate statistically significant differences); and colony morphology on solid medium at (3b) pH 5.0, (3c) pH 5.5, (3d) pH 6.0, and (3e) pH 6.5 at day 35, showing colony diameter (mean $\pm$ SD) (Scale bars = 1 cm).

4. CONCLUSIONS

The optimal *in vitro* nutritional and cultural conditions for the mycelial growth of the *A. javanica* strain KK were influenced by specific factors: $\frac{1}{2}$ MMN at pH of 5.3, supplemented with 0.05% glucose and 0.1% malt extract, at a pH 5.0. The optimization protocol that has been established provides a highly efficient strategy for the mass production of high-quality mycelial inocula in terms of practical application. Ultimately, these discoveries establish a foundation for sustainable forestry management and advanced mycorrhizal technology. This research not only accelerates reforestation efforts and improves tree survival rates by enabling successful artificial seedling inoculation but also establishes a promising path toward the semi-artificial cultivation of this ecologically vital and economically lucrative mushroom, thereby bridging the gap between forest conservation and community livelihood.

5. ACKNOWLEDGEMENT

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D-Tagatose Production Using Recombinant L-Arabinose Isomerase from *Cellulomonas Palmilytica*

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ABSTRACT: D-Tagatose is a rare sugar widely recognized as a low-calorie sweetener and functional food ingredient. This study aimed to produce D-tagatose from D-galactose using recombinant L-arabinose isomerase from *Cellulomonas palmilytica* strain EW123 (CpL-AI) and to optimize the reaction conditions for enzymatic bioconversion. The CpL-AI gene was successfully cloned and heterologously expressed in *Escherichia coli*. SDS-PAGE analysis confirmed recombinant enzyme expression with an approximate molecular weight of 58.98 kDa. Biochemical characterization revealed that CpL-AI exhibited optimal activity at 50 °C and pH 8.0 in 50 mM sodium phosphate buffer. Among the tested metal ions, Mn²⁺ and Co²⁺ significantly enhanced enzyme activity, whereas Zn²⁺ strongly inhibited catalytic activity. Because Mn²⁺ is more suitable for food-related applications, it was selected for further optimization, with 1.0 mM identified as the optimal concentration. D-Tagatose production increased progressively with reaction time and reached a maximum conversion yield of 26.39% after 24 h under optimized conditions. These findings demonstrate that recombinant CpL-AI exhibits favorable catalytic activity for D-tagatose production and highlight its potential for sustainable rare sugar production in the food and biotechnological industries.

Keywords: *Cellulomonas palmilytica*; D-tagatose; Low-calorie Sweetener; L-arabinose Isomerase; Rare Sugar

1. INTRODUCTION

D-Tagatose is a rare sugar that has attracted considerable attention because of its low caloric value and potential application as an alternative sweetener in food systems. Compared with conventional sugars, D-tagatose exhibits favorable physicochemical and metabolic properties, making it a promising ingredient for health-oriented food products [1]. Traditional chemical synthesis of D-tagatose from D-galactose requires high temperatures and alkaline conditions, which often result in low selectivity and the formation of undesirable by-products. In contrast, enzymatic conversion using L-arabinose isomerase (L-AI; EC 5.3.1.4) provides a more selective and environmentally friendly approach under mild reaction conditions [2].

L-AI catalyzes the reversible isomerization of D-galactose to D-tagatose; however, the reaction is thermodynamically limited and typically results in moderate conversion yields. To overcome these limitations, recombinant expression systems such as *Escherichia coli* have been widely employed to produce L-AI with improved catalytic efficiency and scalability. Previous studies have demonstrated that recombinant L-AI can effectively catalyze the conversion of D-galactose to D-tagatose, although enzyme activity and conversion efficiency strongly depend on the enzyme source and reaction conditions [3, 4].

The selection of an appropriate enzyme source is therefore critical. *Cellulomonas palmilytica* is a Gram-positive bacterium isolated from earthworm soil biofertilizer and has been reported as a novel

species with strong lignocellulose-degrading capability, particularly toward oil palm empty fruit bunches (OPEFB). Notably, strain EW123^T possesses numerous genes encoding carbohydrate-active enzymes, including cellulases and hemicellulases, indicating its potential as a valuable source of industrial enzymes for biomass conversion [5]. These characteristics suggest that this microorganism may also serve as a promising source of L-arabinose isomerase for rare sugar production.

In this study, L-arabinose isomerase from *C. palmitytica* strain EW123 (CpL-AI) was heterologously expressed in *E. coli*, followed by biochemical characterization and optimization of reaction conditions for D-tagatose production from D-galactose. This work aims to contribute to the development of efficient and sustainable enzymatic processes for rare sugar production.

2. MATERIALS AND METHODS

2.1 Microorganisms and Plasmid

C. palmitytica strain EW123 (also known as TBRC 11805 and NBRC 114552) was used as the source of L-arabinose isomerase (accession number: UJP39927.1). *E. coli* DH5 α and *E. coli* BL21 (DE3) (New England Biolabs, USA) were used as the cloning and expression hosts, respectively, while pET28a (+) (Novagen, Darmstadt, Germany) was used as the expression vector.

2.2 Gene Cloning, Protein Expression, and Enzyme Purification

The gene encoding CpL-AI (1,509 bp) was amplified from the genomic DNA of *C. palmitytica* strain EW123 using primers containing *Eco*RI and *Hind*III recognition sites (forward primer: 5'-AAA AAGAATTCGTGAGCAAGCCGTACGCCGAC-3' and reverse primer: 5'-AAAAAAGCTTTCAG AACCCCTGGGCCAGGC-3'; *Eco*RI and *Hind*III recognition sites are underlined). The amplified fragment was purified, digested with the corresponding restriction enzymes, and ligated into the pET28a(+) vector previously digested with the same enzymes to construct the pET28CpL-AI plasmid. The ligation mixture was transformed into *E. coli* DH5 α cells. Positive transformants were confirmed by colony PCR and Sanger DNA sequencing (Macrogen, Korea). Recombinant plasmids were extracted using the QIAprep Spin Miniprep Kit (Qiagen). Subsequently, the pET28CpL-AI plasmid was transformed into *E. coli* BL21 (DE3) for recombinant protein expression.

Escherichia coli BL21 (DE3) harboring pET28CpL-AI was cultivated in LB medium supplemented with kanamycin (50 μ g/mL). The culture was incubated at 37 °C with shaking at 200 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.6. Protein expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, followed by incubation at 16 °C for 18 h. Cells were harvested by centrifugation and disrupted by sonication. The cell-free extract was applied to HisTrap™ FF columns (GE Healthcare, Little Chalfont, UK) for affinity purification. The purity of the purified proteins was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [6].

2.3 Protein Determination and Enzyme Assay

All experiments were performed in triplicate. Protein concentration was determined using the Bradford assay (Bio-Rad, USA) with bovine serum albumin as the standard. CpL-AI activity was assayed using 5 g/L D-galactose (TCI, Tokyo, Japan) in 50 mM sodium phosphate buffer (pH 7.0) at 50 °C for 20 min. D-Tagatose production was quantified using Seliwanoff's reagent with D-tagatose (TCI, Tokyo, Japan) as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μ mol of D-tagatose per minute under the assay conditions.

2.4 Optimization of Reaction Conditions for D-tagatose Production

The effects of temperature, pH, metal ions, and reaction time on CpL-AI activity for D-tagatose production were investigated. The effect of pH was evaluated over a pH range of 4.0–10.0 using 50 mM sodium acetate buffer (SAB, pH 4.0–6.0), sodium phosphate buffer (SPB, pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0), and sodium carbonate buffer (SCB, pH 9.0–10.0) at a constant temperature of 50 °C. Temperature optimization was conducted at temperatures ranging from 30 to 70 °C in 50 mM phosphate buffer at pH 7.0.

The effects of metal ions were evaluated at 50 °C in 50 mM SPB (pH 8.0) containing various divalent metal ions, including Co²⁺, Mn²⁺, Mg²⁺, Ni²⁺, Cu²⁺, and Zn²⁺, each at a final concentration of 1 mM. The optimal Mn²⁺ concentration was subsequently determined by varying the concentration from 0 to 5 mM. All reactions were performed using 5 g/L D-galactose and 2.0 U of purified CpL-AI for 20 min.

In addition, the time course of D-tagatose production was monitored at 50 °C for 24 h in 50 mM SPB (pH 8.0) containing 5 g/L D-galactose and 2.0 U purified CpL-AI. Samples were collected periodically, and product formation was quantified as described below.

2.5 Product Analysis

D-Tagatose production was analyzed by high-performance liquid chromatography (HPLC) equipped with an RID-10A refractive index detector (Shimadzu, Japan). D-Galactose and D-tagatose were used as standards. Separation was performed using an HPX-87P column (Bio-Rad) maintained at 80 °C, with LC-MS grade water as the mobile phase at a flow rate of 0.6 mL/min.

3. RESULTS AND DISCUSSION

3.1 Cloning and Expression of CpL-AI

The cloning and expression of the L-arabinose isomerase (*CpL-AI*) gene were successfully achieved in *E. coli*. Recombinant protein expression was analyzed by SDS–PAGE, as shown in Figure 1. A distinct protein band corresponding to approximately 58.98 kDa was observed in the induced recombinant strain, confirming successful expression of the target enzyme.

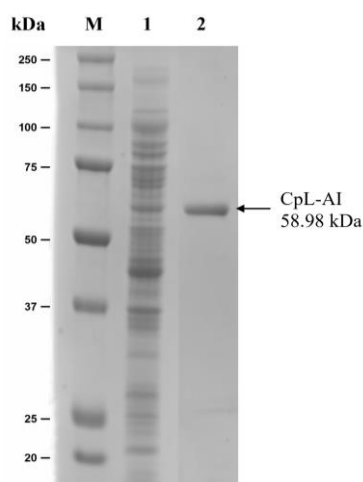


Figure 1. SDS–PAGE analysis of purified recombinant CpL-AI protein. Lane M: protein marker; Lane 1: cell-free extract containing CpL-AI; Lane 2: purified CpL-AI protein.

3.2 Effects of Temperature and pH on the Activity of Recombinant CpL-AI

The effects of temperature and pH on CpL-AI activity were investigated to determine the optimal catalytic conditions of the enzyme. The result showed that enzyme activity increased progressively from 30 to 50 °C, reaching maximum activity at 50 °C (Figure 2a), but sharply decreased at higher temperatures, likely due to thermal denaturation or structural destabilization. Similar temperature profiles have been reported for microbial L-arabinose isomerases involved in D-tagatose production, with optimum temperatures generally ranging from 50 to 65 °C depending on the source organism [2].

The effect of pH showed low enzyme activity under acidic conditions, with maximum activity observed at neutral to slightly alkaline pH (pH 7.0–8.0) (Figure 2b.). Activity gradually decreased under alkaline conditions, possibly due to alterations in the ionization state of amino acid residues involved in catalysis and substrate binding, leading to reduced enzyme stability. The optimum temperature and pH observed in this study are consistent with previous reports on bacterial L-arabinose isomerases, including enzymes from *Thermotoga maritima* and *Bacillus coagulans*, which exhibited high activity at moderate temperatures and near-neutral pH [4, 7]. These conditions are advantageous for food-processing applications because they reduce non-enzymatic browning and by-product formation. Overall, CpL-AI exhibited optimal activity at 50 °C and pH 7.0–8.0, indicating its potential for rare sugar production and industrial bioconversion applications.

3.3 Effects of Metal Ions the Activity of Recombinant CpL-AI

The effects of divalent metal ions on CpL-AI activity were evaluated using Co²⁺, Mn²⁺, Mg²⁺, Ni²⁺, Cu²⁺, and Zn²⁺ (Figure 3a). Metal ions significantly influenced enzyme activity. Co²⁺ showed the strongest activation effect, increasing relative activity to 152.53%, followed by Mn²⁺ (145.45%). Mg²⁺ moderately enhanced activity (114.14%), whereas Ni²⁺ and Cu²⁺ reduced activity to 77.78% and 75.76%, respectively. Zn²⁺ exhibited the strongest inhibitory effect, decreasing activity to 36.36% relative to the control.

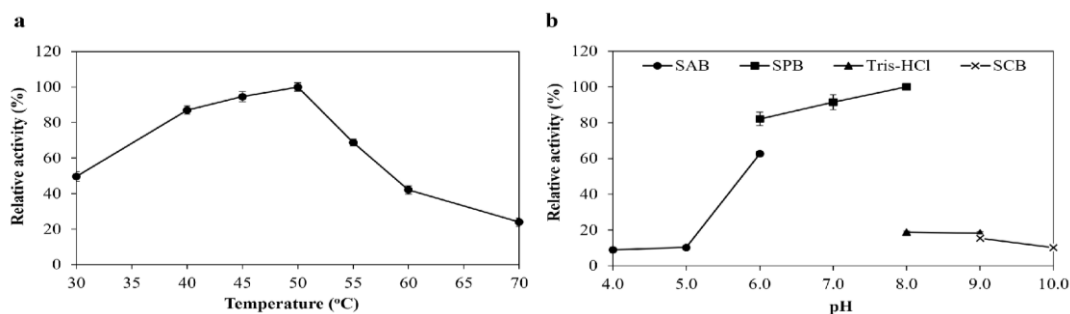


Figure 2. Effects of temperature (a) and pH (b) on the relative activity of recombinant CpL-AI. SAB, sodium acetate buffer; SPB, sodium phosphate buffer; Tris-HCl, Tris-HCl buffer; SCB, sodium carbonate buffer.

These results indicate that CpL-AI is a metal-dependent enzyme requiring divalent metal ions for optimal catalytic activity. The stimulatory effects of Co²⁺ and Mn²⁺ are consistent with previous studies on microbial L-arabinose isomerases, in which Mn²⁺ or Co²⁺ were reported to enhance enzyme activity and stability [4, 8]. In contrast, Zn²⁺ and several other metal ions exhibited weak activation or inhibitory effects [8].

Although both Co²⁺ and Mn²⁺ significantly enhanced CpL-AI activity, Mn²⁺ was selected for further optimization because it is considered safer and more suitable for food-related applications than Co²⁺. Therefore, the effect of Mn²⁺ concentration on enzyme activity was further investigated (Figure 3b). Relative activity increased to approximately 134% and 146% at 0.5 and 1.0 mM Mn²⁺, respectively, but gradually decreased at concentrations above 1.0 mM. The reduced activity at higher Mn²⁺ concentrations

may result from excessive metal ion binding that alters enzyme conformation or interferes with substrate accessibility. Similar concentration-dependent activation patterns have been reported previously [4, 7]. Therefore, 1.0 mM Mn^{2+} was considered the optimal concentration for CpL-AI activity in subsequent experiments.

3.4 Effects of Reaction Time on CpL-AI Activity for D-tagatose Production

The effect of reaction time on D-tagatose production by CpL-AI was evaluated over 24 h of incubation (Figure 4.). D-Tagatose production increased progressively with incubation time, reaching approximately 0.74 and 0.85 g/L after 0.5 and 1 h, respectively, and a maximum of 1.32 g/L after 24 h, corresponding to a conversion yield of 26.39%. Although D-tagatose production continuously increased throughout the reaction period, the reaction rate gradually decreased after prolonged incubation, particularly after 12 h, likely due to substrate depletion, product accumulation, partial enzyme inactivation, or thermodynamic equilibrium of the reversible aldose–ketose isomerization reaction.

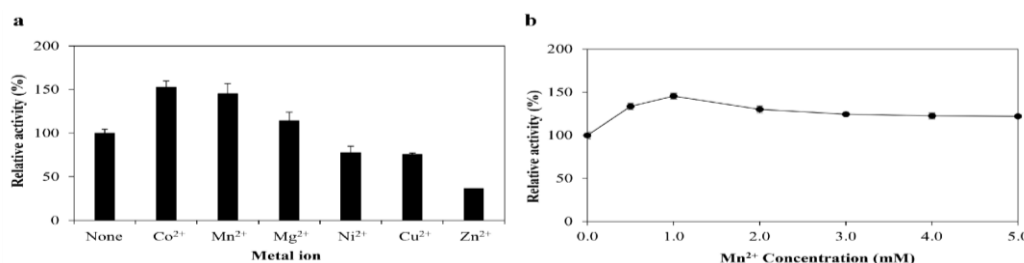


Figure 3. Effects of metal ions (a) and Mn^{2+} concentration (b) on the relative activity of recombinant CpL-AI.

Similar time-dependent increases in D-tagatose production have been reported for recombinant L-arabinose isomerases from *Thermus* sp. IM6501 and *Clostridium hylemonae* [9, 10]. Despite the reduced reaction rate during prolonged incubation, the continuous increase in D-tagatose concentration observed in this study indicates that CpL-AI retained catalytic activity throughout the reaction period. These results suggest that extended reaction time enhances D-tagatose production, although the production rate becomes slower during prolonged incubation. Therefore, optimizing incubation time is important to balance product yield and process efficiency in industrial D-tagatose production.

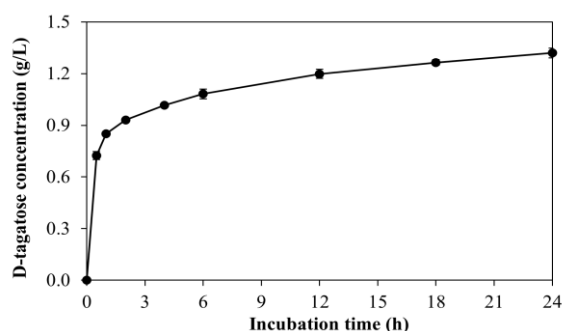


Figure 4. Effects of reaction time on CpL-AI activity for D-tagatose production.

4. CONCLUSIONS

The recombinant CpL-AI from *C. palmitytica* was successfully expressed in *E. coli* and exhibited favorable catalytic properties for D-tagatose production. Enzyme activity was significantly influenced

by temperature, pH, metal ions, and reaction time, with optimal activity observed at 50 °C, pH 8.0, and 1.0 mM Mn²⁺. D-Tagatose production increased progressively during incubation, reaching the maximum concentration of approximately 1.32 g/L after 24 h, corresponding to a conversion rate of 26.39%. The enzyme also maintained catalytic activity throughout the reaction period under moderate reaction conditions. These findings highlight the potential of CpL-AI as a promising biocatalyst for sustainable rare sugar production and provide a basis for further process optimization and industrial applications in the food and biotechnology industries.

5. ACKNOWLEDGEMENT

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Evaluation of Factors Affecting Phase Separation in Pickering Emulsions Stabilized by *Microbacterium esteraromaticum* SBS1-7

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ABSTRACT: *Microbacterium esteraromaticum* SBS1-7, a Gram-positive, non-pathogenic bacterium, has been proven for its ability to degrade monocyclic aromatic hydrocarbons (i.e., BTEX) as well as polycyclic aromatic hydrocarbons (PAHs) such as naphthalene (2 aromatic rings). In order to evaluate an ability of SBS1-7 to degrade phenanthrene (3 aromatic rings), the solvent extraction technique was used to quantify the residual phenanthrene. However, SBS1-7 exhibited a unique ability to stabilize the Pickering emulsions. The highly stable emulsion hindered the solvent extraction and quantification of the residual phenanthrene. This study aimed to develop an extraction technique that can destabilized the emulsion formed by SBS1-7. The SBS1-7 cells exposed with phenanthrene were used for emulsion preparation. Many parameters (i.e., temperature, centrifugation time, NaCl supplementation, and types of extracting solvent) were evaluated in detail. In addition, to confirm the SBS1-7's ability as a Pickering emulsions stabilizer, the emulsion stabilized by Safranin-O stained SBS1-7 was visualized under microscope. The emulsion was destabilized by centrifugation time and NaCl supplementation, while temperature (4 and 8°C) and the change of extracting solvent (*n*-heptane, *n*-octane, *n*-nonane, *n*-decane, *n*-dodecane, *n*-tridecane, and *n*-tetradecane) could not destabilize the emulsion. Furthermore, the stained SBS1-7 cells could be observed on the surface of tetradecane droplets, confirming its ability to stabilize the Pickering emulsions.

Keywords: Emulsion destabilization, *Microbacterium esteraromaticum*, Pickering emulsions

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are the organic compounds composed of multiple aromatic rings. They are released from incomplete combustion, industrial production (e.g., petrochemical, coking coal, cement, and bitumen) [1], as well as natural sources such as volcanic eruptions, forest fires, crude oil, and petroleum [2], leading to contamination of the environment including atmosphere, water, and soil. PAHs have very low water solubility and high hydrophobicity, which contribute to their high persistence in the environment. PAHs are toxic for living organisms as they can bind with DNA and protein, causing the gene mutation, malformation, and cancer [3]. In addition, some PAHs can impair the immune system and can damage red blood cells. To eliminate PAHs from the environment, biodegradation is considered as environmentally friendly and cost-effective alternative to conventional physical and chemical methods [4].

Microbacterium esteraromaticum SBS1-7 is a Gram-positive, short rod shape, non-pathogenic, and non-spore forming bacteria. SBS1-7, isolated from an estuarine sediment, has been proven for its ability to degrade monocyclic aromatic compounds including BTEX (benzene, toluene, ethylbenzene, and three isomers of xylene), as well as PAH such as naphthalene (2 aromatic rings) based on the metabolites detected during biodegradation when the specified compound was supplemented as a sole carbon source [5]. Our preliminary study aimed to evaluate SBS1-7's ability to degrade phenanthrene (3 aromatic rings).

To quantify the residual phenanthrene, solvent extraction using *n*-hexane was performed by adding the solvent into the sample. The mixture was then vortexed and centrifuged, after which the clear upper solvent phase was collected for GC-FID analysis. However, the sample collected on day 7 formed highly stable emulsion even after centrifugation, hindering quantification of the residual phenanthrene.

This study aimed to explore an extraction technique which can destabilize the Pickering emulsion formed by *M. esteraromaticum* SBS1-7. Various parameters, including temperature, centrifugation force, NaCl supplementation, and types of extracting solvent, were investigated to evaluate their effects on emulsion stabilization.

2. MATERIALS AND METHODS

2.1 Chemicals and media

All chemicals used in this study were purchased from well-known manufacturers, including Sigma-Aldrich (Germany), Asia Pacific Specialty Chemical (Australia), and Tokyo Chemical Industry (Japan). Media components were purchased from Lab M (UK). Lysogenic broth (LB) was used for bacteria propagation and maintenance. Mineral salt medium (MM) modified from Rahul [6] was used for cell suspension preparation.

2.2 Preparation of cell suspension

A single colony of *M. esteraromaticum* SBS1-7 was transferred into 5 mL of LB medium (in 50 mL-conical tube). The culture was incubated at 30°C, 120 rpm for 24 h. Next, the inoculum was used to inoculate 50 mL of LB medium (in 250 mL-baffle flask) at 2% v/v. Then, the culture was incubated at 30°C, 120 rpm for 24 h. Cells were harvested by centrifugation at 4,372xg, 4°C for 8 min, washed with sterile normal saline solution (NSS; 0.85% NaCl), and resuspended with MM to obtain a cell suspension with an OD₆₀₀ of 5.0.

2.3 Emulsion preparation

To prepare a highly stable emulsion, 1 mL of cell suspension was transferred into 4 mL-glass vial. Phenanthrene stock (3,000 ppm in DMSO) was added to the cell suspension to obtain a final concentration of 60 ppm. The glass vial was sealed with a butyl rubber septum and screw cap. The culture was incubated at 30°C, 120 rpm for 7 days. The cell suspension was transferred into a 2 mL-microcentrifuge tube. Then, 200 μ L of *n*-tetradecane or *n*-hexane was added. The sample was mixed by vortex for 5 min.

2.4 Effect of factors on emulsion destabilization

The emulsion was used to evaluate factors affecting the destabilization of the Pickering emulsion stabilized by SBS1-7. Tested parameters are summarized in Table 1.

Table 1. Parameters and condition used in this study

Parameter	Value/Type
Temperature	4 and 8°C
Centrifugation time	5 and 10 min
NaCl supplementation	100, 300, and 750 mM
Types of extracting solvent	<i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, <i>n</i> -dodecane, <i>n</i> -tridecane, and <i>n</i> -tetradecane

2.5 Safranin O staining

To clearly observe the bacterial cells under microscope, cells were stained with Safranin O as follows. The cell suspension ($OD_{600} = 5.0$) was diluted with MM to obtain OD_{600} of 0.8. Subsequently, cells were harvested from 1 mL of cell suspension by centrifugation at 11,357xg for 10 min. Safranin O was added for cell staining, followed by gentle mixing, and the suspension was left under static condition for 10 min. After that, the stained cell pellet was washed with sterile NSS until the supernatant became clear or light red. The stained cell pellet was resuspended in 1 mL of MM before being transfer into a glass vial, after which 1 mL of *n*-tetradecane was added. The mixture was mixed by vortex for 2 min. The emulsion formed in the upper solvent phase was collected and visualized under a light microscope.

3. RESULTS AND DISCUSSION

3.1 Effect of temperature

To investigate the effect of temperature on the emulsion formed by SBS1-7, the emulsion was incubated at 4 and 8°C. After 2 days, the emulsion remained stable similar to the control experiment kept at a room temperature. Nonetheless, some of the emulsion stored at 4°C exhibited partial destabilization of the emulsion as shown in Figure 1.

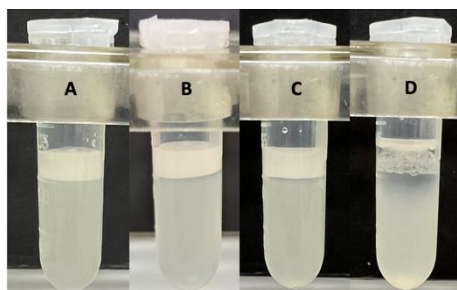


Figure 1. Emulsion stability after a storage for 48 h at (A) room temperature, (B) 8°C, and (C and D) 4°C

3.2 Effect of centrifugation time and NaCl supplementation

Centrifugal separation has been widely used for phase separation by accelerating sedimentation under gravity force [7]. This technique was expected to promote phase separation by sedimenting SBS1-7 cells that stabilized the Pickering emulsion. Sodium chloride (NaCl), on the other hand, is a monovalent

salt commonly used to enhance emulsion stability. NaCl has been reported to increase interfacial absorption, thereby improving emulsion stability [8]. However, excessive NaCl concentration may increase surface tension and reduce surface hydrophobicity, causing the emulsion destabilization [9]. In this study, the emulsion with different disperse phase (i.e., tetradecane and hexane) were used to evaluate the effects of centrifugation time and NaCl concentration on emulsion stability. Emulsion with tetradecane as a disperse phase was destabilized after supplementation with 100 mM NaCl and 10 min centrifugation. On the other hand, the emulsion from hexane exhibited lower stability and was completely destabilized after 5 min of centrifugation without NaCl supplementation (Figure 2). French [10] investigated the effect of NaCl concentration on the stability of Pickering emulsions stabilized by silica particles. The result showed that increasing NaCl concentration led to larger droplets diameters and increased free silica sedimentation, indicating the lower absorption of silica at the oil-water interface, which promoted the particle flocculation and emulsion destabilization.

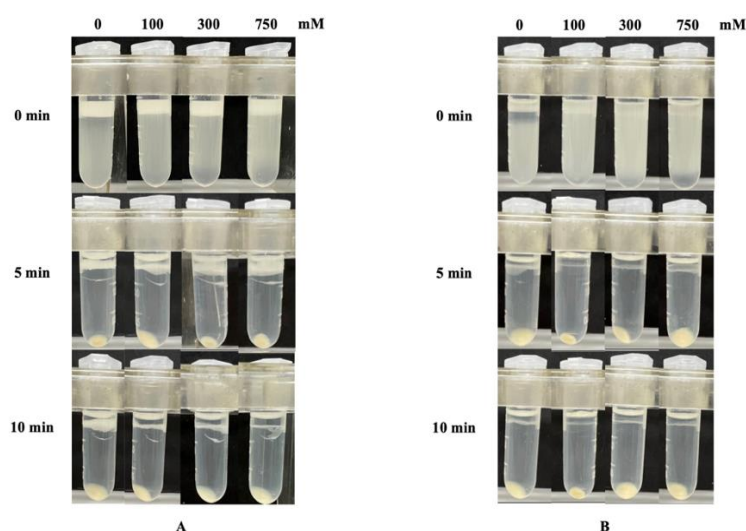


Figure 2. Effect of centrifugation time and NaCl supplementation on Pickering emulsion formed by SBS1-7 using (A) tetradecane and (B) hexane as a disperse phase

3.3 Effect of extracting solvent

To investigate the effect of extracting solvent on emulsion stability, alkanes with carbon atoms ranging from 6 to 14 (C₆-C₁₄) were used for emulsion formation. Stable emulsions were observed in all alkanes tested (Figure 3). In addition, to evaluate emulsion stability, the emulsions formed with each extracting solvent were left under static condition for 24 h, and the emulsification index (EI₂₄) was determined. EI₂₄ was calculated as the percentage of the ratio of emulsion layer height to total liquid height. The result showed that EI₂₄ increased significantly with an increase in carbon atoms of the alkane used (Figure 4). Alkanes with higher carbon numbers exhibit higher viscosity [11]. Increased viscosity can enhance emulsion stability by reducing emulsion fluidity and mobility, thereby decreasing the collision between emulsion droplets and consequently lowering the coalescence rate [12]. Moreover, the lower water solubility of longer chain alkanes significantly reduced Ostwald ripening [13], thereby enhancing emulsion stability.

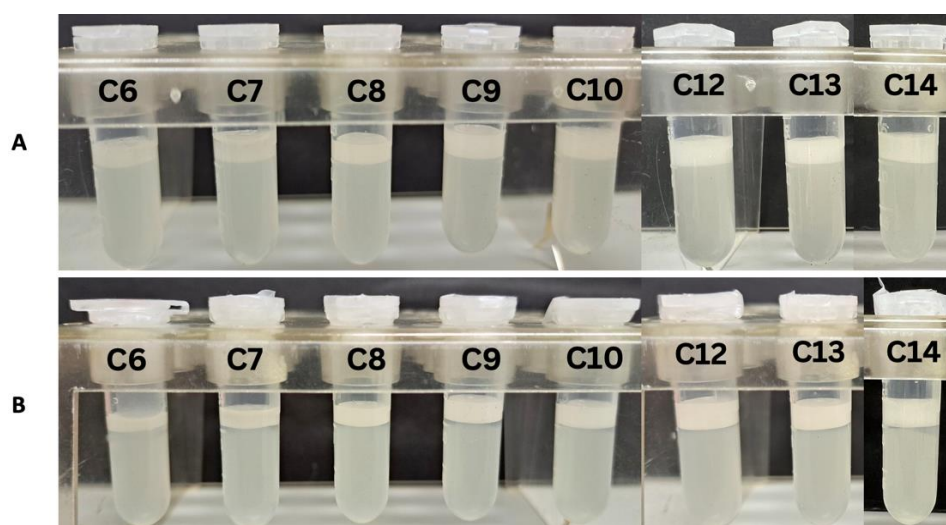


Figure 3. Emulsion stability with alkanes as the disperse phase after (A) 0 hour and (B) 24 hours.

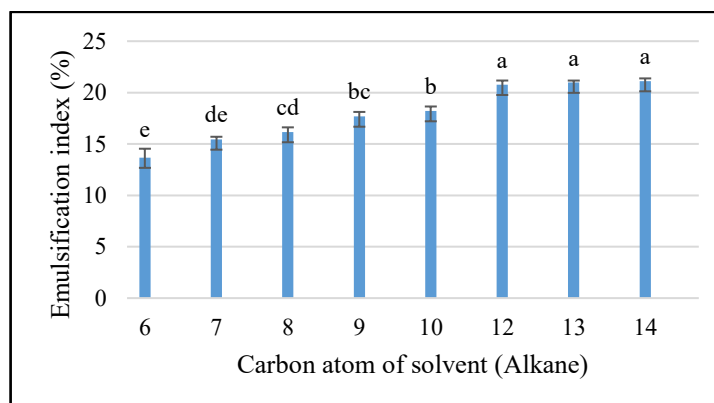


Figure 4. Emulsification index (EI₂₄) of emulsion formed by SBS1-7 with different alkanes

3.4 Safranin O staining

Pickering emulsion is an emulsion stabilized by solid particles, in contrast to traditional emulsions, which are stabilized by surfactants [14]. To confirm the ability of *M. esteraromaticum* SBS1-7 to stabilize Pickering emulsion, the emulsion stabilized by safranin O-stained SBS1-7 cells was visualized under a light microscope. Since safranin O dissolves only in the aqueous phase, the tetradecane would appear colorless. Figure 5 revealed how Safranin O-stained SBS1-7 cells surrounded tetradecane droplets, thereby preventing droplets coalescence. This observation confirms that SBS1-7 migrated to the water-tetradecane interphase and stabilized the Pickering emulsion.

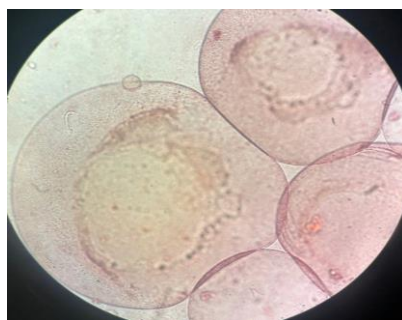


Figure 5. Tetradecane droplets surrounded by Safranin O-stained SBS1-7 cells

4. CONCLUSIONS

M. esteraromaticum SBS1-7 exhibited the ability to stabilize Pickering emulsion. This property interfered with the quantification of residual phenanthrene because the emulsion formed during the extraction step was highly stable, thereby hindering recovery of the solvent phase. Emulsion formation was observed with all extracting solvents evaluated in this study. Among the solvents tested, *n*-hexane was the most suitable solvent for phenanthrene quantification due to its relatively low emulsion stability, whereas *n*-tetradecane was the most suitable solvent for studying emulsion stability.

5. ACKNOWLEDGEMENT

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Identification and Functional Analysis of Serine Racemase Genes in D-Serine Producing Lactic Acid Bacteria

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ABSTRACT: D-Serine (D-Ser) functions as a co-agonist of N-methyl-D-aspartate glutamate receptors in the mammalian brain, and plays critical roles in cortical memory and learning, cerebellar motor skill acquisition, and various neurological disorders. Given these functions, D-Ser holds potential as a unique functional food ingredient; however, it is not approved as a food additive in Japan. On the other hand, D-Ser produced by lactic acid bacteria during food processing is considered a natural product and is exempt from such regulations. In this study, we screened D-Ser-producing lactic acid bacteria and identified *Lactococcus* sp. strain 0710, which exhibits serine racemase activity. The serine racemase gene was cloned and expressed in *Escherichia coli*, and the purified enzyme was characterized. Its primary structure shows homology to bacterial alanine racemases dependent on pyridoxal 5'-phosphate (PLP), a coenzyme form of vitamin B6. The purified enzyme catalyzes the PLP-dependent racemization of both alanine and serine. To clarify the reaction characteristics of this enzyme, the effects of temperature and pH on its activity were evaluated. The results confirmed that this enzyme exhibited the highest activity at 40°C and pH 7.5. Notably, the enzyme features a unique subunit structure: both blue native PAGE and gel filtration chromatography indicated a tetrameric form, contrasting with most previously reported dimeric alanine and serine racemases. Here, we report the enzymological properties of the Ala/Ser racemase from *Lactococcus* sp. 0710 and discuss the structural basis enabling its serine racemization activity.

Keywords: Alanine Racemase, D-amino acid, D-Serine; Serine Racemase

Roles of Aspartate-Beta-Decarboxylases in Aspartate Metabolism of *Acetobacter pasteurianus* SKU1108

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ABSTRACT: Aspartic acid is an important intermediate product in amino acid metabolism and plays various physiological roles in cells. Aspartic acid is involved not only as a constituent of proteins, but also in energy metabolism. Previous studies have shown that acetic acid bacterium *Acetobacter pasteurianus* consumed L-aspartic acid (Asp) and produced L-alanine (Ala) during vinegar brewing process. It is also known that the same amino acid conversion occurs in soy sauce lactic acid bacteria (*Tetragenococcus halophilus*). In *T. halophilus*, the intracellular Asp is decarboxylated by aspartate- β -decarboxylase (AspD) and consumes intracellular H⁺, resulting in the production of Ala and CO₂. The pH difference between inside and outside the cell is used for ATP generation. We hypothesized that the similar ATP generation system coupling the conversion of Asp to Ala could happen in the *A. pasteurianus*. Genome analysis of *A. pasteurianus* SKU1108 identified two putative AspD homologs, AspD1 (529 aa) and AspD2 (538 aa), which shared 62.7% amino acid sequence identity. In addition, a putative aspartate transporter, AspT (563 aa), was identified. Such an efficient energy-generating pathway might be a strategy for acetic acid bacteria to obtain energy efficiently during the fermentation process and is an important factor in understanding the metabolism of acetic acid bacteria. In the present study, we constructed expression plasmid of AspD1 gene and obtained the recombinant enzyme in *Escherichia coli*. Recombinant AspD1 was highly purified using Nickel affinity column chromatography and its enzymatic properties, including the optimal pH and temperature stability, were characterized. The optimal pH and temperature of AspD1 were pH 6.0 and 40°C, respectively. These findings suggest the involvement of AspD1 in Asp metabolism in *A. pasteurianus*.

Keywords: Aspartate- β -decarboxylase; Aspartic acid metabolism; *Acetobacter pasteurianus* SKU110

Structural Basis for the High Catalytic Efficiency of the Iron-Dependent Xylose Isomerase from *Lactococcus lactis* IO-1

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ABSTRACT: Xylose isomerase (XI) is a metalloenzyme found in bacteria and some fungi that converts the pentose sugar D-xylose to D-xylulose through a reversible reaction. Group II XIs are characterized by an extended N-terminus, and complete structural interactions between the core domains and the terminal tails of adjacent subunits are essential to the function of these XIs. Due to its ability to isomerize D-xylose to D-xylulose, XIs offer the distinct advantage for heterologous expression in *Saccharomyces cerevisiae* for developing microbial cell factories that produce several high-value products. Until now, XI from *Piromyces* sp. E2 has been widely used as a reference enzyme due to its strong catalytic activity and efficient expression in yeast. However, the *Lactococcus lactis* IO-1 XI fundamentally challenges this benchmark with a documented K_m of 2.25 mM and a k_{cat} of 184 s⁻¹ that yields vastly superior catalytic velocity. Notably, our crystallographic analysis revealed that this enzyme uniquely coordinates with iron within its active site, offering a potential structural rationale for its high catalytic efficiency. This was further confirmed by metal ion replacement assays, in which the relative activity increased with ferrous ions (Fe²⁺). To the best of our knowledge, this represents the first report of an iron-dependent xylose isomerase. This novelty paves the way for heterologous expression in *S. cerevisiae* for accelerated bioethanol production and sustainable green chemistry. Further anomalous difference Fourier mapping will provide insights on the iron occupancy inside the structural conformation of the active site.

Keywords: Bioethanol production, Metalloenzyme, Xylose isomerase

Development of a Paper-Based Test Strip for Colorimetric Detection of Cholinesterase Activity in Canine Serum

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ABSTRACT: Organophosphate and carbamate pesticide poisoning is a common cause of canine intoxication, and is reflected biochemically by a marked reduction in serum cholinesterase activity. Conventional detection relies on Ellman's assay, which requires freshly prepared reagents, temperature control, and laboratory spectrophotometry, limiting its use in field settings. This study aimed to develop a portable paper-based test strip for the colorimetric detection of reduced cholinesterase activity following toxicant exposure in blood samples of dogs presented for necropsy. The strip employed a two-zone dip-format design in which 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was immobilized in a chitosan gel on one zone and butyrylthiocholine iodide (BuChI) substrate was stabilized with polyvinylpyrrolidone (PVP-40) and sucrose on a separate zone, with a physical gap separating the two reagents to prevent premature reaction during storage. Upon application of serum mixed with phosphate buffer (pH 8.0), active cholinesterase hydrolyzed BuChI to thiocholine, which reacted with DTNB to produce a yellow product quantified by image analysis (ImageJ). Key parameters were systematically optimized, including DTNB concentration, incubation temperature, and sample volume. A DTNB concentration of 0.08 M dissolved in absolute ethanol provided the strongest and most stable signal, and observable colorimetric reaction within 2-3 minutes. The optimized prototype produced a clear, measurable yellow signal correlating with enzyme activity. Analytical validation of reduced cholinesterase activity is planned by spiking healthy canine serum in vitro with organophosphate and carbamate and comparing the strip against the reference Ellman's assay, with clinical canine serum samples to be incorporated subsequently. The shelf-life stability of the assembled strip will also be determined under room-temperature storage. These findings demonstrate the feasibility of a rapid, low-cost, and field-deployable strip for screening cholinesterase activity, supporting its potential application in the preliminary screening of pesticide poisoning in dogs.

Keywords: Canine serum, Cholinesterase, Ellman's assay, Paper-based strip, Pesticide poisoning

Valorization of Soybean Processing Residue as an Alternative Nitrogen Source for Enhanced Dark Fermentative Biohydrogen Production

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ABSTRACT: Dark fermentative biohydrogen production from agricultural residues has emerged as a promising strategy for sustainable renewable energy generation. Among the critical factors affecting hydrogen fermentation, nitrogen sources play an essential role in microbial growth, intracellular metabolism, enzyme biosynthesis, and substrate degradation efficiency. However, conventional nitrogen supplements, such as yeast extract and peptone, are expensive and limit the economic feasibility of industrial-scale biohydrogen production. This study investigated soybean meal (SBM), an abundant agro-industrial byproduct, as an alternative low-cost organic nitrogen source for biohydrogen production by *Thermoanaerobacterium saccharolyticum* TH05 under thermophilic anaerobic conditions at 55 °C. Soybean meal contained high levels of protein (47.78%), nitrogen (7.32%), hemicellulose (9.32%), and cellulose (5.76%), indicating its suitability as both a nitrogen source and a carbon precursor. Efficient degradation of these complex polymers by extracellular enzymes is essential for releasing fermentable sugars and supporting biological hydrogen production. The results revealed that SBM concentrations ranging from 0% to 8% (w/v) were evaluated, with 4% SBM achieving the highest hydrogen yield of 15.3 ± 0.47 mL H₂ g⁻¹ TS after 120 h. This was accompanied by increased ethanol (1.47 g L⁻¹) and butyric acid (1.70 g L⁻¹) production. Residual nitrogen decreased from 1.52 to 0.04 g L⁻¹ in the 4% SBM treatment, indicating efficient nitrogen assimilation. Enhanced amylase, cellulase, and xylanase activities further promoted carbohydrate hydrolysis and substrate conversion. In contrast, excessive SBM supplementation reduced hydrogen productivity, likely due to nitrogen overload and metabolic imbalance. These findings demonstrate the strong potential of soybean meal as a sustainable and cost-effective organic nitrogen source for thermophilic biohydrogen production while simultaneously promoting agricultural waste valorization.

Keywords: Biohydrogen, Dark fermentation, Soybean meal, Organic nitrogen source, *Thermoanaerobacterium saccharolyticum*, Waste valorization

1. INTRODUCTION

Hydrogen presents a pivotal solution to reducing carbon dioxide emissions and combating climate change^[1]. Boasting a high energy density (120 MJ kg⁻¹) and exceptional versatility, it can replace fossil fuels in energy-intensive sectors such as transportation, chemical production, and manufacturing^[2]. Previous research has shown that using industrial waste as a feedstock can lead to sustainable and cost-effective biohydrogen production. Consequently, current research focuses on utilizing agricultural waste

or lignocellulosic materials to replace carbon, nitrogen, and mineral sources in biohydrogen production^[3]. Optimizing nitrogen sources represents another crucial global development for biohydrogen production. Current nitrogen sources are expensive, required in large quantities, and typically derived from animal or plant tissue extracts like peptone, yeast, beef, or malt extract^[4]. To reduce process costs at an industrial scale, these should be replaced with readily available, cost-effective, and protein- or carbohydrate-rich alternatives^[5]. Nitrogen-containing nutrients—including ammonia, vitamins, and proteins—are vital for cell replication, maintenance, intracellular metabolism, and the enzymatic digestion of substrates, thereby significantly influencing fermentation efficiency^[6]. Consequently, utilizing appropriately treated, ammonia-rich residues such as animal manure is highly viable; they are widely accessible, and their repurposing mitigates the high disposal costs and pollution risks associated with agricultural waste^[7]. For this reason, it is necessary to thoroughly investigate the nitrogen requirements of hydrogen-producing strains and their effects on bacterial and enzyme metabolism.

Driven by the need to minimize chemical dependency and environmental pollution, research has increasingly focused on alternative bionitrogen sources, with soybean meal (*Glycine max* L.) (SBM) emerging as a prominent candidate. As the primary by-product of soybean oil extraction, SBM is a vital crop globally and within Thailand, widely utilized across animal nutrition, food processing, and biofuels due to its low cost, high protein content (40%–50%), and well-balanced amino acid profile^[8]. Processing involves grinding, drying, and thermally treating soybeans to extract oil, leaving behind a nutrient-rich solid residue^[9]. Beyond proteins, SBM contains structural carbohydrates that can serve as precursors for hydrogen production, including starch (4.66%–7%), cellulose (2%–4%), and hemicellulose (8%–10%)^[10]. While various components of the soybean plant have been investigated for biofuels—such as SBM serving as a core substrate for *Thermotoga maritima* (0.0326 g H₂ g⁻¹ glucose)^[11], while soybean straw yielded 60.2 ml H₂ g⁻¹ soybean straw using anaerobic bacteria^[12]. However, there have been no clear reports of using SBM as a nitrogen source for hydrogen production. Therefore, developing and optimizing culture media formulations using SBM as a nitrogen alternative represents a vital frontier in enhancing dark anaerobic biohydrogen fermentation.

2. MATERIALS AND METHODS

2.1 Determination of SBM

The chemical composition of SBM was determined using an acid hydrolysis-based procedure. Briefly, 0.1 g of dried SBM was mixed with 2 mL of 72% (w/v) H₂SO₄ and incubated at room temperature for 1 h. The hydrolysate was then diluted with 16 mL of distilled water and autoclaved at 121 °C for 1 h. After cooling, the mixture was filtered through a pre-weighed G2 glass filter. The residue was washed with Milli-Q water, dried at 100 °C to constant weight, and weighed. Glucose and xylose in the filtrate were analyzed by HPLC. Protein, ash, fat, and nitrogen contents were analyzed by AOAC method^[13].

2.2 Culture medium

Thermoanaerobacterium saccharolyticum strain TH05 from microbial collection of Excellent Center of Enzyme Technology and Microbial Utilization was cultivated in Basal medium (BM7-CO₂) 1 L contained 0.45g K₂HPO₄, 0.45g KH₂PO₄, 0.9g NaCl, 0.9g (NH₄)₂ SO₄, 5g Yeast extract powder, 0.12g CaCl₂·2H₂O, 0.18g MgSO₄·7H₂O, 1% Resazurine 50 µL, 4g Na₂CO₃, 0.5g cysteine hydrochloride. The BM7-CO₂ medium was adjusted to pH 7.2, and then flushed with CO₂ gas to obtain anaerobic condition. The medium was autoclaved at 121 °C for 15 minutes and kept for use^[14]. Soybean meal 3% (w/v) was used as the carbon source.

5 mL of microbial suspension at exponential growth phase ($OD_{660}=0.5$) was transferred into 45 mL BM7 medium supplemented with 3% glucose as a carbon source to a final concentration of 30 g L⁻¹ for inoculum. Medium was dispensed anaerobically in a 120 mL serum bottle capped with a rubber stopper and closed with aluminum caps. The headspace was replaced with carbon dioxide gas. Gas compositions were analyzed by GC- TCD after 24 h. To investigate the potential of substrate utilization of strain TH05 that comprised 3% (w/v) Glucose, Xylose, Starch, Cellulose, Xylan and SBM.

2.3 Hydrogen production from SBM

Batch hydrogen production experiments were performed in a SBM at a final concentration of 0 - 8% (w/v) (for nitrogen source as 0.07 - 0.28% nitrogen), which was used as the carbon and nitrogen source. The BM7-CO₂ was adapted without (NH)₄ SO₄ and Yeast extract powder. The batch fermentation was performed in 50 mL working volume, at 55°C, 120 h. Each experiment detected the gas composition and collected samples for organic acid, enzyme production, and nitrogen remaining.

2.4 Analytical methods

“Volatile fatty acids and alcohols

Preparation of liquid samples was conducted by centrifuging the fermentation broth at 10,000 rpm for 5 min, then the supernatants were filtered through a 0.2 µm nylon membrane prior. The supernatant was determined alcohol (ethanol, butanol), and organic acid by Gas Chromatography - Flame Ionization Detector (GC-FID).

Biogas composition

Hydrogen and carbon dioxide were measured by using a gas chromatograph equipped with a thermal conductivity detector (TCD). The volume of biogas was measured with a wet syringe and the composition was analyzed by gas chromatography GC-8APT with thermal conductivity detector (TCD), Shimadzu, Japan. Operating temperature was set at 120°C for injector and detector. Argon as a carrier gas and the activated charcoal packed column were used for the analysis.

Enzyme activity

The Amylase, Cellulase and Xylanase activity (U mL⁻¹) were measured by estimation of reducing sugars liberated from substrate Starch, CMC and Xylan, respectively. The substrate and enzyme solutions in a water bath at 50°C, and detected reducing sugar by Somogyi&Nelson reagent. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of reducing sugar in 1min.

Statistical Analysis

Data are expressed as the mean ± SD of three independent experiments. Statistical differences among treatment groups were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. SBM composition

SBM is an abundant byproduct after oil extraction. Soybean meal is widely used across the food industry, animal feed, and biofuel production^[15]. It is by far the dominant protein supplement used in livestock, and SBM also contains a significant amount of fiber and protein. The composition of SBM

may vary depending on the origin and cultivar of soybean, environmental growing conditions, and processing conditions. On the wet weight basis, SBM consists of 48–50% protein, 30–32% carbohydrate, 5–6% ash, and 2–3% fat^[16]. Composition of the carbohydrate components is given in Table 1.

Table 1. Characterization of SBM.

Composition	Typical content (% w/w)	
	Loman and Ju, 2016	This study
Cellulose	2 - 4	5.76 ± 0.36
Hemicellulose	8 - 10	9.32 ± 1.03
Lignin	0.5 - 1	0.34 ± 0.03
Starch	0.5 - 1.5	2.37 ± 0.15
Pectin	10 - 12	9.82 ± 1.54
Soluble oligosaccharide	10 - 12	7.43 ± 1.69
Protein	50 - 52	47.78 ± 1.65
Ash	5-6	9.82 ± 1.54
Fat	2-3	ND
Nitrogen (N)	7.0 – 7.7	7.32 ± 0.87

3.2. Biohydrogen performance of *T. saccharolyticum* strain TH05

T. saccharolyticum is a thermophilic, anaerobic bacterium highly prized in biotechnology for its ability to ferment a wide array of sugars (including hexoses and pentoses) into hydrogen and other metabolites. Through dark fermentation, it efficiently converts biomass-derived compounds into clean energy^[17]. This study showed hydrogen production from monosaccharide glucose and xylose at 26.68±1.06 and 19.73±0.81 mL H₂ g⁻¹ TS from a 50 mL working volume, respectively (Table 2.).

Starch produced the hydrogen yield (16.96±0.39 mL H₂ g⁻¹ TS), followed by xylan (15.14±0.01 mL H₂ g⁻¹ TS) and SBM (10.82±1.76 mL H₂ g⁻¹ TS). Xylan and soybean meal contain more structurally complex hemicellulosic and lignocellulosic components that require multiple hydrolytic enzymes for degradation. Nevertheless, the relatively high hydrogen production from soybean meal demonstrates the capability of *T. saccharolyticum* TH05 to utilize untreated agricultural biomass efficiently. Soybean meal not only supplied fermentable carbohydrates but also provided organic nitrogen, amino acids, vitamins, and minerals that supported microbial growth and enzyme production. These results indicate that complex agricultural residues can serve as promising low-cost substrates for thermophilic dark fermentation, particularly when microorganisms possess strong extracellular hydrolytic enzyme systems capable of converting polysaccharides into bioavailable sugars for hydrogen production.) (Table 2.).

Table 2. Hydrogen production and yields, and metabolites from 3% (w/v) various substrates by *Thermoanaerobacterium saccharolyticum* strain TH05 in BM7-CO₂ medium (initial pH 7.0) under anaerobic condition at 55 °C (working volume 50 mL)

Substrates	Cumulative H ₂ content (%)	Cumulative H ₂ production (mL ⁻¹)	Cumulative H ₂ yields (mL H ₂ g ⁻¹ TS)	Metabolites (g L ⁻¹)			
				Ethanol	Butanol	Acetate	Butyrate
Glucose	40.02±0.79 ^a	190±1.60 ^a	26.68±1.06 ^a	1.92±0.05 ^a	0.19±0.02 ^a	1.54±0.43 ^a	1.39±0.43 ^a
Xylose	29.60±0.36 ^a	150±1.21 ^a	19.73±0.81 ^a	1.30±0.08 ^b	0.18±0.01 ^b	0.78±0.21 ^b	0.50±0.20 ^c
Starch	25.44±0.14 ^b	140±0.59 ^b	16.96±0.39 ^b	1.85±0.10 ^a	0.28±0.01 ^a	0.43±0.02 ^b	0.76±0.05 ^b
Xylan	22.77±0.05 ^b	130±0.01 ^b	15.14±0.01 ^b	0.57±0.65 ^c	0.08±0.09 ^c	0.00±0.00 ^d	0.00±0.00 ^d
Cellulose	3.31±143 ^d	45±0.96 ^d	2.21±0.64 ^d	0.71±0.01 ^c	0.09±0.01 ^c	0.10±0.08 ^c	0.72±0.17 ^b
Soybean meal	16.23±0.06 ^c	120±2.65 ^c	10.82±1.76 ^c	0.83±0.11 ^b	0.17±0.01 ^b	0.94±0.04 ^a	0.33±0.09 ^c

Note: Different lowercase letters above the bars indicate significant differences among treatments based on one-way ANOVA followed by Tukey's HSD test ($p < 0.05$).

3.3. Batch dark thermophilic hydrogen production using SBM

Nutrients or micronutrients must be provided as a supplement for optimum microbial activities for biohydrogen conversion from carbohydrate-rich substrates^[18]. SBM supplementation significantly influenced hydrogen production by *T. saccharolyticum* TH05 (Figure 1 A–D.). Among all tested conditions, 4% SBM produced the highest cumulative hydrogen yield, reaching 8.8±0.34 to 15.3±0.47 mL H₂ g⁻¹ TS at 24 to 120 h, respectively, which was markedly higher than the control (0% SBM) and other SBM concentrations. Hydrogen production rapidly increased during the first 24 - 48 h, indicating active microbial metabolism and efficient substrate utilization. In contrast, excessive SBM loading (6–8% w/v) resulted in lower hydrogen yields, with final yields of 11.5±1.57 and 12.0±0.89 mL H₂ g⁻¹ TS, respectively, despite higher nitrogen availability, suggesting that excessive nitrogen negatively affected hydrogen-producing metabolism. Nitrogen analysis showed that residual nitrogen under 4% SBM decreased from 1.52 g L⁻¹ at the initial stage to only 0.04 g L⁻¹ after 120 h, indicating efficient nitrogen assimilation. Similarly, residual nitrogen under 2% SBM decreased from 0.66 to 0.07 g L⁻¹. However, substantial residual nitrogen remained in the 6% and 8% SBM treatments, with final concentrations of 0.82 and 1.65 g L⁻¹, respectively, reflecting excess nitrogen supply beyond microbial requirements. Metabolite analysis further demonstrated that 4% SBM promoted the highest ethanol (1.47 g L⁻¹). In comparison, 2% SBM produced lower concentrations of ethanol (1.34 g L⁻¹) and butyric acid (1.23 g L⁻¹), while 6–8% SBM showed intermediate metabolite production.

The enhanced hydrogen production under moderate SBM supplementation was also correlated with increased extracellular enzyme activities. Amylase, cellulase, and xylanase activities were highest at 4% SBM, suggesting improved hydrolysis of complex carbohydrates into fermentable sugars that supported hydrogen production. However, enzyme activities decreased at higher SBM concentrations, possibly due to substrate inhibition or nitrogen overload. The relatively low butanol concentrations observed under all conditions (0.21 - 0.57 g L⁻¹) indicated that fermentation remained primarily in the acidogenic phase rather than shifting toward solventogenesis. Overall, the results demonstrate that SBM can serve as an effective low-cost organic nitrogen source for biohydrogen production, with 4% SBM providing the optimal balance between nutrient availability, enzymatic hydrolysis, and metabolic activity for maximizing hydrogen yield by *T. saccharolyticum* TH05.

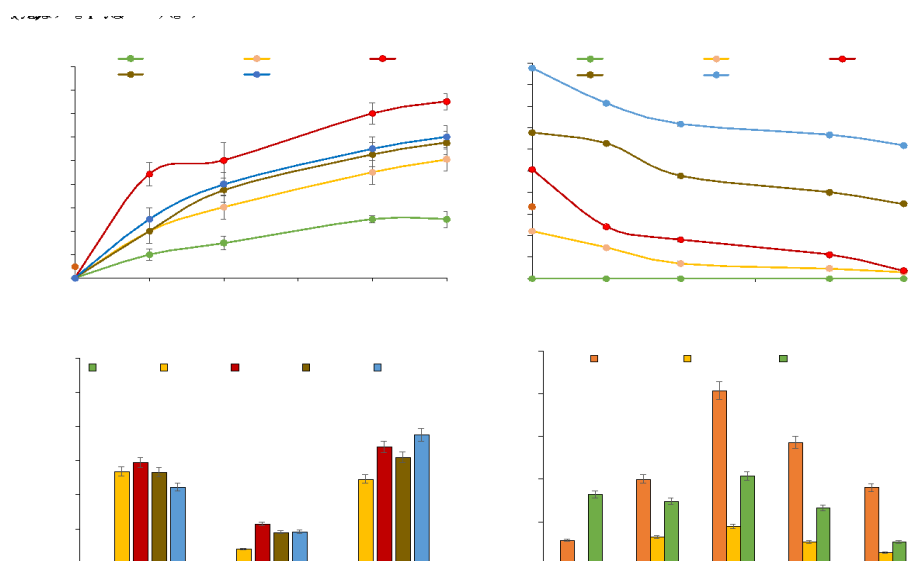


Figure 1. Hydrogen production (A), nitrogen content (B), alcohol and organic acid production (C) and enzyme activity (D) from SBM using dark fermentation under anaerobic thermophilic at 55 °C (0-120 h) by *T. saccharolyticum* TH05.

Different lowercase letters above the bars indicate significant differences among treatments based on one-way ANOVA followed by Tukey's HSD test ($p < 0.05$).

4. CONCLUSIONS

SBM demonstrated strong potential as an inexpensive and sustainable nitrogen source for thermophilic dark fermentative hydrogen production by *T. saccharolyticum* TH05. Moderate SBM supplementation significantly enhanced hydrogen production, metabolite formation, and extracellular enzyme activities. The optimum condition was achieved at 4% SBM, producing 15.3 ± 0.47 mL H₂ g⁻¹ TS with efficient nitrogen assimilation and increased metabolite formation and enzyme activities. Excessive SBM supplementation negatively affected hydrogen productivity due to excess residual nitrogen and possible metabolic inhibition. Overall, the study highlights the feasibility of utilizing soybean processing residue as an alternative nutrient source for cost-effective and environmentally sustainable biohydrogen production.

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Isolation and Identification of Thermoacidophilic Bacteria for Accelerated Food Waste Degradation

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ABSTRACT: Food waste is a major global environmental concern due to its contribution to greenhouse gas emissions, resource depletion, and economic losses. Conventional composting systems often generate unpleasant odors because they operate under neutral to alkaline conditions. This study aimed to isolate and identify thermoacidophilic bacteria with potential application in acidulocomposting, a composting process conducted under acidic and thermophilic conditions (pH 4.0–6.5 and 50–60°C) to improve degradation efficiency and reduce odor emissions. Six thermoacidophilic bacterial isolates were obtained from soil samples and screened for extracellular enzyme production on substrate-specific agar media at pH 4.5 and 50°C. Enzymatic activity was determined using the Enzymatic Index (EI). Xylanase activity was detected in all isolates, with BCMD42 exhibiting the highest activity (EI = 1.71). Cellulase, protease, and lipase activities were variably detected among the isolates, with G2.2 and H2.1 showing the highest cellulase (EI = 1.38) and lipase (EI = 2.00) activities, respectively, while no amylase activity was observed. Molecular identification based on full-length 16S rRNA gene sequencing revealed that isolate B313 was closely related to *Tuberibacillus calidus* (99.93% similarity), whereas isolates C1C, G2.2, H2.1, and C1A were closely related to *Alicyclobacillus pomorum* (99.06–99.50% similarity). In addition, isolate BCMD42 showed 98.64% similarity to *Alicyclobacillus herbarius*, suggesting that it may represent a genetically distinct strain within the genus *Alicyclobacillus*. These findings demonstrate the potential of thermoacidophilic bacteria as promising candidates for acidulocomposting by producing extracellular enzymes involved in the degradation of lignocellulosic and organic substrates. Their application may contribute to the development of more efficient and environmentally friendly food waste composting systems with reduced odor emissions.

Keywords: Acidulocomposting, Bacterial identification, Extracellular enzymes, Food waste degradation, Thermoacidophilic bacteria

1. INTRODUCTION

Food waste has become a critical environmental, economic, and social challenge worldwide as a consequence of rapid urbanization, population growth, and changing consumption patterns. According

to the Food and Agriculture Organization (FAO), approximately 1.3 billion tons of food are discarded as waste annually, representing nearly one-third of all food produced for human consumption [1]. The decomposition of food waste in landfills generated methane, a potent greenhouse gas, accounting for approximately 8–10% of global anthropogenic emissions [2]. In Thailand, increasing amounts of food waste from households, restaurants, markets, and food industries are commonly disposed as an open dump, creating environmental concerns [3]. Food waste is rich in biodegradable compounds such as carbohydrates, proteins, lipids, and lignocellulosic materials, making it a suitable substrate for microbial degradation and resource recovery. Various waste treatment technologies have been developed, such as anaerobic digestion, incineration, and composting. Among these, composting is widely recognized as an environmentally friendly and cost-effective method for converting organic waste into nutrient-rich soil amendments through microbial activity [4]. Conventional composting systems typically operate under neutral to slightly alkaline conditions and require frequent aeration, moisture adjustment, and turning to maintain process stability [5]. However, these systems often involve long processing periods and may generate ammonia and volatile sulfur compounds that cause unpleasant odors. Acidulocomposting is a modified composting approach that operates under acidic and thermophilic conditions (pH 3.5–6.5 and 50–70 °C) [6]. Under these conditions, ammonia volatilization and the growth of many odor-producing microorganisms are reduced, while elevated temperatures accelerate hydrolysis and enhance pathogen inactivation [6]. The successful implementation of acidulocomposting depends on thermoacidophilic microorganisms capable of thriving under low pH and high temperature. These organisms produce acid-tolerant and thermostable extracellular enzymes such as amylase, cellulase, xylanase, protease, and lipase, which catalyze the degradation of complex organic compounds present in food waste [7]. Such enzymes maintain catalytic activities under harsh conditions where enzymes from mesophilic microorganisms are often unstable.

Among thermoacidophilic bacteria, *Alicyclobacillus* has attracted considerable attention. Members of this genus are Gram-positive, endospore-forming bacteria that contain ω -alicyclic fatty acids, which confer enhanced membrane stability under acidic and thermal stress [8]. Several species have been reported to produce hydrolytic enzymes with potential applications in industrial bioprocesses and environmental biotechnology [8]. Although thermoacidophilic bacteria have been studied in various countries, information on indigenous thermoacidophilic bacteria in Thailand and their application in food waste degradation remains limited. Therefore, this study aimed to isolate thermoacidophilic bacteria from environmental samples, evaluate their extracellular enzyme production related to food waste degradation, and identify promising isolates by 16S rRNA gene sequencing for potential use in efficient, odor-reduced, and sustainable acidulocomposting systems.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation

Soil samples were collected from rice fields, palm plantations, and mangrove sediments using sterile tools and transported under cool conditions for immediate processing. For enrichment of thermoacidophilic microorganisms, 1 g of each soil sample was inoculated into 60 mL of selective BAT and YSG media (HiMedia Laboratories, Mumbai, India) adjusted to pH 4.5 and incubated at 50 °C with shaking at 160 rpm for 14 days. Direct isolation was also performed by suspending 0.5 g of soil in 0.85% saline solution, followed by serially dilution (10^0 – 10^{-3}) and spread plating on BAT and YSG agar plates. Plates were incubated at 50 °C for 48 h. Purified isolates were maintained on agar plates at 4 °C and preserve in 25% (v/v) glycerol at –80 °C for long-term storage.

2.2 Enzyme Production Test

Extracellular enzyme production was evaluated using Minimal Salt Medium (MSM) agar containing 1% specific substrates; starch for amylase, carboxymethyl cellulose (CMC) for cellulase, xylan for xylanase, skim milk with peptone for protease, and tributyrin for lipase activity. The basal medium consisted of KH_2PO_4 (1.0 g/L), K_2HPO_4 (1.0 g/L), NH_4NO_3 (1.0 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L), NaCl (0.5 g/L), CaCl_2 (0.02 g/L), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g/L), and agar (15–20 g/L), with the pH adjusted to 4.5 before sterilization at 121 °C for 15 min. Bacterial isolates were scraped from agar plates, suspended in sterile saline solution, and adjusted to an optical density (OD) of 0.5–1.0. The bacterial suspensions were spot inoculated onto the agar plates and incubated at 50 °C. Enzymatic activity was determined by the formation of hydrolysis halos surrounding the colonies. Starch hydrolysis was detected using iodine solution, cellulase and xylanase activities by Congo red staining followed by rinsing by 1 M NaCl rinsing, protease activity by clear zones on skim milk agar, and lipase activity by halo formation on tributyrin agar. Enzymatic activity was expressed as the Enzymatic Index (EI), calculated as the ratio of halo zone diameter to colony diameter.

2.3 Bacterial Identification

Gnomic DNA was extracted from bacterial isolates using a chloroform-based method involving cell lysis with lysozyme and sodium dodecyl sulfate (SDS), followed by chloroform–isoamyl alcohol extraction and ethanol precipitation. The purified DNA was resuspended in sterile distilled water or TE buffer for subsequent molecular analysis. The nearly full-length 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGG CTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), as previously described by Lane (1991) [10]. The PCR reaction mixture contained template DNA, primers, dNTPs, reaction buffer, MgCl_2 , and Taq DNA polymerase. PCR amplification was performed with an initial denaturation at 94–95 °C, followed by 30–35 cycles of denaturation, annealing, and extension, with a final extension at 72 °C. The amplified PCR products were sequenced, and the resulting 16S rRNA gene sequences were compared with reference sequences in the National Center for Biotechnology Information (NCBI) database using the BLASTn algorithm for bacterial identification based on sequence similarity [11].

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Thermoacidophilic Bacteria

A total 9 soil samples were collected for the isolation of thermoacidophilic bacteria. Following enrichment and direct isolation under acidic and thermophilic conditions, bacterial colonies were obtained on BAT and YSG media at pH 4.5 and 50 °C. Based on distinct colony morphology and stable growth under thermoacidophilic conditions, six isolates were selected for further characterization.

Among the selected isolates, C1C, H2.1, C1A, and G2.2 demonstrated stable growth under optimal pH 4.5 incubated at 50 °C for 48 h on YSG medium, whereas BCMD42 and B313 grew successfully on BAT medium. The ability of these isolates to grow well under acidic and elevated temperature conditions confirmed their optimal growth under thermoacidulophilic environments. Colony morphology of the isolates varied in terms of pigmentation, shape, texture, and size as summarized in Table 1. Most isolates formed round colonies with white, cream or cream-to- yellow pigmentation. Isolates B313, G2.2, and H2.1 exhibited shiny colony surfaces, whereas C1C showed a matte appearance. In contrast, C1A and BCMD42 showed erose colony morphology with dull and semi-matte textures, respectively. Colony sizes ranged from 0.3 to 0.6 mm, with C1A producing the largest colonies and BCMD42 the smallest.

Sequence analysis of PCR products and comparison with sequences available in the NCBI database revealed that isolates B313 showed 99.93% similarity to *Tuberibacillus calidus*, whereas isolates C1C, G2.2, H2.1, and C1A were closely related to *Alicyclobacillus pomorum* exhibiting 99.06-99.50% similarity. In addition, isolate BCMD42 demonstrated 98.64% similarity to *Alicyclobacillus herbarius* (Table 2.).

Table 1. Colony morphology of thermoacidophilic bacteria

Isolate	Colony color	Colony morphology	Colony texture	Colony size
B313	Cream to yellow	Round	Shiny	0.5 mm
C1C	White	Round	Matte	0.5 mm
G2.2	White	Round	Shiny	0.4 mm
H2.1	Cream to Yellow	Round	Shiny	0.5 mm
C1A	Cream	Erose	Dull	0.6 mm
BCMD42	Yellow	Erose	Semi matte	0.3 mm

Source: Colony morphology characteristics were described according to Harley and Prescott (2002) [9]. Type strain information and taxonomic nomenclature were referenced from the List of Prokaryotic Names with Standing in Nomenclature (LPSN).

Table 2. The identification of thermoacidophilic bacterial isolates using 16S rRNA gene sequencing

Isolated colonies	Closest species	% Similarity	Accession number
B313	<i>Tuberibacillus calidus</i> 606b	99.93	PZ444240
C1C	<i>Alicyclobacillus pomorum</i> NBRC 100861	99.21	PZ444239
G2.2	<i>Alicyclobacillus pomorum</i> NBRC 100861	99.50	PZ444243
H2.1	<i>Alicyclobacillus pomorum</i> NBRC 100861	99.50	PZ444242
C1A	<i>Alicyclobacillus pomorum</i> NBRC 100861	99.06	PZ444247
BCMD42	<i>Alicyclobacillus herbarius</i> C-ZJB-12-79	98.64	PZ444248

The phylogenetic analysis based on full-length 16S rRNA gene sequences further confirmed the taxonomic placement of the isolates within the genera *Alicyclobacillus* and *Tuberibacillus* (Figure 1a). Isolates C1C, C1A, G2.2, and H2.1 clustered closely with *A. pomorum*, while B313 formed a distinct cluster with *T. calidus*, indicating a close evolutionary relationship with this thermoacidophilic species. Isolate BCMD42 clustered with *A. herbarius* but formed a relatively distinct branch compared with other reference strains. The relatively lower sequence similarity of BCMD42 compared with commonly accepted species-level threshold (98.7-99.0%) suggests possible genetic divergence within the genus *Alicyclobacillus* and warrants further phylogenetic and phenotypic characterization. In addition, BCMD42 exhibited broad extracellular enzyme activities, particularly strong xylanase and cellulase activities, highlighting its potential application in acidulocomposting systems.

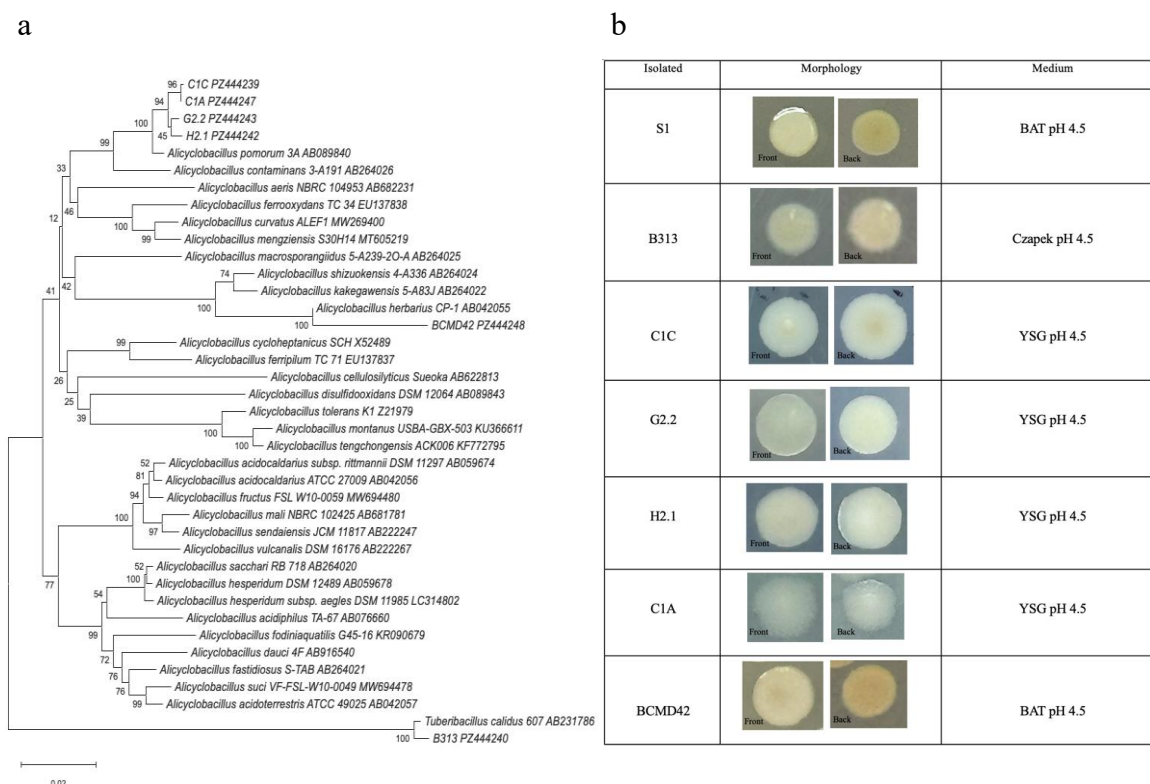


Figure 1. (a) Phylogenetic tree based on full-length 16S rRNA gene sequences showing the relationships between the thermoacidophilic bacterial isolates and closely related reference strains retrieved from the NCBI database. Phylogenetic analysis was conducted using the Neighbor-Joining method with 1,000 bootstrap replicates to evaluate the reliability of the tree topology. Numbers at the nodes represent bootstrap values (%) indicating confidence in branch clustering. The scale bar represents 0.02 nucleotide substitutions per site. **(b)** Colony morphology of the thermoacidophilic bacterial isolates grown on selective agar media under thermoacidophilic conditions.

3.2 Extracellular Enzyme Production of Thermoacidophilic Bacterial Isolates

Extracellular enzyme production profiles of the isolates are summarized in Tables 3 and 4. All isolates produced at least one extracellular enzyme, although the enzymatic profiles varied among isolates. Xylanase activity was detected in all isolates, indicating their potential for degradation of hemicellulosic compounds commonly present in plant-based food waste. Among the isolates, BCMD42 exhibited the highest xylanase activity with an EI value of 1.71, followed by C1C (1.57) and B313 (1.50).

Cellulase activity was observed in isolates G2.2, C1A, and BCMD42. G2.2 showing the highest cellulase activity with an EI value of 1.38, followed by BCMD42 (1.33) and C1A (1.25). Protease activity was detected only in B313 and H2.1, with H2.1 showing the highest protease EI value of 1.52. Lipase activity was observed in S1 and H2.1, where H2.1 demonstrated the strongest lipase activity with the highest EI value recorded in this study (2.00), followed by S1 (1.86). No amylase activity was detected in any isolate under the tested conditions.

Overall, the results demonstrated that isolates possess multiple extracellular enzymes associated with degradation of cellulose, hemicellulose, proteins, and lipids. In particular, BCMD42 and H2.1 exhibited strong enzymatic potential, suggesting their suitability for application in acidulocomposting and accelerated food waste biodegradation systems.

Table 3. Determination of enzymatic index of extracellular enzyme activities

Isolate	Amylase	Xylanase	Cellulase	Protease	Lipase
S1	-	1.25	-	-	1.86
B313	-	1.50	-	1.1	-
C1C	-	1.57	-	-	-
G2.2	-	1.25	1.38	-	-
H2.1	-	1.20	-	1.52	2
C1A	-	1.20	1.25	-	-
BCMD42	-	1.71	1.33	-	-

Table 4. Extracellular hydrolytic enzyme production of thermoacidophilic bacterial isolates

Isolate	Amylase	Xylanase	Cellulase	Protease	Lipase
S1	-	+	-	-	+
B313	-	+	-	+	-
C1C	-	+	-	-	-
G2.2	-	+	+	-	-
H2.1	-	+	-	+	+
C1A	-	+	+	-	-
BCMD42	-	+	+	-	-

Note: (+) Positive enzyme production; (–) no enzyme production detected.

4. CONCLUSIONS

This study successfully isolated and identified thermoacidophilic bacteria with potential applications in acidulocomposting for food waste degradation. All isolates exhibited extracellular enzyme activities involved in the degradation of cellulose, hemicellulose, proteins, and lipids, although their enzymatic profiles varied among isolates. Among them, isolates BCMD42 and H2.1 demonstrated the greatest enzymatic potential, indicating their suitability for efficient biodegradation under acidic and thermophilic conditions. Molecular identification based on 16S rRNA gene sequencing revealed that the isolates were closely related to *Tuberibacillus calidus*, *Alicyclobacillus pomorum*, and *Alicyclobacillus herbarius*. Overall, these findings demonstrate that thermoacidophilic bacteria are promising microbial agents for sustainable food waste management. Their application in acidulocomposting systems has the potential to enhance degradation efficiency while reducing odor emissions, thereby contributing to the development of more efficient and environmentally friendly composting technologies.

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Evaluation of Culture–Substrate Compatibility and Hydraulic Retention Time for Enhanced Biohydrogen Production

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ABSTRACT: Biohydrogen is a promising carbon-neutral energy carrier with high energy density. In Thailand, abundant organic waste and biomass residues remain underutilized. Dark fermentation provides an advantageous technology for energy conversion. This study evaluated biohydrogen production potential using pure cultures (*Klebsiella aerogenes* (a.k.a. *Enterobacter aerogenes*) and *Clostridium butyricum*) and anaerobic digestion (AD) sludge across various substrates, including glucose, glycerol, and oil, under mesophilic conditions in batch mode. Results indicated each culture achieved its maximum hydrogen yield when paired with its optimal substrate, with glucose and glycerol being optimal for *C. butyricum* (1.0 mol H₂/mol glucose) and *K. aerogenes*, respectively. Furthermore, this study developed a semi-continuous fermentation process to investigate the impacts of Hydraulic Retention Time (HRT) at 2 – 4 days. The results demonstrated that a 2-day HRT successfully stabilized the system pH, leading to a 47% yield increase to 1.5 mol H₂/mol glucose. This optimized yield represents 76% of the theoretical potential for the dominant butyrate metabolic pathway. These established operational conditions provide a critical baseline for biomass applications to evaluate commercial feasibility of industrial scale biohydrogen production in the future.

Keywords: Biohydrogen, Dark fermentation, Hydraulic retention time, Semi-continuous fermentation

Valorization of Corn Tassel Agro-Residues: Polyphenol Recovery and Cosmetic Potential via Conventional and Ultrasound-Assisted Extractions

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ABSTRACT: This study aimed to valorize corn tassel residues (T-CARs) as a sustainable source of bioactive ingredients for cosmetic applications. T-CARs were extracted using two extraction methods: conventional extraction (CE, 24 h) and ultrasound-assisted extraction (UAE, 30 min), utilizing three solvents (deionized water, 80% methanol, and 80% ethanol). The extracts were evaluated for their polyphenol (total phenolic–TPC and flavonoid–TFC contents) and cosmetic potentials, including antioxidant, anti-tyrosinase and anti-acnes activities. Additionally, cytotoxicity against human keratinocyte (HaCAT) cells was determined to ensure safety for cosmetics. Results indicated that CE generally yielded higher polyphenol contents compared to UAE. The significantly highest TPC was found in the methanolic and ethanolic CE extracts (791.9 ± 6.3 and 773.7 ± 17.2 mg GAE/g extract, respectively) while the ethanolic CE extract exhibited the highest TFC (570.8 ± 3.8 mg QE/g extract). Evaluation of cosmetic potential showed a diverse bioactivity profile among the extracts. The ethanolic UAE extract showed the highest ABTS and FRAP antioxidant activities ($4,171.5 \pm 47.8$ and 749.3 ± 15.4 mg TEAC/g extract, respectively; $p < 0.05$), whereas the methanolic and aqueous CE extracts exhibited the highest DPPH scavenging (756.8 ± 21.7 mg TEAC/g extract; $p < 0.05$) and anti-tyrosinase activity (23.9 ± 3.6 %inhibition; $p < 0.05$), respectively. Furthermore, the ethanolic CE extracts demonstrated significant anti-microbial efficacy against *Cutibacterium acnes* as anti-acne (MIC 12.5 mg/mL and MBC 25.0 mg/mL). The ethanolic corn tassels CE extract showed no toxicity at concentrations of 0.5 and 1.0 mg/mL ($> 90\%$ cell viability). In conclusion, this study validates the valorization of T-CARs, especially the ethanolic CE extract, as safe and promising bioactive ingredients for cosmetic applications.

Keywords: Agro-residues, Corn Tassels, Cosmetic, Polyphenols, Valorization

1. INTRODUCTION

Corn (*Zea mays*), known as maize, is one of the main causes of the smog crisis, and tiny particle matter (e.g., PM 2.5) that recur yearly, affecting health problems such as respiratory, cardiovascular, skin and eye diseases in Northern Thailand [1]. Approximately 60% of corn agro-residues (CARs), referring to the parts of corn that are not commercially valuable, are eliminated by burning. CARs consist of its stalks (~48% of the total dry mass), leaves (28%), tassels (~1%), husks (8%), and cobs (15%). Besides, agro-residues can be reduced by production such as animal feed, fertilizer, or fuel briquette [2]. Recently, many studies have focused on agricultural and industrial waste including CARs searching for natural antioxidants for health and value-added cosmetics applications. Previous studies showed that CARs extracts contained phenolic compounds and exhibited antioxidant activities [3-5]. Moreover, these previous studies mostly used conventional extraction methods, such as maceration, steam distillation

and shaking for CARs bioactive extraction. While these methods are advantageous due to their simplicity and cost-effectiveness, ultrasound-assisted extraction (UAE) offers several advantages, including simple instrument setup, reduced extraction time, and low energy consumption. This facilitates the extraction of valuable components such as polyphenols, carotenoids, and other bioactives into the solvent. [6,7]. However, the study on these cosmetic activities of CARs extracts especially in tassels part using green extraction has little been investigated. Therefore, this study aimed to determine polyphenols (total phenolic–TPC and flavonoid–TFC contents) and cosmetic activities (antioxidant, anti-acne and anti-tyrosinase activities) of corn tassels agro-residues (T-CARs) extracts using two different methods (conventional and ultrasound-assisted extractions). Moreover, the cytotoxicity of the T-CARs extracts was investigated to ensure safety for cosmetic applications. The T-CARs may be used as a sustainable source of bioactive ingredients, thereby promoting agricultural waste valorization and contributing to a circular economy in the cosmetic industry.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and solvents were analytical grade. ABTS (2,2'-azino-bis (3-ethylbenz thiazoline-6-sulfonic acid)), aluminum chloride (AlCl_3), dibasic phosphate, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, ethanol, gallic acid, quercetin, kojic acid, trolox, L-DOPA, and tyrosinase from mushroom were purchased from Sigma Chemical (St. Louis, MO, USA). Folin-Ciocalteu reagent, ferric chloride, monobasic phosphate, potassium acetate (CH_3COOK) and sodium carbonate (Na_2CO_3) were purchased from Merck (Darmstadt, Germany).

2.2 Plant Preparation and Extraction

Corn (*Zea mays*) agro-residues (CARs) were collected from Chiang Rai, Thailand. These tassels CARs (T-CARs) samples were separated, cleaned with water and dried in a hot air oven at 45°C until a constant sample weight. The dried sample was milled into powder and stored in a desiccator until use.

T-CARs were extracted by two different methods using conventional shaking extraction-CE and ultrasound assisted extraction-UAE with three different solvents (DI water, 80% methanol and 80% ethanol) at a ratio of sample: solvent (1:10 w/v). For CE, sample was extracted by incubator shaker at 200 rpm at room temperature for 24 hours, whereas, UAE extract was prepared by using ultrasonicator at 40 kHz at room temperature for 30 minutes. All extracts from both extractions were filtered by Whatman filter paper No.1. Organic solvents were removed by rotary evaporator at 50°C. T-CARs extracts were freeze-dried and kept at -20°C until used.

2.3 Determination of Polyphenols

Total Phenolic Contents (TPC) of T-CARs extracts were measured according to Thitipramote *et al.*, [8] with slight modification. Briefly, 20 μL of T-CARs extracts were reacted with 100 μL of 0.2 M Folin-Ciocalteu reagent and 80 μL of 7.5% Na_2CO_3 . The mixture was left to stand in the dark for 30 minutes at room temperature. The absorbance was measured at 765 nm using a microplate reader. Gallic acid was used as a reference standard. The results were expressed as mg gallic acid equivalents (GAE)/g extract.

Total Flavonoid Content (TFC) of T-CARs extracts were measured according to Thitipramote *et al.*, [8] with slight modification. Briefly, 25 μL of T-CARs extracts were mixed with 75 μL of ethanol, 140 μL of DI water, 5 μL of 10% (w/v) AlCl_3 , and 5 μL of 1M CH_3COOK , respectively. The mixture was incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 415 nm.

Quercetin was used as a reference standard, and the results were expressed as mg quercetin equivalents (QE)/g extract.

2.4 Determination of Cosmetic Potentials

Cosmetic potentials of the T-CARs extracts were investigated in terms of antioxidant activity for anti-aging, anti-tyrosinase activity for whitening and anti-microbial activity against *Cutibacterium acnes* for anti-acnes.

2.4.1 Antioxidant Activity

DPPH Radical Scavenging Activity (DPPH) was performed as previously described Thitipramote *et al.*, [9]. Briefly, 10 μ L of T-CARs extracts were mixed with 190 μ L of DPPH reagent. The reaction mixture was left to stand in the dark for 30 minutes at room temperature. The absorbance was measured at 515 nm. Trolox solution was used as a reference standard. The results were expressed as mg Trolox equivalent antioxidant capacity (TEAC)/g extract. **ABTS Radical Scavenging Activity (ABTS)** as previously described Thitipramote *et al.*, [9]. 10 μ L of T-CARs extracts was added to 190 μ L of the diluted ABTS solution. The reaction was performed in the dark at room temperature for 15 minutes. The absorbance was measured at 734 nm. Trolox solution was used as a reference standard. The results were expressed as mg Trolox equivalent antioxidant capacity (TEAC)/g extract.

Ferric Reducing Antioxidant Power (FRAP) was measured as previously described Thitipramote *et al.*, [9]. Briefly, 10 μ L of T-CARs extracts were mixed with 190 μ L of FRAP solution. The mixture was kept in the dark for 15 minutes at room temperature, and then the absorbance was measured at 593 nm using a microplate reader. Trolox solution was used as a reference standard. The results were expressed as mg Trolox equivalent antioxidant capacity (TEAC)/g extract.

2.4.2 Anti-Tyrosinase Activity

Tyrosinase inhibition assays were performed with L-DOPA as substrate according to Wang *et al.*, [10]. Briefly, the reaction mixture contained 15 μ L of mushroom tyrosinase (2500 U/ mL), in 685 μ L phosphate buffer (0.05 M, pH 6.5) was mixed with 200 μ L of T-CARs extract. Then 100 μ L of L-DOPA (5 mM) was added to the reaction mixture and incubated at 37°C for 15 min. Subsequently, the absorbance was measured at 492 nm using a microplate reader. Kojic acid was used as positive control. The percentage of tyrosinase inhibition was calculated.

2.4.3 Anti-Microbial Activity Against *Cutibacterium acnes*

Minimal inhibitory concentration (MIC) is the lowest concentration of an antibacterial agent, completely preventing visible growth of the test strain of an organism [11]. MICs were assessed in 96-well plates using three replications of each microdilution assay being conducted. To modify the work of Domingos *et al.*, [12], the CARs extracts were diluted by a serial dilution of 0.097 – 50.00 mg/mL in brain heart infusion broth (BHI broth). Ampicillin and Phenoxyethanol are also diluted concentrations of 0.048 – 25.00 μ g/mL. After dilution, each bacterial strain suspension was adjusted to 0.5 on the McFarland scale, added in 96-well plates for 100 μ L. Finally, the plates were incubated at 37°C for 4 days. After incubation, 30 μ L of resazurin 0.015% was added to all wells and further incubated for 2 hours to observe the color change [13].

Minimum bactericidal concentration (MBC) is determined by directly plating the contents of wells with concentrations greater than the MIC value [11]. According to Elshikh's work [15], subcultures were made of four wells that did not show bacterial growth (two wells with higher MICs value and one wells which lower MIC value). The bacteria were grown in BHI and incubated at 37°C for 4 days. The MBC was determined as the minimum concentration of the tested samples that had no bacterial population in the BHI plates.

2.5 Determination of Cytotoxicity

Ethanollic T-CARs extract was tested for cell toxicity on a Human immortalised non-tumorigenic keratinocyte cell line (HaCAT) (CLS Cell Lines Service, Eppelheim, Germany) using the MTT assay. HaCAT were cultured in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% heat-inactivated FBS, 50 IU/mL of penicillin, and 50 µg/mL of streptomycin. Cells (1×10^4 cells/mL) were cultured in 24 well plate at 37°C in 5% CO₂ at 95% humidity. Samples were cytotoxically tested at concentrations of 0.5 and 1 mg/mL. A 1% Triton X was used as a standard reference. Results were expressed as relative cell viability (%).

2.6 Statistical Analysis

All data measurements were expressed as mean $\pm$ standard deviation. Data were statistically analyzed using statistic program (IBM SPSS version 21.0). A significant difference was considered at the level of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Extractable Yield of the T-CARs Extracts

The extractable yield of the T-CARs extracts were shown in Table 1. Comparing both extraction methods, the extracts from conventional extraction (CE) had higher yield than these UAE extracts at same solvent. The highest yield was obtained from methanolic CE extract (10.25% of dry basis). The structural and chemical diversity of bioactive compounds essentially complicates their comprehensive extraction. Because polar and nonpolar compounds require solvents of corresponding polarities, extraction efficiency is highly dependent on both the method and the solvent [14]. Previous studies have shown that extracting corn silk with various solvents (e.g., water, acetone, alcohols, ethyl acetate, and hexane) results in significantly different yields and compositions [15,16]. Therefore, determining the optimal solvent system and extraction method is essential for recovering phytochemicals from plant materials for cosmetic applications.

3.2. Polyphenols of the T-CARs Extracts

Both phenolic and flavonoids compounds are the major components found in corn maize [17]. This study demonstrated that T-CARs extracts from both methods exhibited significantly the total phenolic-TPC and flavonoid-TFC contents (Table 1). For TPC results, it ranged from 476.2 to 791.9 mg GAE/g extract that the T-CARs extracts from CE were significantly higher than the UAE extract with the same solvents (Table 1). The significantly highest TPC was found in methanolic and ethanolic CE extracts (791.9 ± 6.3 and 773.7 ± 17.2 mg GAE/g extract, respectively); whereas the lowest TPC was occurred from aqueous UAE extract (476.2 ± 19.4 mg GAE/g extract).

For TFC, ethanolic extracts had higher flavonoid than those of extract with two other solvents in the same extraction method ($p < 0.05$), that the greatest TFC was found in ethanolic CE extracts (570.8 ± 3.8 mg QE/g extract). This study also demonstrated that the conventional extraction method tended to exhibit phenolic compounds greater than the UAE method. This might be the difference in extraction time, where the conventional method (24 hours) is used 48 times more than the UAE method (30 minutes). Previous study showed that the ethanolic and methanolic extracts of corn tassels had higher total phenolic content than other solvents [17]. Flavonoids, commonly found in plants, are an indispensable part of the human diet, were found in corn silk and corn husks. [18,19].

3.3 Cosmetics Potentials

Antioxidant activity describes the ability of redox molecules in biological systems to scavenge free radicals, of which excessive free radicals lead to cellular damage, mutation, cell death, and accelerated aging [7]. There is increasing interest in natural antioxidants for cosmetic ingredients as natural antioxidants

that could protect the skin against various harmful factors, including ultraviolet radiation and free radicals, which promote healthy skin. The DPPH, ABTS, and FRAP assays were performed to determine the antioxidant capacity of the CARs extracts was shown in Table 1.

T-CARs extracts showed DPPH radical scavenging activity ranged from 189.2 to 756.8 mg TEAC/g extract. The alcoholic (ethanol and methanol) extracts from CE exhibited the significant high DPPH activity and the greatest DPPH was found in methanol CE extract 756.8 ± 21.7 mg TEAC/g extract, ($p < 0.05$). Furthermore, T-CARs extracts were tested antioxidant activity by ABTS assay and its ranged from 2,330.3 to 4,171.5 mg TEAC/g extract. Comparing between solvents in T-CARs extracts, the ethanolic extracts exhibited the higher ABTS antioxidant activity than those other solvent at same extraction method ($p < 0.05$). Moreover, reducing antioxidant power by FRAP assay exhibited ranged from 164.8 to 749.3 mg TEAC/g extract. Comparing solvents, the results indicated that the higher FRAP antioxidant activity in T-CARs extracts were significantly found in the alcoholic (ethanol and methanol) extracts than aqueous extract at same extraction ($p < 0.05$). Results suggested that alcoholic extracts tended to be higher antioxidant than aqueous extracts.

Table 1. Extractable yield, polyphenols [total phenolic (TPC) and flavonoid (TFC) contents] and Antioxidant activities (DPPH, ABTS and FRAP activities) of T-CARs extracts.

Extraction methods	Solvents	Yield of extracts (% dry basis)	Polyphenols		Antioxidant activities		
			TPC (mg GAE/g extract)	TFC (mg QE/g extract)	DPPH (mg TEAC/g extract)	ABTS (mg TEAC/g extract)	FRAP (mg TEAC/g extract)
CE	DI water	6.55	734.4 ± 19.5 _b	54.0 ± 5.0 ^c	350.8 ± 8.5 ^d	$3,117.3 \pm 70.3$ _d	496.1 ± 16.1 _e
	80% Methanol	10.25	791.9 ± 6.3 ^a	249.2 ± 2.9 ^c	756.8 ± 21.7 _a	$3,780.5 \pm 22.8$ _c	723.8 ± 7.7 ^b
	80% Ethanol	7.63	773.7 ± 17.2 _a	570.8 ± 3.8 ^a	550.8 ± 9.7 ^b	$4,059.6 \pm 42.3$ _b	662.5 ± 12.5 _c
UAE	DI water	5.98	476.2 ± 19.4 _e	55.4 ± 5.2 ^c	462.4 ± 18.7 _c	$2,330.3 \pm 84.4$ _e	164.8 ± 9.2 ^f
	80% Methanol	2.88	510.3 ± 19.5 _d	212.9 ± 1.4 ^d	189.2 ± 17.2 _e	$3,054.1 \pm 64.1$ _d	595.5 ± 6.8 ^d
	80% Ethanol	3.37	661.9 ± 8.4 ^c	513.3 ± 4.6 ^b	326.2 ± 34.4 _d	$4,171.5 \pm 47.8$ _a	749.3 ± 15.4 _a

Note: Values are given as mean $\pm$ S.D. (n=5) Different superscript letters (a-e) in the same column indicate significant differences in values ($p < 0.05$, ANOVA, Duncan's test).

Tyrosinase as the rate-limiting enzyme for the biosynthesis of melanin, catalyzes the oxidation of the substances that transformed to melanin. Therefore, tyrosinase inhibitors gained a lot of use in cosmeceuticals for use as whitening agents. The results of anti-tyrosinase activity from T-CARs extracts were shown in Table 2. Although the results showed that kojic acid had the highest anti-tyrosinase activity (68.2 ± 0.1 %inhibition, $p < 0.05$) at a concentration of 400 μ g/mL, the aqueous of T-CARs extract using the conventional method had the greatest significant inhibitory tyrosinase activity at a concentration of 4 mg/mL (23.9 ± 3.6 %Inhibition, $p < 0.05$). Previous studies showed that by-products of corn exhibited anti-tyrosinase activity. Wang *et al.*, [10] showed that hydrolysate in post-washing liquor of steam-exploded corn stalk exhibited anti-tyrosinase activity with an IC₅₀ value of 2.17 mg/mL. Yucharoen *et al.*, [20] demonstrated that the ethanolic extract of corn silk showed strong tyrosinase inhibition with an IC₅₀ value of 12.45 μ g/mL that could benefit the development of commercial cosmetic products. However, there was no study on corn leaves and tassels.

Table 2. Anti-tyrosinase activity of T-CARs extracts with two methods and three different solvents extractions.

Extraction methods	Solvents	Concentration	Anti-tyrosinase activity (% Inhibition)
Positive control	Kojic acid	0.4 mg/mL	68.2 ± 0.1 ^a
CE	DI water	4 mg/mL	23.9 ± 3.6 ^b
	80% Methanol	4 mg/mL	14.5 ± 2.4 ^c
	80% Ethanol	4 mg/mL	14.9 ± 2.4 ^c
UAE	DI water	4 mg/mL	17.5 ± 2.7 ^c
	80% Methanol	4 mg/mL	3.4 ± 1.9 ^c
	80% Ethanol	4 mg/mL	9.3 ± 3.7 ^d

Note: Values are given as mean ± S.D. (n = 5). Different superscribe letters (a-e) in the same column indicate significant differences in values ($p < 0.05$, ANOVA, Duncan's test). ND = Not detectable

Results of polyphenols and antioxidants suggested that the T-CARs extracts with alcoholic solvents (methanol and ethanol) gave higher TPC, TFC and antioxidant capacity. However, methanol is known for its high toxicity and is generally unacceptable for use in cosmetic formulations and the ethanolic extraction uses as a green solvent that is environmentally and consumer friendly. Moreover, ethanolic extraction from CE method had highest polyphenol. Therefore, the ethanolic extracts of T-CARs especially from CE extraction were selected for subsequent antimicrobial and cytotoxicity tests.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Cutibacterium acnes* of tested samples.

Groups	Samples	Concentration	MIC	MBC
Positive control	Ampicillin (AMP)	10 µg/mL	0.39 ± 0.00	0.39 ± 0.00
		25 µg/mL		
CARs	Phenoxyethanol (PE)	mg/mL	4.00 ± 0.00	4.00 ± 0.00
	Tassels-Ethanol (CRT-ET)	1 mg/mL	12.50 ± 0.00	25.00 ± 0.00
		5 mg/mL		
		25 mg/mL		
		50 mg/mL		

Note: Values are given as mean ± S.D. (n = 5). Different superscript letters (a-c) in the same column indicated significant differences values (ANOVA, Duncan's, $p < 0.05$). ND = Not detectable

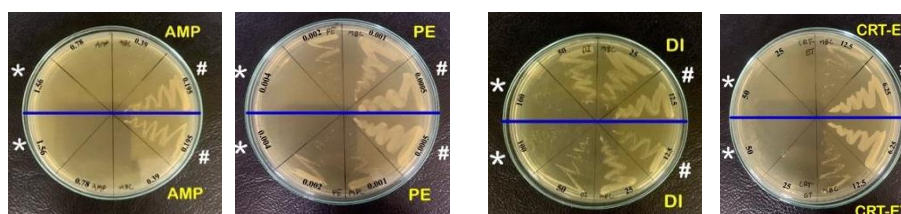


Figure 1. Minimum Bactericidal Concentration (MBC) against *C. acnes* of the T-CARs extracts with ethanol (d; CRT-ET) and Asterisks (*) indicate the highest concentration to the lower concentration (#). AMP=Ampicillin; PE=Phenoxyethanol; DI=Deionized water

Minimum Inhibitory Concentration (MIC) against *Cutibacterium acnes* of T-CARs extracts was shown in Table 3. The CRT-ET showed the MIC value at a concentration of 12.50 mg/mL. Moreover, positive control was strongly effective for antimicrobial properties, that MIC values were 0.39 µg/mL of ampicillin and 4.00 mg/mL of phenoxyethanol (Table 3). Previous studies on corn silk agro-residue extract showed the antimicrobial against *C. acnes* (15.625 mg/mL) [20] *Staphylococcus epidermidis* (125 mg/mL) [20] *Escherichia coli* (125 mg/mL) [21] *S. aureus* (125 mg/mL) [21]. Thus, corn residue

especially corn tassels residue (T-CARs) demonstrates the potential of antibacterial *C. acnes* that can be used to cosmetic applications.

Results of MBC values against *C. acnes* from T-CARs extracts showed that all samples extracted with ethanol (CRT-ET) demonstrated the highest effectiveness at a concentration of 25.00 mg/mL. In the control group, ampicillin showed MBC values of 0.39 µg/mL and phenoxyethanol showed MBC values of 4.00 mg/mL (Table 3; Figure 1).

The results of this study showed antibacterial activity of *Zea mays* against *C. acnes*. According to previous studies, CARs extract contains saponins, tannins, flavonoids, and terpenoids that have antibacterial activity by destruction of cell membranes [22]. Therefore, tassels of corn extract might be associated with their potent antibacterial activity.

3.4 Cytotoxicity of T-CARs Extracts

These ethanolic extracts of CARs were tested with the human keratinocyte (HaCAT) cell line at concentrations of 0.5 and 1 mg/mL for 48 hours. The percentage of cell viability more than 90% was classified to be no toxic for the cell. The results showed that cell viability of all ethanolic extracts in each part of CARs (at 0.5 and 1 mg/mL) was higher than 90% (Table 4). The highest cell viability was found in the CE ethanolic corn Tassels extract at 0.5 mg/mL ($128.1 \pm 19.9\%$; $p < 0.05$). The results suggested that tassel extract can induce proliferation of HaCAT cells and no toxic. Triton X-100 as negative control showed low cell viability ($10.8 \pm 0.6\%$). Thus, the ethanolic extracts of CARs might be safe for use as an active ingredient in cosmetic products or other related products.

Table 4. Cytotoxicity of the ethanolic extracts of T-CARs at a concentration 0.5 and 1 mg/mL.

Groups	Samples	Concentration	MIC	MBC
Positive control	Ampicillin (AMP)	10 µg/mL	0.39 ± 0.00	0.39 ± 0.00
	Phenoxyethanol (PE)	25 µg/mL	4.00 ± 0.00	4.00 ± 0.00
CARs	Tassels-Ethanol (CRT-ET)	1 mg/mL	12.50 ±	25.00 ±
		5 mg/mL	0.00	0.00
		25 mg/mL		
		50 mg/mL		

Note: Values are given as mean ± S.D. (n=3), Different superscript letters (a-d) in the same column indicate significant differences in values ($p < 0.05$, ANOVA, Duncan's test).

4. CONCLUSIONS

Tassel corn agro-residues (T-CARs) extracts, especially using alcoholic (methanol and ethanol) as solvent from conventional-CE extraction; composed of higher polyphenols (phenolic and flavonoid) and antioxidant activities as anti-aging. Moreover, ethanolic CE extracts also inhibited the anti-microbial against *Cutibacterium acnes* as anti-acne and no toxicity in HaCAT cells at concentrations of 0.5 and 1 mg/mL (> 90% cell viability). However, the aqueous T-CARs extracts showed the anti-tyrosinase activity at concentration 4 mg/mL. Results suggested that T-CARs extracts especially from CE extraction can be value-added by being used as a natural active ingredient in cosmetic applications and other related products.

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Antioxidant and Antimicrobial Activities of Crude Extracts from *Phellinus* sp.**Thanpitcha Jenkit¹, Pattamawadee Yanatatsaneejit², Wichanee Bankeeree³, and
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ABSTRACT: *Phellinus* sp., a member of the Hymenochaetaceae family, is a medicinal mushroom traditionally used in East Asia, which has gained significant attention for its diverse pharmacological properties. Bioactive compounds isolated from *Phellinus* sp., including polysaccharides and polyphenolic compounds such as hispolon, caffeic acid, and inoscavin A, have been reported to exhibit immunomodulatory, antioxidant, antibacterial, and anticancer activities. In this study, *Phellinus* sp. was extracted using three solvent systems: distilled water (DW), 60% ethanol, and 95% ethanol. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method, with gallic acid as the standard reference, yielding values of 63.73 ± 3.49 , 94.13 ± 3.84 , and 35.88 ± 4.36 mg GAE/g extract for the DW, 60% ethanol, and 95% ethanol extracts, respectively. The 60% ethanol extract showed the highest TPC among all tested extracts. Antioxidant activities were evaluated using DPPH radical scavenging and ABTS assays, with gallic acid as the standard reference. The 95% ethanol extract demonstrated the strongest DPPH scavenging activity ($IC_{50} = 28.98 \pm 2.04$ µg/mL), while the DW extract showed the strongest ABTS activity ($IC_{50} = 13.70 \pm 0.58$ µg/mL). Antimicrobial activity was assessed using the agar well diffusion method at concentrations ranging from 50 to 200 mg/mL. All extracts exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. For antifungal activity, the extracts exhibited inhibitory activity against *Aspergillus* sp. but not against *Penicillium* sp. under the same conditions. These findings suggest that *Phellinus* sp. extracts possess notable antioxidant, antibacterial, and antifungal activities, highlighting their potential as natural bioactive sources for the development of functional products and future therapeutic applications.

Keywords: Antimicrobial; Antioxidant; Bioactive compounds; Medicinal mushroom; *Phellinus* sp.

1. INTRODUCTION

Good health is fundamental to quality of life, but the misuse and overdose of antibiotics have increasingly weakened this foundation by driving the emergence of antimicrobial resistance (AMR). The World Health Organization [1] report states that antimicrobial resistance has become one of the most pressing global public health threats of our time, with bacterial AMR directly responsible for an estimated 1.27 million deaths worldwide in 2019 and being associated with approximately 4.95 million deaths in the same year. The 2022 Global Antimicrobial Resistance and Use Surveillance System (GLASS) [2] report documented median resistance rates of 42% for third-generation cephalosporin-resistant *Escherichia coli* and 35% for methicillin-resistant *Staphylococcus aureus* (MRSA) across 76 countries.

The Organization for Economic Co-operation and Development (OECD) projects a double surge in resistance to last-resort antibiotics by 2035, while the World Bank estimates AMR could impose over US\$1 trillion in additional healthcare costs by 2050. In response to this escalating crisis, researchers have increasingly turned to natural bioactive compounds as alternative or complementary antimicrobial agents. Among the diverse range of natural sources explored, medicinal mushrooms have attracted considerable scientific attention for their rich bioactive constituents, including polysaccharides and polyphenolic compounds, which have demonstrated broad-spectrum antimicrobial properties. *Phellinus* sp., a genus of bracket fungi belonging to the family Hymenochaetaceae has been traditionally employed in East Asian medicine [3].

Key bioactive compounds isolated from *Phellinus* sp. include polysaccharides and phenolic constituents such as hispolon, caffeic acid, gallic acid, chlorogenic acid, and inoscavin A. Hispolon, a phenolic compound isolated extracted from species including *P. igniarius*, *P. linteus*, and *P. lonicerinus*, has been reported to have anticancer, anti-inflammatory, antioxidant, and antibacterial activities. Phenolic compounds from *Phellinus* spp. have further demonstrated antibacterial efficacy against *Staphylococcus aureus* and *Escherichia coli*, two major pathogens of clinical concern.

Despite the growing evidence supporting the pharmacological potential of *Phellinus* sp., studies systematically evaluating the influence of different solvent systems on the extraction efficiency and biological activities of this mushroom remain limited. The selection of extraction solvent is a critical determinant of bioactive yield, as compounds of varying polarity are selectively solubilized by different solvents, directly shaping the composition and activity of the resulting extracts. Therefore, this study aimed to compare the extraction efficiency of three solvent systems: distilled water, 60% ethanol, and 95% ethanol, for the recovery of bioactive compounds from *Phellinus* sp., and to evaluate the total phenolic content, antioxidant activity, and antimicrobial properties of the extracts.

2. MATERIALS AND METHODS

2.1 Extraction of *Phellinus* sp.

The powdered fruiting body of *Phellinus* sp. was extracted using three solvent systems. Distilled water (DW) extraction was performed at a solid-to-liquid ratio of 1:60 (w/v) and heated for 6 hours using a Soxhlet extractor. Extractions with 60% ethanol and 95% ethanol were conducted at a solid-to-liquid ratio of 1:40 (w/v) and heated at 60°C for 6 hours using a Soxhlet extractor. The crude extracts were collected and dried by lyophilization for *Phellinus* sp. DW extract, whereas the 60% ethanol and 95% ethanol extracts were concentrated using a rotary evaporator. The dried extracts were weighed to calculate the extraction yield and were at -20°C until further use.

2.2 Preliminary compound screening

2.2.1 Evaluation of total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu (FC) reagent, following a method modified from the protocol described by Syukriya [4]. The crude extracts were dissolved in distilled water at appropriate concentrations and mixed with the FC reagent at a FC reagent-to-water ratio of 1:3 for 5 minutes. After that, sodium carbonate (10% w/v) was added to the mixture and incubated for 60 minutes at room temperature in the dark conditions. The absorbance of the mixture was measured at 765 nm using a UV-Vis spectrophotometer. TPC was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry crude extract (mg GAE/g crude dry extract)

2.3 Antioxidant activities

2.3.1 Determination with ABTS

The radical cation of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS^{•+}) modified from the protocol described by Kurniawan [5] was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate at the ratio of 1:1 (v/v) and allowing the mixture to stand for 16 hours in the dark until the absorbance had stabilized. The ABTS^{•+} stock solution was diluted with distilled water to an absorbance of 0.700 ± 0.050 at 734 nm prior to use. The assay was conducted by mixing ABTS^{•+} solution with crude extracts at different concentrations at a solvent-to- ABTS^{•+} solution ratio of 1:3 (v/v). After incubation for 10 minutes in the dark, the absorbance of the mixture was measured at 734 nm. The antioxidant activity of crude samples was calculated relative to the absorbance of ABTS^{•+} solution without extract. The percentage of radical inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 100}{A_{\text{control}}} \quad (1)$$

Where A control was the absorbance of the ABTS^{•+} solution without the extract and A sample was the absorbance of the sample with ABTS^{•+} solution. The half-maximal inhibitory concentration (IC₅₀) was defined as the concentration of extract required to inhibit 50% of the initial ABTS^{•+} radical activity. The blank was a solvent solution of the sample, the negative control was the sample solvent mixed with ABTS^{•+} solution, and the positive control was gallic acid with ABTS^{•+} solution.

2.3.2 Determination with DPPH

The radical scavenging activity was modified from the protocol described by Syukriya [4]. The extracts were serially diluted, and mixed with 0.3 mM DPPH solution at a solvent-to-DPPH solution ratio of 1:1 (v/v), then incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The percentage of radical inhibition was calculated using equation (1). Where A control was the absorbance of the DPPH solution without the extract, and A sample was the absorbance of the mixture containing the extract and DPPH solution. The blank was the sample solvent alone, the negative control was the sample solvent with the DPPH solution, and the positive control was gallic acid mixed with the DPPH solution.

2.4 Antimicrobial activities

2.4.1 Determination of antibacterial activity

The antibacterial activity of *Phellinus* sp. extracts was evaluated using the agar well diffusion method, following a protocol modified from Balouiri [6]. *Escherichia coli* and *Staphylococcus aureus* were used as representative Gram-negative and Gram-positive bacterial strains, respectively. Each strain was initially cultured on nutrient agar (NA). Colonies were then inoculated into nutrient broth (NB) and incubated at 37°C for 16 hours under aerobic conditions. After that, the bacterial suspension was adjusted to an absorbance of approximately 0.100 ± 0.050 at 600 nm, corresponding to approximately 1×10^8 CFU/mL, by subculturing in NB and incubating for 3 hours under the same conditions. A volume of 100 µL of the bacterial suspension was spread onto NA plates, and wells of 8 mm diameter were created using a cork borer. *Phellinus* sp. extracts were dissolved in 10% DMSO at concentrations ranging from 50 to 200 mg/mL, and 100 µL of each concentration was loaded into each well. The plates were incubated aerobically at 37°C for 24 hours. After which antibacterial activity was determined by measuring the diameter of inhibition zones using a Vernier caliper. Neomycin was used as the positive control for *E. coli* and *S. aureus*,

while 10% DMSO was used as the negative control. All experiments were conducted in triplicate.

2.4.2 Determination of antifungal activity

The antifungal activity of *Phellinus* sp. extracts was evaluated using the agar well diffusion method, following a protocol modified from Tran [7]. *Aspergillus* sp. and *Penicillium* sp. were used as representative fungal test strains. Each strain was initially cultured on Potato Dextrose Agar (PDA) and incubated at 25°C. Colonies were then transferred to PDA slants and incubated at 25°C for 7 days. A spore suspension of approximately 1×10^8 spores/mL was prepared, and 100 μ L was spread onto PDA plates. Wells of 8 mm diameter were created using a cork borer. *Phellinus* sp. extracts were dissolved in 10% DMSO at concentrations ranging from 50 to 200 mg/mL, and 100 μ L of each concentration was loaded into each well. The plates were incubated aerobically at 25°C. Observe and record preliminary readings at 24 hours, with final, definitive measurements taken at 48 to 72 hours. Antifungal activity was determined by measuring the diameter of inhibition zones using a Vernier caliper. Mancozeb 80%WP 2.5 mg/mL served as a positive control for both *Aspergillus* sp. and *Penicillium* sp., while 10% DMSO was used as the negative control. All experiments were conducted in triplicate.

3. Statistical analysis

All experiments were performed in triplicate, and the data were shown as their mean $\pm$ standard deviation (SD). The significance of differences between samples in the same group was examined by one-way analysis of variance (one-way ANOVA) with Tukey's HSD post-hoc test at P-values less than 0.05 ($p < 0.05$) using IBM SPSS 28.

3. RESULTS AND DISCUSSION

3.1 Extraction of *Phellinus* sp.

The extraction yields of *Phellinus* sp. obtained using different extraction solvents are presented in Table 1. Among the tested solvents, the 60% ethanol extract showed the highest extraction yield, followed by the distilled water (DW) extract. The lowest extraction yield was observed in the 95% ethanol extract. These results suggest that 60% ethanol was the most effective solvent for extracting compounds from *Phellinus* sp. under the experimental conditions used. The 60% ethanol provides an intermediate polarity that solubilizes both polar and moderately polar compounds while also enhancing cell wall permeability through its water content. DW, being highly polar, primarily extracts hydrophilic compounds but is less effective for moderately polar constituents, resulting in an intermediate yield. In contrast, 95% ethanol has insufficient free water to hydrate and dissolve polar compounds, and its low water activity limits cell wall swelling, resulting in the lowest yield. [8]

Table 1. Yield of *Phellinus* sp. Extracts

Yield (%)	DW extract	60% ethanol extract	95% ethanol extract
	5.72 $\pm$ 0.52	10.42 $\pm$ 0.97	3.10 $\pm$ 0.09

Note: All data were represented as the mean $\pm$ standard deviation (SD) of these independent experiments.

3.2 Total phenolic content of *Phellinus* sp. extracts

The total phenolic content (TPC) varied significantly depending on the extraction solvent. The 60% ethanol extract exhibited the highest phenolic content at 94.13 ± 3.84 mg GAE/g dry weight, followed by the DW (distilled water) extract at 63.73 ± 3.47 mg GAE/g dry weight. The lowest TPC was observed in the 95% ethanol extract at 35.88 ± 4.36 mg GAE/g dry weight. In this study, the 60% ethanol proved to be the most efficient solvent. The synergy between water and ethanol creates an environment with intermediate polarity, which is often optimal for solubilizing a broad spectrum of phenolic compounds [9-10]. On the other hand, pure or high-concentration solvents such as 95% ethanol often show reduced efficiency for polar compounds because their polarity may be too low to effectively solubilize the polyphenol. Similarly, while distilled water is highly polar and excellent for extracting water-soluble compounds, it may not be enough on its own to extract the full range of semi-polar phenolic species, resulting in lower total yield compared to the optimized ethanol-water mixture. [11]

3.3 Antioxidant activities

The antioxidant activities of *Phellinus* sp. extracts were evaluated using ABTS^{•+} and DPPH assays, with results expressed as IC₅₀ (μg/mL) (Figure 1). In the ABTS^{•+} assay, the DW extract demonstrates the strongest antioxidant activity, showing the lowest IC₅₀ value of 13.71 ± 0.81 μg/mL. In contrast, the 95% EtOH extract showed the weakest activity, with the highest IC₅₀ of 21.77 ± 0.44 μg/mL. Statistically significant differences ($p < 0.05$) were observed among all three solvent types in the ABTS^{•+} assay. In the DPPH assay, the 95% EtOH extract exhibited the highest antioxidant capacity, demonstrated by its lowest IC₅₀ = 28.98 ± 2.04 μg/mL, which was significantly lower than 60% ethanol and DW extracts. However, no statistically significant difference was found between the 95% ethanol and DW extracts. Variation in IC₅₀ values is a common phenomenon in phytochemical studies, as different solvents extract different classes of bioactive compounds (e.g., phenolics, flavonoids, or polysaccharides) that have distinct radical-scavenging mechanisms. The strong performance of the DW extract in the ABTS^{•+} assay suggests that water-soluble compounds, such as certain polar phenolic acids or mushroom polysaccharides, are highly effective at neutralizing the ABTS^{•+} radical. In contrast, the superior performance of the 95% ethanol extract in the DPPH assay indicates that this less polar solvent more effectively extracted a concentrated profile of compounds capable of donating hydrogen atoms to stabilize the DPPH radical, while the comparable activity of the 60% ethanol and DW extracts suggests these two share a similar hydrogen-donating compound profile, distinct from that of 95% ethanol. [12]

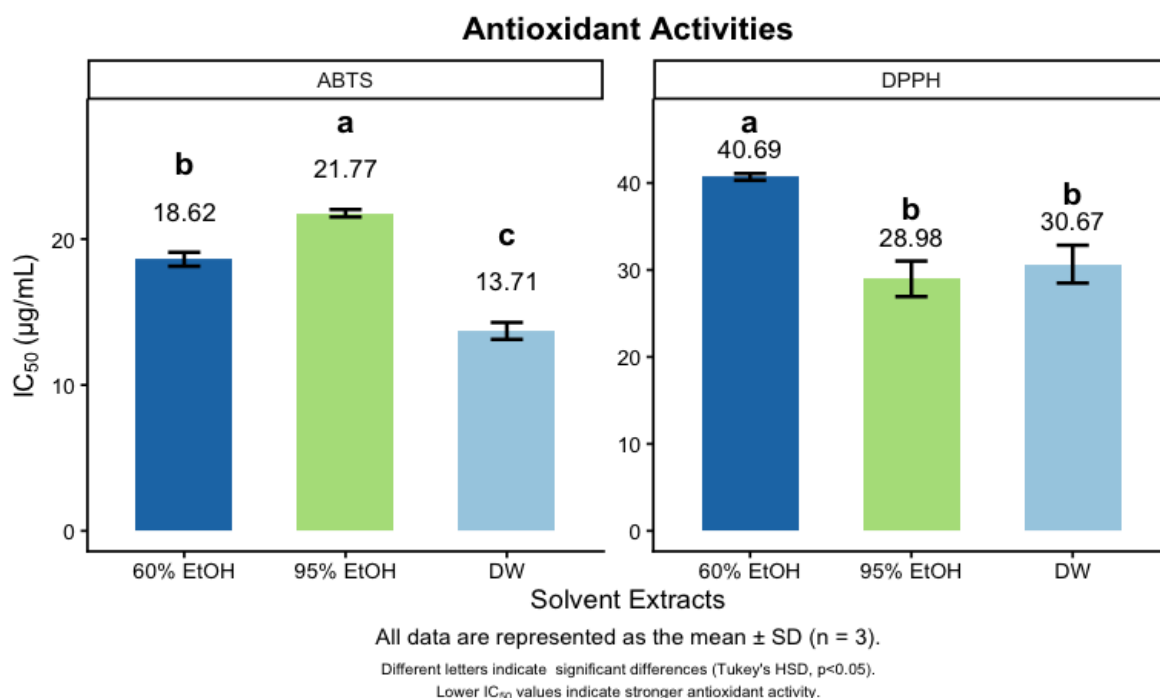


Figure 1. All data are represented as mean ± SD (n = 3). Different letters indicate significant differences among solvent extracts within the same assay ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test).

3.4 Antimicrobial activities

3.4.1 Antibacterial activity

All extracts showed a dose-dependent increase in antibacterial activity against both *E. coli* and *S. aureus*. Notably, *S. aureus* (Gram-positive) appeared more susceptible to the extracts than *E. coli* (Gram-negative), as evidenced by the presence of inhibition zones at the lowest concentration (50 mg/mL) for all solvents. On the other hand, *E. coli* showed resistance to the DW extract at all tested concentrations, while the ethanolic extracts (60% ethanol and 95% ethanol) were effective only at concentrations ≥ 100 mg/mL.

Table 2. Antibacterial activity of *Phellinus* sp. extracts

	Inhibition zone (mm)							
	<i>Escherichia coli</i> *				<i>Staphylococcus aureus</i> **			
Concentration (mg/mL)	50	100	150	200	50	100	150	200
DW	ND	ND	ND	ND	+	++	++	+++
60% ethanol	ND	+	++	+++	+	++	++	+++
95% ethanol	ND	+	++	+++	+	++	++	+++

Note: * + is 10.00-12.00 mm, ++ is 12.01-14.00 mm, and +++ is more than 14.01 mm

** + is 10.00-16.00 mm, ++ is 16.01-23.00 mm, and +++ is more than 23.01 mm

3.4.2 Antifungal activity

All extracts show activity against *Aspergillus* sp., with all solvents showing inhibitory effects at all tested concentrations (50–200 mg/mL). However, no activity was observed against *Penicillium* sp. for any of the extracts, the solvent or the concentration used.

Table 3. Antifungal activity of *Phellinus* sp. extracts

	Inhibition zone (mm)							
	<i>Aspergillus</i> sp.				<i>Penicillium</i> sp.			
Concentration (mg/mL)	50	100	150	200	50	100	150	200
DW	ND	+	++	+++	ND	ND	ND	ND
60% ethanol	+	++	++	+++	ND	ND	ND	ND
95% ethanol	+	++	++	+++	ND	ND	ND	ND

Note: * + is 10.00-16.00 mm, ++ is 16.01-23.00 mm, and +++ is more than 23.01 mm

The observed antimicrobial efficacy may be attributed to the previous total phenolic content (TPC) findings, as phenolic compounds are documented for their ability to disrupt microbial cell membranes and interfere with bacterial enzyme systems. [13]

The greater susceptibility of *S. aureus* compared to *E. coli* can be explained by the fact that the structural characteristics of gram-negative bacteria; *E. coli* has an outer lipopolysaccharide membrane that acts as a physical barrier, limiting the penetration of hydrophobic compounds into the cell. [14] The failure of the distilled water (DW) extract to inhibit *E. coli* suggests that the active antibacterial components of *Phellinus* sp. are likely hydrophobic in nature, requiring ethanol as a solvent to achieve sufficient extraction efficiency and cellular penetration.

Regarding antifungal activity, the selective inhibition of *Aspergillus* sp. and the absence of activity against *Penicillium* sp. suggest a high degree of specificity in the bioactive metabolites of *Phellinus* sp. This selectivity may be due to differences in cell wall composition or to detoxification mechanisms specific to each fungal genus. [15]

4. CONCLUSIONS

Phellinus sp. is a valuable source of bioactive compounds with significant antioxidant and antimicrobial potential, though the optimal solvent depended on the property assessed. The 60% ethanol extract gave the highest yield and total phenolic content, while the DW extract showed the strongest antioxidant activity (lowest IC₅₀ in both ABTS and DPPH assays), indicating that phenolic content alone does not determine radical-scavenging capacity. All extracts exhibited dose-dependent antibacterial activity, with greater susceptibility in *S. aureus* than *E. coli*, and consistent antifungal activity against *Aspergillus* sp., though none were effective against *Penicillium* sp. Future studies should identify the specific compounds within each extract to clarify their contribution to these bioactivities. Furthermore, the potent antioxidant and antimicrobial properties of these extracts indicate they could be used for industrial or pharmacological potential and integrated into the development of functional food products, such as fruit wraps or healthy snack coatings, where they may serve as natural additives to enhance both nutritional value and shelf-life stability.

5. ACKNOWLEDGEMENT

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Cloning, Heterologous Expression, and Purification of Sphingomyelinase of *Staphylococcus epidermidis*

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ABSTRACT: Sphingomyelinase (SMase) is an enzyme that hydrolyzes sphingomyelin into ceramide and phosphocholine via the sphingomyelin hydrolysis pathway. Ceramide is a major lipid component of the stratum corneum (SC) and plays a crucial role in maintaining skin barrier integrity and epidermal homeostasis. Previous studies have demonstrated that SMase is produced by several bacterial species, including *Staphylococcus aureus* and *Staphylococcus epidermidis*. Unlike the cytotoxic β -toxin produced by *S. aureus*, SMase derived from *S. epidermidis* has been reported to enhance ceramide production and skin barrier homeostasis. This study, therefore, aimed to clone, express, and purify recombinant SMase encoded by the *SMase* gene of *S. epidermidis*. The *SMase* gene was amplified by polymerase chain reaction (PCR) and ligated into the pET-32a(+) expression vector at *Bam*HI and *Xho*I restriction sites, and transformed into *Escherichia coli* TOP10 for recombinant plasmid amplification. Successful amplification of the *SMase* gene was confirmed by the presence of a DNA fragment of approximately 1,005 bp after restriction enzyme digestion. Recombinant protein was expressed in *E. coli* BL21 (DE3) and optimized using different IPTG concentrations and induction times at 16 °C, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed a protein band corresponding to the expected molecular weight of SMase (~56 kDa), with the highest expression observed following induction with 0.2 mM IPTG for 12 h. The recombinant SMase protein was purified by Ni-NTA affinity chromatography with stepwise imidazole gradient elution (30 mM-1 M), and the highest protein yield was obtained in the 200 mM imidazole fraction. These findings demonstrate the successful production of recombinant *S. epidermidis* SMase for future studies, including biochemical and functional characterizations.

Keywords: *Staphylococcus epidermidis*; Sphingomyelinase; Expression; Purification, pET-32a (+)

1. INTRODUCTION

Sphingomyelinase (SMase) is a key enzyme that catalyzes the hydrolysis of sphingomyelin into ceramide and phosphocholine via the sphingomyelin hydrolysis pathway [1]. The resulting ceramide serves not only as an essential structural component of the lipid lamella in the stratum corneum (SC) but also acts as a lipid second messenger involved in cell differentiation, proliferation, and apoptosis [2]. In skin physiology, alterations in ceramide levels are closely associated with skin barrier integrity and transepidermal water loss (TEWL), highlighting the critical role of SMase in maintaining epidermal homeostasis [3].

Ceramides play an essential role in maintaining skin hydration and skin barrier homeostasis by regulating lipid lamellar organization and reducing TEWL [4, 5]. Decreased ceramide levels are strongly

associated with impaired skin barrier function and the progression of chronic inflammatory skin diseases, including atopic dermatitis and psoriasis [6]. Although topical ceramide-containing formulations are widely used in skincare products and cosmetics, these products mainly function as exogenous supplementation and are unable to effectively stimulate endogenous ceramide biosynthesis. Consequently, their effects on skin barrier restoration are often short-term and unsustainable. Therefore, alternative approaches capable of naturally enhancing ceramide production have gained increasing attention [7].

Previous studies have demonstrated that SMase is produced by several bacterial species, including *S. aureus* and *S. epidermidis*. However, the SMase of *S. aureus*, known as β -toxin, has been associated with virulence and cytotoxic effects, whereas *S. epidermidis* produces a non-cytotoxic SMase that contributes to ceramide production and skin barrier homeostasis. Importantly, *S. epidermidis*-derived SMase has been shown to increase ceramide levels and reduce TEWL without significant cytotoxic or hemolytic effects, suggesting its potential application in enhancing skin barrier function and cosmeceutical development [3].

Although a previous study has demonstrated the beneficial role of *S. epidermidis*-derived SMase on skin barrier homeostasis, reports on its recombinant production and purification remain limited. Therefore, the present study aimed to construct a recombinant expression system for *S. epidermidis* SMase, optimize its heterologous expression in *E. coli* BL21 (DE3), and purify recombinant SMase for future biochemical characterization and functional validation.

2. MATERIALS AND METHODS

2.1 Bacterial Culture and DNA Extraction

S. epidermidis ATCC 12228 was cultured on Luria Bertani (LB) agar and incubated at 37°C overnight. Bacterial cells were collected for DNA extraction using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia) according to the manufacturer's instructions.

2.2 PCR Amplification

The *SMase* gene was amplified by polymerase chain reaction (PCR) in a total reaction volume of 50 μ L that contained 1 μ L of PlatinumTM SuperFiTM II DNA Polymerase, 50 ng of genomic DNA isolated from *S. epidermidis*, 2.5 μ L each of 10 μ M forward and reverse primers, 10 mM dNTP mix, 5x SuperFiTM II Buffer, and nuclease-free water.

The primer sequences used for amplification of the *S. epidermidis SMase* gene were as follows:

SPH-SE_fwd (5'-CGAGGATCCATGAAACGAGGTGTAACAATATTG-3')

SPH-SE_rev (5'-GACCTCGAGCTATTCATTTGATGCATAGCCAG-3')

PCR amplification was performed under the following thermocycling conditions: initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 62°C for 10 seconds, extension at 72°C for 30 seconds, final extension at 72°C for 5 minutes, and the reaction was subsequently maintained at 4 °C. The PCR amplicons were analyzed by 1% agarose gel electrophoresis and purified using the GF-1 Ambiclean PCR & Gel DNA CLEAN-UP Kit (Vivantis, Malaysia) according to the manufacturer's instructions.

2.3 Restriction Enzyme Digestion

The PCR-amplified *SMase* gene from *S. epidermidis* and pET32a(+) were digested with *Bam*HI and *Xho*I restriction endonucleases in a total reaction volume of 50 μ L containing 5 μ L of 10x buffer, 1 μ L of *Bam*HI, 1 μ L of *Xho*I, and 43 μ L of amplified DNA fragment. The reaction mixture was incubated at

37°C for 2 hours, followed by heat inactivated at 65°C for 20 minutes. Aliquots (5 µL) of the reaction mixture were analyzed by 0.75% agarose gel electrophoresis, stained with SYBR Safe, and visualized under a blue light transilluminator to confirm complete digestion.

2.4 Ligation

The digested *SMase* gene fragment was ligated into the pET-32a(+) vector (Synbio Technologies, LLC, Monmouth Junction, NJ, USA) in a ligation mixture that contained the following components: 3:1 (insert: vector), 10x ligation buffer, T4 DNA ligase, and nuclease-free water to a final volume of 50 µL. The ligation mixture was incubated at 26 °C for 18 hours.

2.5 Heat-Shock Transformation

The recombinant plasmid pET-32a(+)-SMase (10 µL) was added to 100 µL of chemically competent *Escherichia coli* TOP10 cells (Thermo Fisher Scientific, Cat. No. C404010) in sterile microcentrifuge tubes and incubated on ice for 30 minutes. The mixtures were heat-shocked at 42°C for 90 seconds, followed by incubation on ice for 5 minutes. Subsequently, 1 mL of LB broth was added, and the cells were incubated at 37 °C for 1-2 hours. Finally, 50-100 µL of the transformation mixture was spread onto LB agar plates containing 50 µg/mL ampicillin and incubated overnight at 37 °C. Following plasmid confirmation, the recombinant plasmid pET-32a(+)-SMase was transformed into chemically competent *E. coli* BL21(DE3) cells (ECOSTM 21, Yeastern Biotech, Cat. No. FYE207-5VL) for recombinant protein expression using the same transformation method.

2.6 Plasmid Extraction and Restriction Enzyme Digestion

After transformation into *E. coli* TOP10 cells, a single colony was selected from LB agar plates, inoculated into 30 mL of LB broth containing 50 µg/mL ampicillin, and incubated overnight at 37 °C with shaking at 250 rpm. After overnight incubation, the recombinant plasmid pET-32a(+)-SMase was extracted using a commercial plasmid extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions and analyzed by 0.75% agarose gel electrophoresis to confirm the presence of the recombinant plasmid. Subsequently, the extracted recombinant plasmid was digested with *Bam*HI and *Xho*I restriction enzymes using the same protocol described in the restriction enzyme digestion step.

2.7 Protein Expression

After the clones were verified by double digestion with restriction enzymes, a single colony of *E. coli* BL21(DE3) harboring pET32a(+)-SMase was inoculated into 50 mL of LB broth containing 50 µg/mL ampicillin and incubated overnight at 37 °C with shaking at 250 rpm. Subsequently, 500 µL of the overnight culture was transferred into 49.5 mL of fresh LB broth and incubated at 37 °C with shaking at 250 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.6. Protein expression was then induced by adding isopropyl β-D-1- thiogalactopyranoside (IPTG) to a final concentration of 0 mM (control), 0.075 mM, 0.1 mM, and 0.2 mM, respectively. The cultures were further incubated at 16 °C with shaking at 250 rpm for 8 and 12 hours, to determine the optimal induction time. Following induction, bacterial cells were harvested by centrifugation, and the resulting cell pellets were lysed using lysis buffer. The lysed cell suspensions were incubated at room temperature for 30 minutes, followed by centrifugation. The supernatant was collected to analyze the expression level of the SMase protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% separating gel. The optimum IPTG concentration and induction time were selected for protein purification using immobilized metal affinity chromatography (IMAC).

2.8 Protein Purification

The 6×His-tagged SMase protein was purified using Ni-NTA affinity chromatography. Before sample loading, the Ni²⁺-Sephacrose beads were equilibrated with equilibration buffer (EQ buffer), followed by addition of binding buffer (containing EQ buffer and 15 mM imidazole). Subsequently, the protein was loaded onto the column and washed with wash buffer (containing EQ buffer and 20 mM imidazole) to remove nonspecifically bound proteins. The SMase protein was then eluted stepwise with 30 mM, 50 mM, 100 mM, 120 mM, 200 mM, and 500 mM imidazole. All eluted fractions were collected and analyzed by 10% SDS–PAGE.

3. RESULTS AND DISCUSSION

3.1 Construction of Recombinant Plasmid pET-32a(+)-SMase

The recombinant plasmid pET-32a(+)-SMase was constructed by inserting the *SMase* gene of *S. epidermidis* into the pET-32a(+) expression vector at the *Bam*HI and *Xho*I restriction sites, as illustrated in Figure 1. The pET-32a(+) vector contains a T7 promoter, a thioredoxin fusion tag, and 6x His-tag at 5' end of the inserted gene, which facilitate IPTG-inducible expression and efficient purification of the recombinant SMase protein.

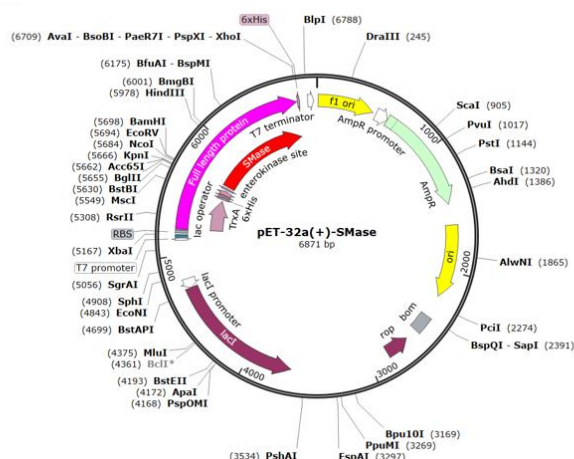


Figure 1. Cloning map of the recombinant plasmid pET-32a(+)-SMase using SnapGene viewer to illustrate the insertion of *SMase* gene into the *Bam*HI and *Xho*I restriction sites.

3.2 PCR Amplification

The *SMase* gene of *S. epidermidis* was successfully amplified by PCR, as demonstrated by 1% agarose gel electrophoresis (Figure 2). A clear single DNA band corresponding to approximately 1,005 bp was observed in lanes 1 and 2, which was consistent with the expected size of the amplified *SMase* gene fragment. This finding confirms the successful amplification of the target gene. The amplified *SMase* gene was subsequently used for restriction enzyme digestion and cloning into the pET-32a(+) vector.



Figure 2. Agarose gel electrophoresis (1% agarose) of PCR-amplified *SMase* gene fragments. Lane M: 100bp DNA Ladder; Lane 1-2: PCR products of the *SMase* gene (~1,005 bp).

3.3 Restriction Enzyme Digestion

The *SMase* gene and pET32a(+) vector were digested with *Bam*HI and *Xho*I endonucleases for 2 hours. The digested fragments were analyzed by 0.75% agarose gel electrophoresis (Figure 3). DNA fragments corresponding to the digested pET-32a(+) vector (~5.8 kb) and *SMase* gene insert (~1.0 kb) were observed. These results confirmed the successful digestion of both insert and vector. The successful digestion produced DNA fragments that were suitable for subsequent ligation and construction of the recombinant pET-32a(+)-*SMase* plasmid.

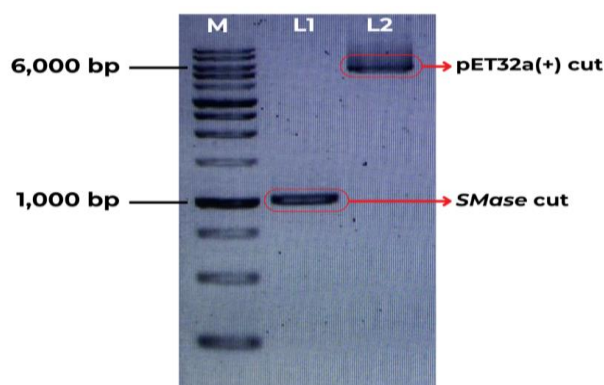


Figure 3. Agarose gel electrophoresis (0.75% agarose) of restriction enzyme digestion. Lane M: 1kb DNA ladder; Lane 1: digested *SMase* gene insert; Lane 2: digested pET32a(+) vector

3.4 Selection and Verification of Recombinant Plasmid by Restriction Enzyme Digestion

The recombinant plasmid pET32a(+)-*SMase* was digested with *Bam*HI and *Xho*I under the same conditions used for restriction enzyme digestion and analyzed by 0.75% agarose gel electrophoresis (Figure 4). Digested plasmids from colonies L2, L3, and L4 showed DNA fragments of approximately 1.0 kb and 5.8 kb, corresponding to the *SMase* gene insert and the pET32a(+) vector, respectively. The presence of the expected insert and vector fragments confirmed the successful ligation of the *SMase* gene into the pET-32a(+) vector. Positive recombinant clones were subsequently selected for protein

expression. These results confirmed that the selected colonies contained the recombinant plasmid pET32a(+)-SMase for subsequent protein expression.

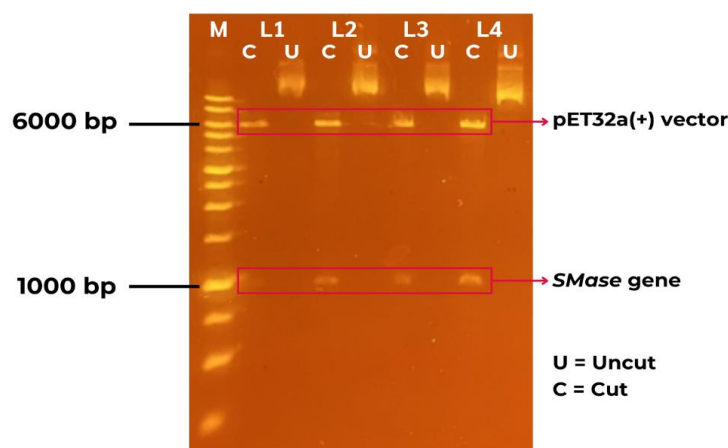


Figure 4. Agarose gel electrophoresis (0.75% agarose) of cut plasmid pET32a(+)-SMase by *Bam*HI and *Xho*I restriction enzyme digestion. Lane M: 1 kb DNA ladder; lanes L1–L4 (uncut): undigested recombinant plasmids; lanes L1–L4 (cut): digested recombinant plasmids.

3.5 Protein Expression

Recombinant SMase expression was induced with different IPTG concentrations (0, 0.075, 0.1, and 0.2 mM) at 16 °C for 8 and 12 hours and analyzed by SDS-PAGE (Figure 5). A protein band corresponding to the expected molecular weight of SMase (~56 kDa), including thioredoxin and 6xHis tags encoded by the pET32a(+) vector, was observed. The most intense protein band was detected following induction with 0.2 mM IPTG for 12 h, and this condition was selected for subsequent protein purification. These results confirmed the successful expression of recombinant SMase in *E. coli* BL21(DE3).

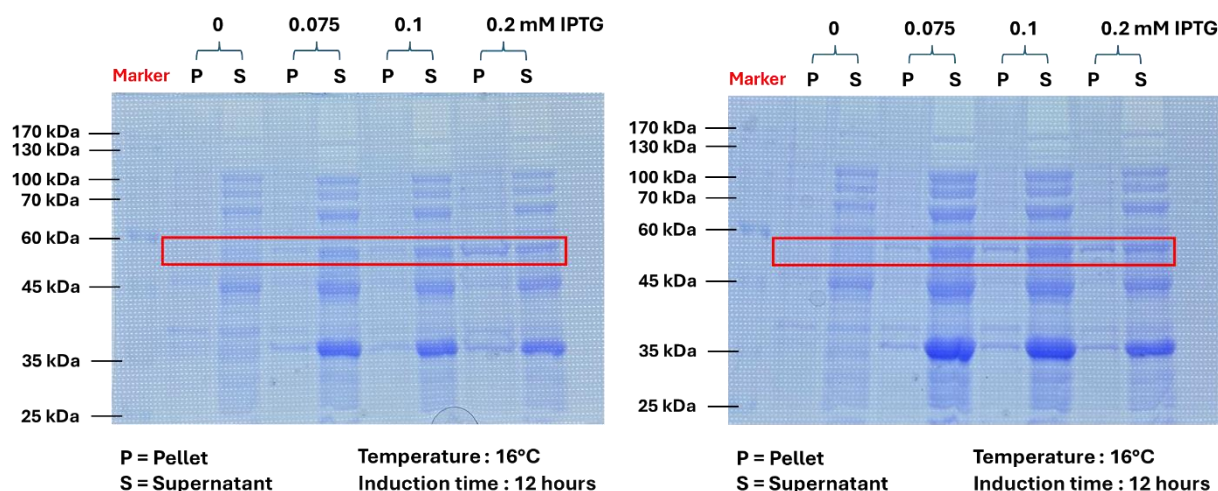


Figure 5. SDS-PAGE analysis of recombinant SMase expression in *E. coli* BL21(DE3) induced with different IPTG concentrations (0, 0.075, 0.1, and 0.2 mM) at 16 °C for 8 and 12 h. P: pellet fraction; S: supernatant fraction.

3.6 Protein Purification

Recombinant SMase protein was purified using Ni-NTA affinity chromatography and analyzed by SDS-PAGE (Figure 6). A protein band corresponding to the expected molecular weight of SMase, including thioredoxin and 6xHis tags (~56 kDa), was detected in the elution fraction using 200 mM imidazole fraction. Only minimal amounts of the target protein were detected in the flow-through and washing fractions, suggesting a high affinity between the recombinant SMase and the immobilized nickel ions. These findings demonstrate that Ni-NTA affinity chromatography is an effective purification strategy for recombinant SMase. However, the enzymatic activity of the purified recombinant SMase was not evaluated in this study and remains to be investigated in future studies.

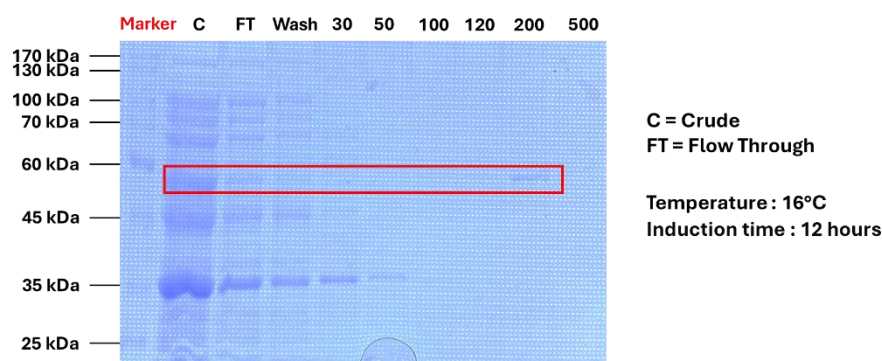


Figure 6. SDS-PAGE analysis of recombinant SMase protein purified by Ni-NTA affinity chromatography. M: protein marker; C: crude cell lysate; FT: Flow-through fraction; Wash: washing fraction; 30-500: elution fractions.

4. CONCLUSIONS

The *SMase* gene encoding sphingomyelinase from *Staphylococcus epidermidis* was successfully cloned into the pET-32a(+) expression vector and heterologously expressed in *E. coli* BL21(DE3), with the highest expression observed following induction with 0.2 mM IPTG for 12 h at 16 °C. The recombinant protein was subsequently purified using Ni-NTA affinity chromatography, and the highest purity was recovered in the 200 mM imidazole fraction. These findings provide a basis for the production of recombinant SMase. However, this study did not assess the enzymatic activity of the purified recombinant SMase, which will need to be examined in subsequent research.

5. ACKNOWLEDGEMENT

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Optimizing Mycelium Pellet Morphology and Its Impact on Bioactive Compound Production via a Taguchi Image Analysis Framework

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ABSTRACT: Mycelial pellet morphology is a key determinant of transport phenomena and productivity in submerged fungal fermentations, yet quantitative frameworks for its control remain underdeveloped. This study presents an integrated framework combining Taguchi experimental design, automated image analysis in FIJI, and multivariate analysis to optimize mycelial pellet morphology in *Pleurotus* spp. and to evaluate its impact on polysaccharides and triterpenoids production. The Taguchi L₉ orthogonal array was employed to evaluate the effects of four key medium components (olive oil, CaCO₃, yeast extract, and Tween 80) at three levels each. Fiji-based image analysis enabled the extraction of quantitative descriptors, including pellet count, circularity, and Feret diameter. Analysis of signal-to-noise (S/N) ratios identified the optimal condition (4mL olive oil, 0.25g CaCO₃, 0.5g yeast extract, and 0.5mL Tween 80) with pellet morphology significantly higher circularity 0.84 ± 0.05 , an increase in triterpenoid content of 1.38 ± 0.11 mg/dL, and polysaccharide yields of 1.96 ± 0.4 mg/dL. PCA explained 83% of total variance, with PC1 (44%) driven by productivity markers (circularity: 0.40; polysaccharides: 0.39; triterpenoids: 0.39) and negatively by pellet size (−0.36), while PC2 (30%) captured biomass-related traits (pellet count: 0.50; biomass: 0.46; circularity: 0.38). Circularity's strong positive loadings on both PCs (0.40 and 0.38) establish it as the most integrative morphological descriptor, positively correlates with all bioactive compounds, and inversely correlates with pellet size, thereby confirming that small, highly circular pellets optimize mass transfer and productivity. The findings establish a quantitative relationship between pellet morphology and fermentation performance in *Pleurotus* spp. and demonstrate that an integrated design-of-experiments approach with image-based analysis provides a reproducible, data-driven framework for morphological assessment and process optimization in submerged fungal fermentation.

Keywords: Circularity; FIJI; Principal component analysis; Submerged fermentation; Triterpenoids

Nucleotide Variations in the Middle Region of Melanocortin 4 Receptor Gene and Their Potential Association with Obesity Risk in the Thai Population

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ABSTRACT: At present, the analysis of specific gene variations can be used for the prognosis of obesity. The MC4 receptor protein, encoded by the *MC4R* gene, plays a key role in the leptin-melanocortin pathway, which regulates satiety and energy balance in humans. This research aimed to evaluate polymorphisms in the middle region of the *MC4R* gene and investigate the possible correlation between these gene variations and obesity in the Thai population. DNA was collected from 114 non-diabetic Thai volunteers, consisting of 60 normal-weight individuals (Body Mass Index [BMI] 18.5–22.9 kg/m²) and 54 obese individuals (BMI equal to or greater than 25.0 kg/m²). The middle region of the *MC4R* gene (positions 5,555–6,166) was amplified, producing a specific PCR product of 612 base pairs. Nucleotide sequence analysis of the 114 samples revealed two heterozygous SNPs exclusively in obese volunteers, identified as G5726A and T6100C. The G5726A mutation, also identified in previous study, results in a valine-to-isoleucine substitution at position 103 (V103I) in the MC4 receptor; however, as a conservative missense mutation, it is unlikely to significantly impact the chemical properties or the function of the protein. Conversely, the T6100C mutation is silent, causing no change to the amino acid sequence or protein function. Based on preliminary data from this study, it is recommended to increase the sample size in both groups and extend the analysis to encompass the entire *MC4R* gene. This approach will enhance the accuracy, reliability, and validity of assessing the potential of this gene for predicting obesity risk in the Thai population.

Keywords: Leptin - melanocortin pathway, *MC4R* gene; Nucleotide variations, Obesity, Thai population

1. INTRODUCTION

According to World Health Organization (2024), reports showed that 1 in 8 people worldwide suffered from obesity. In 2022, it was found that more than 1 billion people were obese, and 43% of adults globally were overweight. The rate of overweight adults worldwide has more than doubled since 1990. At the same time, the rate of overweight and obesity among children and adolescents aged 5-19 has increased fourfold [1]. In Thailand, a report in 2023 found that the percentage of Thai people who are overweight and obese has risen to 48.35%. Moreover, children and adolescents with obesity are five times more likely to become adults with this condition [2]. The significance of obesity is not only a personal health issue but also impacts society and the overall economy. It is a cause of various diseases that increase the risk of chronic non-communicable diseases such as cardiovascular disease, diabetes, and certain types of cancer. It also affects the country's medical expenditure. Therefore, reducing the incidence of obesity is an important global and national issue that requires systematic planning and development of preventive and treatment measures [1,3]. Obesity results from many factors, including genetics, environment, and lifestyle behaviors. Advances in genetic technology and the analysis of gene variations have played an important role in the recent study of obesity [3].

The Melanocortin 4 Receptor gene (*MC4R*) plays an important role in producing receptor proteins in the leptin-melanocortin pathway, which regulates food intake and energy homeostasis in the body. The hormone leptin, secreted by fat cells, binds to the leptin receptor and sends signals to pro-opiomelanocortin (POMC) neurons located in the hypothalamus of the brain. These neurons then release alpha-melanocyte-stimulating hormone (α -MSH). When α -MSH binds to the Melanocortin 4 receptor, it inhibits the production of proteins related to increased appetite, resulting in reduced hunger and increased energy expenditure. Therefore, the interaction between the Melanocortin 4 receptor and the leptin-melanocortin pathway helps maintain energy balance in the body and effectively controls body weight [4].

Nucleotide sequence variations within the *MC4R* gene may affect the leptin-melanocortin pathway and, hence, affect the appetite and energy expenditure within the body. In 1998, Vaisse et al. demonstrated that nucleotide variations in the *MC4R* gene led to changes in the amino acid structure of the MC4 receptor. These changes caused the receptor to malfunction, preventing the hypothalamus from effectively regulating food intake and energy expenditure, which ultimately contributed to obesity in affected individuals [5]. In 2024, Metzger et al. investigated the Phe51Leu mutation in the MC4 receptor in mice, of which the amino acid sequences were identical to humans. Their study showed that the Phe51Leu variant had reduced function compared to the normal MC4 receptor. Furthermore, mice carrying the Phe51Leu mutation consumed more food than normal mice, leading to an increase in body weight by approximately 21% to 29% compared to healthy controls. Based on these findings, the researchers suggested that the Phe51Leu mutation may be linked to hyperphagia, characterized by an intense and uncontrollable hunger [6]. Thus, studying genetic diversity in the *MC4R* gene by analyzing single nucleotide polymorphisms (SNPs), which are changes in a single base position in the gene, may help predict the risk of obesity.

This research analyzed nucleotide variations in the middle region of the *MC4R* gene in obese Thai individuals compared to a normal-weight control group. The analysis focused on the positions 5555-6166 of the gene to develop an understanding of how nucleotide sequence variations in this gene impact obesity. This knowledge aims to assist in assessing the risk and prognosis of obesity, as well as in determining appropriate prevention strategies for the Thai population in the future.

2. MATERIALS AND METHODS

Ethical statement

This study was reviewed and approved by the Ethics Committee of Chiang Mai University, Chiang Mai, Thailand (approval code: CMUREC 65/106 (2022)).

Population sampling

This research involved 114 Thai participants, including 60 individuals classified as normal weight with a Body Mass Index or BMI (body weight (kg)/height²(m)) between 18.50 and 22.99 kg/m², and 54 individuals considered obese with a BMI equal to or above 25.00 kg/m². All participants were aged between 18 and 60 years and did not have diabetes. Participants with a BMI of 23.0–24.9 kg/m², corresponding to the overweight category, were excluded to maintain distinct normal-weight and obese cohorts and reduce potential confounding in the association analyses.

DNA isolation from hair roots

Due to the COVID-19 pandemic, this study used hair roots as an alternative source for DNA isolation instead of the usual buccal cells. Five hair roots, each measuring 1 centimeter in length, were collected from each participant. The roots were cleaned with 70% ethanol and left to dry thoroughly

before DNA isolation was carried out using the PrimeWay Genomic DNA Extraction Kit from 1st BASE Co., Ltd. (Singapore) according to the manufacturer's recommendations, with the pre-lysis incubation time of 24 hours.

Primer designation

Primers MC4R-F1 and MC4R-R1 (Table 1) were designed using the reference sequence from the National Center for Biotechnology Information (NCBI) to amplify the middle region of the *MC4R* gene. These primers target the region spanning positions 5,555-6,166 of the *MC4R* gene. The primers were synthesized by Bio Basic, Markham, Ontario, Canada.

Table 1. Nucleotide sequences of primers designed for *MC4R* gene amplification

Primer name	Primer sequence (5' to 3')	T _m (°C)	Amplicon Size (bp)
MC4R-F1	GTCTCTCCTGAGGTGTTTGTG	65.0	612
MC4R-R1	GATGGTCAAGGTAATCGCTCC	65.0	

PCR amplification of the *MC4R* gene

The amplification of the *MC4R* gene was conducted in a 50 µL PCR reaction using an optimal annealing temperature of 65.8°C, as determined by gradient PCR. The reaction mixture included 50 ng of DNA template, 1× KOD One™ master mix (Toyobo, Osaka, Japan), and 0.5 µM of each primer. PCR cycling was performed using a Thermo Fisher Scientific thermocycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 95°C for 10 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 65.8°C for 30 seconds, and extension at 68°C for 1 minute; followed by a final extension at 68°C for 10 minutes. The PCR amplicons were analyzed on a 1.2% agarose gel (Markham, Ontario, Canada) using a 1 kb Plus DNA ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as the size marker and visualized under a UV transilluminator (UVP, UK) using RedSafe™ (iNtron Biotechnology, Seongnam-si, Gyeonggi-do, South Korea) DNA staining.

Nucleotide sequence analysis

PCR amplicons were submitted to First BASE Laboratories Sdn Bhd (Malaysia) for purification and nucleotide sequence analysis, which was performed using the Big-Dye terminator method with the MC4R-F1 primer. The nucleotide sequences obtained in this study were aligned and compared with the reference sequence from NCBI using BioEdit sequence alignment editor version 7.7.1 to identify single nucleotide polymorphisms (SNPs).

Simulating the translation of the nucleotide sequence into an amino acid sequence

ExPASy (<https://web.expasy.org/translate/>) was used to simulate the translation of the nucleotide sequence into an amino acid sequence, illustrating how variations in the nucleotide sequence result in amino acid changes in the MC4 receptor protein. Clustal Omega (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>) software was employed to perform alignment and comparison between MC4 receptor variants and the NCBI reference sequence.

3. RESULTS AND DISCUSSION

Identification of SNPs in the middle region of the *MC4R* gene

The analysis of electropherograms from all 114 samples identified two forms of single nucleotide polymorphisms (SNPs), both of which were found exclusively in obese volunteers. Both SNPs exhibited heterozygous characteristics, indicating that the genotypes contained allele pairs with different bases. The first SNP form, observed in five obese volunteers with BMI values of 25.64, 26.44, 27.47, 38.82, and 53.15, displayed overlapping peaks of a black peak representing guanine (G) and a green peak representing adenine (A), as illustrated in Figure 1. The second SNP form was identified in one sample from a volunteer with a BMI of 27.53. This variant exhibited overlapping peaks of a blue peak representing cytosine (C) and a red peak representing thymine (T), as shown in Figure 2. Interestingly, SNPs were observed only in obese volunteers, while no nucleotide variation was detected in normal-weight volunteers. When analyzed separately, the G5726A mutation showed a significant association with obesity (Fisher's exact test, $p = 0.0215$). In contrast, the T6100C was not significantly associated with obesity (Fisher's exact test, $p = 0.4737$).

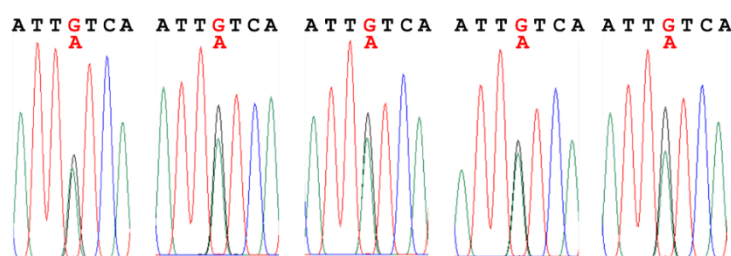


Figure 1. Heterozygous SNP G5726A identified in five obese volunteers

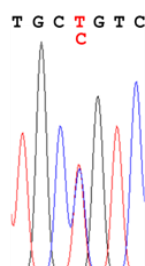


Figure 2. Heterozygous SNP T6100C identified in one obese volunteer

Alterations in amino acid sequence due to *MC4R* gene variants

The nucleotide substitution from guanine to adenine at position 5726 (G5726A) and the substitution from thymine to cytosine at position 6100 (T6100C) occurred within the coding region of the *MC4R* gene. This coding region is responsible for encoding the MC4 receptor protein.

The alignment of the 162 amino acids from the middle region of the MC4 receptor protein in samples from this study with the full-length MC4 receptor protein sequence of 332 amino acids from NCBI revealed a match corresponding to amino acid positions 79 to 240 of the MC4 receptor protein. The G5726A mutation resulted in the substitution of valine with isoleucine at position 103 (V103I), as illustrated in Figure 3. The G5726A mutation is classified as a conservative missense mutation,

indicating that the change in amino acid at this position does not markedly affect its chemical properties. Both valine (Val) and isoleucine (Ile) are non-polar aliphatic amino acids with hydrocarbon side chains (R groups) that are uncharged and nonpolar. Consequently, this substitution is unlikely to significantly influence the chemical characteristics or functional role of the MC4 receptor protein. However, the function of the MC4 receptor protein with the V103I variant should be further investigated.

On the other hand, the alignment of the amino acid sequence in the middle region of the MC4 receptor protein with the T6100C mutation, compared to the full-length MC4 receptor protein sequence of 332 amino acids from NCBI, revealed no change in the amino acid sequence. This indicates that the T6100C mutation is a silent mutation, meaning it alters the genetic code but does not affect the type of amino acid produced. Both codon translations result in the same amino acid, so the mutation does not impact protein function. Specifically, the nucleotide changes from thymine to cytosine at position 6100 alters the codon from GCU to GCC, both of which encode alanine (Ala) at position 227 of the full MC4 receptor protein sequence, as shown in Figure 3.

NCBI	MVNSTHRGMHTSLHLWNRSSYRLHSNAESLGKGYSDGGCYEQLFVSPEVFVTLGVISLL	60
G5726A	-----	0
NCBI	ENILVIVAIAKNKLNHSPMYFFICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVN	120
G5726A	-----MYFFICSLAVADMLVSVSNGSETIIITLLNSTDTDAQSFTVN	42
	*****;*****	
NCBI	IDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACVTS	180
G5726A	IDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMTVKRVGIIISCIWAACVTS	102

NCBI	GILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFLMARLHIKRIAVLPGTGAIRQGAN	240
G5726A	GILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFLMARLHIKRIAVLPGTGAIRQGAN	162

NCBI	MKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFNLYLILIMCNSIIDPL	300
G5726A	-----	162
NCBI	IYALRSQELRKTFKEIICCYPLGGCLDSSRY	332
G5726A	-----	162

Figure 3. Comparison of the amino acid sequence in the middle region of the MC4 receptor protein with the G5726A mutation to the full-length amino acid sequence of the MC4 receptor protein from NCBI

Potential of Melanocortin 4 Receptor Gene Variations as Prognostic Markers for Obesity

Several publications have reported that the G5726A mutation can be found in both normal-weight and obese individuals. Moreover, the V103 mutation in the MC4 receptor protein occurs in more than one percent of the normal-weight population [7]. Additionally, it has been reported that the V103 mutation in the MC4 receptor may help protect against obesity. This is because the V103I mutation affects the response of the *MC4R* gene to agouti-related protein (AGRP), which normally plays a role in stimulating appetite. Studies have shown that the response of the *MC4R* gene to AGRP is reduced, potentially leading to decreased appetite and a lower risk of developing obesity [8,9]. Therefore, based on previous studies, the V103I variant is unlikely to be associated with obesity. However, in this study, the G5726A mutation was absent from the normal-weight group, whereas it was detected only in obese individuals, resulting in a significant association with obesity as determined by Fisher's exact test ($p = 0.0215$). Conversely, despite the smaller number of 54 obese participants, this SNP was found in five individuals, resulting in an amino acid substitution in the MC4 receptor protein. However, due to the relatively small sample sizes in both the normal-weight and obese groups, increasing the number of participants is

recommended to ensure statistically reliable results.

Additionally, several other studies have reported mutations at different *MC4R* gene positions that impact the function of receptor in various ways and may influence obesity risk across diverse populations worldwide. In 2010, Cole et al. reported the T5933C mutation, which causes an amino acid substitution from cysteine (Cys) to arginine (Arg) at position 172 of the full MC4 receptor protein [10]. This mutation is a non-conservative missense mutation; however, its effect on obesity development was not explored. Therefore, because the coding region of the *MC4R* gene spans positions 5420–6418 (999 bp), while only the middle region (positions 5555–6166; 612 bp) was analyzed in this study, future investigations should extend the analysis to encompass the entire coding sequence as well as the regulatory regions of the *MC4R* gene. This broader approach may provide a more comprehensive understanding of the potential association between *MC4R* genetic variation and obesity risk in the Thai population.

Integrating the results of this study with insights from literature reviews, nucleotide variations have been identified within the *MC4R* gene at positions 5,555–6,166. The effects of these variations on MC4 receptor function require further investigation, as they may contribute to obesity risk in diverse populations worldwide.

4. CONCLUSIONS

This study analyzed nucleotide sequence variations in the Melanocortin 4 Receptor (*MC4R*) gene between obese Thai individuals and a normal-weight control group, focusing on positions 5,555–6,166. The aim was to preliminarily assess polymorphisms of this gene and their potential influence on obesity, with the goal of creating a database to predict risk and guide effective prevention strategies for the Thai population. Analysis of the nucleotide sequence in the middle region of the *MC4R* gene revealed two heterozygous SNP forms, G5726A and T6100C, which were observed exclusively in obese individuals. The G5726A mutation, primarily observed in previous investigations, results in a conservative missense mutation and is not expected to significantly impact the function of the MC4 receptor protein. In contrast, the T6100C mutation is a silent mutation that does not alter the amino acid sequence and, therefore, does not affect the function of the protein. Based on the preliminary data from analyzing nucleotide variations at positions 5,555–6,166 of the *MC4R* gene in 114 samples in this study, two SNP forms were found in six obese volunteers. Therefore, it is recommended to increase the sample size in both groups and expand the analysis to cover the entire *MC4R* gene. This will improve the accuracy, reliability, and validity of assessing the potential use of this gene for predicting obesity risk in the Thai population.

5. ACKNOWLEDGEMENT

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Expression and Purification of Recombinant *SpCas9*-HF1-Plus Protein in *Escherichia coli* *LysY/I^q* for High Precision Gene Editing

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ABSTRACT: The high-fidelity Cas9 variant, *SpCas9*-HF1-plus, is a powerful tool for precise genome editing. It significantly reduces off-target effects compared to wild-type Cas9. However, the application of this technology in fungi via Cas9 ribonucleoprotein (RNP) complexes requires the production of high-purity recombinant proteins. This study focuses on the production, optimization, and purification of *SpCas9*-HF1-plus with 3× NLS (Nuclear Localization Sequence) in recombinant *Escherichia coli* *lysY/I^q*. The *SpCas9*-HF1-plus gene with 3× NLS was successfully cloned from pET-FLAG-*SpCas9*-HF1-plus by PCR and ligated with a pET28a vector. The resulting plasmid named pET28a-*SpCas9*-HF1-plus was introduced into an expression host, *E. coli* *lysY/I^q*. For expression of heterologous protein, the recombinant *E. coli* was induced with 0.4 mM IPTG, and results from these expression trials showed that the highest yield among the conditions tested was achieved at an induction temperature of 18 °C for 24 h. The recombinant protein was purified using Ni-NTA affinity chromatography, with the target protein effectively eluting at imidazole concentrations between 100–200 mM. SDS-PAGE analysis revealed a prominent band at approximately 170 kDa, matching the theoretical molecular weight. The purified protein was further confirmed by Western blot analysis using an anti-6× His tag antibody. In summary, this established production process provides a reliable platform for producing the Cas9 protein, serving as a crucial foundation for genome editing in fungi in the future.

Keywords: CRISPR/Cas9, *SpCas9*-HF1-plus, Protein purification

A Multipurpose Antibody Format for Flexible Assay Development Using Cell-Based and Cell-Free Production

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ABSTRACT: Antibody reagents used in diagnostics often require separate optimization for each assay format or detection reporter, which can limit rapid development and increase production burden. This study aimed to develop a multipurpose antibody format that can be produced by both *Escherichia coli*-based expression and cell-free protein synthesis (CFPS) and then applied across multiple assay platforms. The antibody format was designed to maintain antigen recognition while enabling flexible coupling with different detection modules. In the cell-based route, recombinant proteins were produced in *Escherichia coli*, recovered from inclusion bodies, refolded using an automated dialysis-based process developed in our group, and purified to obtain functional reagents at milligram scale. The purified antibodies retained antigen-binding activity and were successfully used in conventional enzyme-linked immunosorbent assay (ELISA) and simplified one-step ELISA which does not require secondary antibodies. In parallel, CFPS enabled rapid preparation of assay-ready antibody reagents directly from DNA templates. The cell-free products showed antigen-dependent signals in plate-based assays and were also applicable to fluorescence-based immunostaining after simple mixing with a reporter component. These results demonstrate that the proposed antibody format can bridge scalable production and rapid on-demand preparation while supporting reporter exchange and multiple analytical applications. The platform may contribute to flexible diagnostic reagent development, particularly when rapid customization and assay adaptability are required.

Keywords: Antibody engineering, Cell-free protein synthesis, Diagnostic assays, *Escherichia coli*, Multipurpose antibody

Thermo-Responsive Morphological Evolution and Rheological Behavior of Wax/Butter-Based Phase Change Microparticles

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ABSTRACT: Phase change materials (PCMs) are materials capable of storing and releasing thermal energy via reversible phase transitions, such as solid-liquid and liquid-gas transitions, over a specific temperature range. Owing to their thermal energy storage and release capacity, as well as their temperature-regulating properties, PCMs have been widely investigated in various industrial and scientific fields, including agriculture, cosmetics, biomedicine, and pharmaceuticals. In particular, their ability to undergo changes in physical properties in response to subtle temperature variations demonstrates their potential as stimuli-responsive delivery systems for the precise control of active ingredient release. In this study, wax (W)/butter (B)-based PCM microparticles with sizes of approximately 1-2 μm were successfully fabricated. This study focused on investigating how temperature-dependent shape transformation is associated with the rheological and thermal properties of the material. At 30 °C, the PCM particles exhibited a distinct anisotropic morphology, whereas increasing the temperature to 50 °C resulted in a transformation into an isotropic spherical morphology. This morphological evolution is considered to arise from the complex interplay among viscosity, interfacial tension, and solidification kinetics during particle formation. In addition, the composition and thermal properties of the fabricated microparticles were analyzed, and their temperature-dependent viscoelastic behavior was further evaluated. Rheological analysis confirmed that the viscosity and viscoelastic moduli decreased with increasing temperature, which is consistent with the observed morphological transformation of the PCM particles. Overall, the developed W/B-based PCM microparticles exhibit thermo-responsive shape transformation and temperature-dependent rheological behavior, highlighting their potential as functional materials for thermally controlled delivery systems. Furthermore, their inherent biodegradability and sustainability make the PCM microparticles attractive eco-friendly materials for future cosmetic and pharmaceutical formulations.

Keywords: Phase Change Materials (PCMs), Wax-Based Microparticles, Temperature-Responsive Morphological Evolution, Viscoelasticity; Rheology

Skin-Adhesive Illite-Containing Amphiphilic Hydrogel Composite Patches for Wound Care

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ABSTRACT: Hydrogel-based wound dressings have attracted considerable attention owing to their high water content, biocompatibility, and ability to maintain a moist wound environment. Despite these advantages, achieving both robust mechanical properties and reliable skin adhesion remains a significant challenge for hydrogel wound dressings. In this study, skin-adhesive tough amphiphilic hydrogel composite patches containing illite, a natural clay mineral abundant in the Yeongdong region of Chungcheongbuk-do, South Korea, were developed for wound dressing applications. The N,N-dimethylacrylamide (DMAAm)-based amphiphilic hydrogel composite patches containing illite were synthesized by varying the contents of hydrophobic moieties and illite particles using UV radical polymerization. The resulting amphiphilic network, composed of hydrophilic and hydrophobic polymer phases and size-controlled illite particles, provided a balanced combination of hydration, mechanical flexibility, and skin-interfacial adhesion. In addition, the incorporation of illite particles enhanced network integrity and introduced additional functionalities into the composite structure, including antibacterial activity, adsorption capability toward wound-associated harmful substances, and UV protection. This inorganic–polymer hybrid design provides a simple, scalable fabrication strategy for functional hydrogel composite patches. The developed illite-containing amphiphilic hydrogel patches exhibited promising characteristics as skin-adhesive wound dressing materials, thereby providing a versatile platform for future biomedical applications.

Keywords: Amphiphilic hydrogel Composites, Illite, Skin-Adhesion, Wound Dressings, UV Radical Polymerization

AI-Based Oral Lesion Detection System with Integrated Online Chatbot

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ABSTRACT: Many individuals struggle to correctly identify oral ulcers, often leading to incorrect self-treatment that can worsen their condition. To address this issue, this study introduces an accessible AI-driven LINE Official Account chatbot, Smile Bot, designed to provide instant and user-friendly oral lesion screening. The system classifies oral conditions into five primary categories: Canker Sores, Oral Thrush, Herpes, Oral Cancer, and Normal tissue, allowing users to seek the correct medical treatment. To develop the core classification engine, a dataset comprising 409 training images, 113 validation images, and 109 testing images was utilized. The study evaluated and compared four pretrained convolutional neural network architectures: VGG16, ResNet50, Xception, and EfficientNetB0. For each architecture, the pretrained convolutional layers were frozen, and a new classifier was trained on the custom dataset. Among the tested networks, EfficientNetB0 demonstrated the best performance, achieving the highest overall accuracy of 70.64%, with a precision of 0.6948, a recall of 0.6950, and an F1 score of 0.6929. The model's supremacy was further supported by a confusion matrix showing high values along the main diagonal. Google Colab was used for code development, and the model was successfully integrated with the LINE application using Ngrok, a channel secret, and a channel access token. To use the tool, users simply upload a photo of their oral ulcer to the Smile Bot chat. The bot immediately replies with the predicted lesion type, a classification, confidence percentage, and a Grad-CAM visualization map showing exactly which part of the image the AI analyzed. Future work will focus on gathering more data to improve accuracy, preventing overfitting, expanding the system to detect a wider variety of oral diseases, and providing causes and treatment advice within the chat. To reach users without social media, plans also include expanding the platform to web-based and application-based interfaces.

Keywords: AI; Chatbot, Convolutional Neural Networ, Detection for Oral Diseases, EfficientNetB0

Deciphering the Tissue-Specific Molecular Imprint of Oral Mesenchymal Stem Cells: A Comparative RNA-Seq Analysis

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ABSTRACT: Owing to their multilineage differentiation capabilities, mesenchymal stem cells (MSCs) serve as a cornerstone in regenerative medicine. However, the tissue origin fundamentally dictates their functional traits. This present study explores to decipher the transcriptomic heterogeneity between stem cells from human exfoliated deciduous teeth (SHED) and alveolar bone mesenchymal stem cells (ABMSCs) to establish precise cell-selection criteria for oral tissue engineering. High-throughput RNA sequencing was performed on biological replicates (n = 3 per group) obtained from independent human tissue donors, allowing biological variation between donors to be captured and improving the robustness and reproducibility of the findings. Differentially expressed genes (DEGs) were identified ($\log_2FC \geq 2$, $p < 0.05$) and analyzed via DAVID and STRING bioinformatics tools. Transcriptomic analysis identified 477 upregulated and 220 downregulated transcripts in ABMSCs relative to SHED. ABMSCs exhibited elevated genes involved in immunomodulatory responses and cytokine-mediated signaling, whereas suppressed genes (upregulated in SHED) were enriched in neurogenic pathways. Importantly, STRING analysis revealed distinct topological structures and hub proteins between groups, confirming that ABMSCs and SHED operate via fundamentally different molecular machinery. Collectively, our data demonstrates that the tissue origin of oral MSCs establishes distinct molecular signatures that shape their biological properties and therapeutic potential. The identification of clearly divergent regulatory networks provides important insights into the functional characteristics of MSCs derived from different tissue sources and serves as a critical foundation for developing targeted and highly specific regenerative therapies for oral and maxillofacial tissues.

Keywords: Alveolar bone, Mesenchymal Stem Cells, RNA sequencing, Regenerative tissue, SHED

Development of a Graphene-Modified Quartz Crystal Microbalance Biosensor for Non-Invasive Prostate Cancer Detection

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ABSTRACT: Prostate cancer is one of the most common cancers among men worldwide, and early diagnosis is essential for improved treatment outcomes and survival rates. This preliminary study presents a graphene oxide-coated quartz crystal microbalance (GO-QCM) biosensor for label-free detection of distal-less homeobox-1 (DLX1), a biomarker associated with prostate cancer progression. The objective of this work was to investigate the feasibility of a graphene-modified QCM platform for sensitive and reusable DLX1 detection. Gold-coated QCM sensors were modified with graphene oxide to improve surface area and biomolecular interaction. DLX1 single-stranded DNA probes were immobilized onto the GO-QCM surface through EDC/NHS coupling chemistry. Biosensor performance was evaluated using DLX1 target concentrations ranging from 1 nM to 10 μ M by monitoring resonance frequency changes during hybridization. The results confirmed an immobilization of the DLX1 probe through resonance frequency reduction caused by mass loading on the sensor surface. Concentrations from 10 nM to 10 μ M produced negative frequency shifts that confirmed successful probe-target hybridization, whereas weak hybridization behavior was observed at a concentration of 1 nM. The strongest response was observed at a concentration of 1 μ M. In addition, the biosensor demonstrated regeneration capability following NaOH treatment, which indicated potential reusability for repeated detection cycles.

Keywords: DLX1 biomarker, DNA biosensor, Graphene oxide, Prostate cancer, Quartz crystal microbalance

***mecA* and *dnaG* Gene Knockout in *Staphylococcus aureus* via CRISPR-Cas9 System as a Model for Methicillin-Resistant *Staphylococcus aureus* Inhibition**

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ABSTRACT: The rapid rise of antibiotic-resistant *Staphylococcus aureus* (*S. aureus*) poses a significant global public health challenge. Although *S. aureus* commonly colonizes the skin and respiratory tract of approximately 30% of healthy individuals without causing disease, it can cause infections ranging from mild skin and soft tissue infections to severe, life-threatening illnesses when it enters the body through wounds or other breaches in host defenses. Conventional antibiotics often lack specificity, resulting in disruption of beneficial microbiota and contributing to the spread of antimicrobial resistance. This study explores the potential of CRISPR-Cas9 as a programmable antimicrobial strategy by targeting two genes in *S. aureus*: *mecA*, which confers methicillin resistance in methicillin-resistant *S. aureus* (MRSA), and *dnaG*, which encodes a DNA primase essential for bacterial DNA replication. Following CRISPR-Cas9 treatment, PCR amplification and gel electrophoresis were used to assess the targeted genomic regions for evidence of editing. The observed PCR results were consistent with successful modification of the target loci, supporting the feasibility of CRISPR-Cas9-mediated gene targeting in *S. aureus*. These findings demonstrate the potential of CRISPR-Cas9 as a targeted antimicrobial platform against antibiotic-resistant *S. aureus*. Additional validation, including DNA sequencing and functional assays, will be necessary to confirm gene disruption and evaluate treatment specificity and its effects on non-target microbial communities.

Keywords: Antibiotic resistance, CRISPR Cas-9, *S. aureus*, Knockout, Gene editing

An AI-Based System for Facial Expression Classification: A Comparative Study of Model Training Across Various Emotions

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ABSTRACT: This study addresses the diagnostic challenges of monitoring depression, a major mental health disorder affecting millions worldwide, by developing a facial emotion recognition system designed to track emotional changes across therapy sessions. Current therapy outcome assessments heavily rely on subjective clinical interviews and self-report questionnaires, which are time-consuming and heavily strain limited psychiatric resources, particularly in countries like Thailand. To provide mental health professionals with objective, quantitative metrics, this research evaluated an AI framework to classify seven distinct emotions. Utilizing a dataset of 2,800 images partitioned into training (60%), validation (20%), and testing (20%) subsets, deep learning architectures, including VGG16, ResNet, EfficientNetB0, and Xception, were trained for 16 epochs, with Xception serving as the core model. The models demonstrated excellent stability with no signs of overfitting or underfitting, achieving an overall average accuracy of approximately 90% and average F1 scores ranging between 0.85 and 0.89. Explainable AI heatmaps successfully verified that the models focused accurately on key facial regions relevant to emotion detection. Among the categories, happiness was classified with the highest precision, while disgust yielded the lowest performance. By converting qualitative facial expressions into quantifiable data, this system reduces clinical workloads, supports psychiatrists with objective evaluation tools, and enhances the overall accessibility of mental health monitoring. Future work will focus on integrating real-time, frame-by-frame processing to continuously analyze emotional states within clinical, hospital, and psychiatric treatment environments, offering medical professionals comprehensive longitudinal insights for optimized patient care.

Keywords: Depression, Deep Learning, Emotion Recognition, Explainable AI, Xception

Development of an AI-Based LINE Chatbot for Preliminary Classification of Common Pediatric Skin Conditions

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ABSTRACT: This study addresses the diagnostic challenges posed by overlapping symptoms among common pediatric skin conditions, including Heat Rash, Molluscum Contagiosum, Ringworm, Seborrheic Dermatitis, Urticaria, and Normal Skin, by developing an artificial intelligence (AI)-based framework for preliminary classification. A dataset of 655 images was divided into training (398), validation (162), and testing (95) subsets. Four pre-trained convolutional neural network architectures (VGG16, Xception, EfficientNetB0, and ResNet50) were evaluated using transfer learning over 25 epochs. ResNet50 demonstrated the best overall performance, achieving a test accuracy of 82.22%, a precision of 0.8225, a recall of 0.8222, and an F1-score of 0.8188. The model achieved strong classification performance for Molluscum Contagiosum and Normal Skin, with F1-scores of 0.88 and 0.87, respectively. In contrast, Heat Rash remained the most challenging class (F1-score = 0.64) because of its visual similarity to other inflammatory skin conditions, highlighting the need for targeted class-weighting strategies and focal loss to improve classification performance for visually similar classes in future work. Training curves indicated slight overfitting, with validation accuracy stabilizing at approximately 80–86%. To translate the model into an accessible screening tool, the ResNet50 model was integrated into a LINE Chatbot. By uploading an image of the affected skin, users receive an immediate preliminary prediction, a confidence score indicating the estimated probability of the predicted class, an explainable AI heatmap highlighting the image regions influencing the prediction, and basic care recommendations. The chatbot clearly communicates that its predictions are intended solely for preliminary screening and should not be considered a substitute for professional medical diagnosis. Future work will expand the dataset to include more diverse skin tones and implement k-fold cross-validation to provide a more statistically robust model evaluation, thereby improving model robustness, generalizability, and clinical reliability.

Keywords: Image classification, LINE Chatbot, Pediatric skin conditions, ResNet50, Transfer learning

Computational Evaluation of Understudied Second-Shell Residues Surrounding the Catalytic Triad of PETase

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ABSTRACT: Polyethylene terephthalate (PET) pollution is a growing environmental challenge due to the persistence of plastic waste in natural ecosystems. With over 380 million tonnes of plastic produced annually and enzymatic degradation offers a promising low-energy recycling strategy, improving the thermostability of poly(ethylene terephthalate) hydrolase (PETase) is essential for industrial applications, particularly in tropical regions such as Southeast Asia. While previous engineering efforts have focused on active-site residues and known thermostability hotspots, the role of second-shell residues surrounding the catalytic triad remains poorly understood. This study aimed to identify and evaluate understudied second-shell residues as potential engineering targets in PETase. The crystal structure of PETase (PDB: 5XJH) was analyzed using PyMOL to identify residues surrounding the catalytic triad (Ser160, Asp206, and His237). Candidate residues were selected based on spatial proximity, secondary structure, and literature screening. Nine understudied residues were subjected to computational mutagenesis using DynaMut2 to predict changes in protein stability ($\Delta\Delta G$). Among the single mutations evaluated, the least destabilizing effects were observed for A89P (-0.29 kcal/mol) and A202P (-0.32 kcal/mol). Five rationally selected double-mutant combinations were additionally assessed, all of which were predicted to further reduce stability, with $\Delta\Delta G$ values ranging from -0.26 to -1.30 kcal/mol. These results suggest that the second-shell environment surrounding the PETase catalytic triad is structurally constrained and sensitive to amino-acid substitutions. Most single and double mutations decreased predicted stability, indicating that these residues contribute to maintaining enzyme structure. This study establishes a computational elimination framework that narrows the mutational search space by identifying second-shell positions with limited tolerance to substitution, thereby supporting more targeted strategies for future PETase engineering and sustainable plastic biodegradation.

Keywords: Computational mutagenesis, Plastic biodegradation, Poly (ethylene terephthalate) hydrolase (PETase), Protein stability, Second-shell residues

Deep Learning-Based Classification of Oral Diseases

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ABSTRACT: Oral lesions, including fibroma, hand, foot, and mouth disease (HFMD), mouth ulcers, mucocoeles, and oral cancer, are common health conditions that affect the mouth and can cause pain, discomfort, and difficulty eating or speaking. While some lesions are harmless, others may indicate serious diseases such as oral cancer, making early detection and accurate diagnosis important for maintaining oral health and improving patient outcomes. Currently, oral lesions are primarily diagnosed through visual examination by healthcare professionals, which can be challenging and time-consuming due to the similar visual appearances of different lesions, potentially leading to delayed diagnosis and treatment. Therefore, this study investigates the effectiveness of various pretrained models to identify the best-performing model for further development. The dataset consisted of 1,270 images, which were divided into 972 training images, 148 validation images, and 150 test images. Four pre-trained models: EfficientNetB0, ResNet50, VGG16, Xception, were compared for oral lesion classification. Each model was trained for 35 epochs using the same training, validation, and test sets. Their performances were evaluated and compared based on accuracy, F1 score, confusion matrix analysis, and training quality. ResNet50 achieved the best overall performance, with an accuracy of 83.33% and an F1 score of 0.8344, while EfficientNetB0 achieved an accuracy of 78.21% and an F1 score of 0.7439. Xception achieved an accuracy of 78.00% and an F1 score of 0.7870, whereas VGG16 achieved an accuracy of 60.66% and an F1 score of 0.600. For the oral cancer class, the selected ResNet50 model achieved a sensitivity (recall) of 84.0%, specificity of 96.8%, showing promising performance for oral cancer screening while maintaining a low false-positive rate. Heatmaps verified that the models focused on relevant oral lesion areas, though some confusion occurred between mouth ulcers and mucocoeles due to their similar visual characteristics. Nevertheless, the model is intended as a screening support tool rather than a definitive diagnosis system, and its predictions should be interpreted in conjunction with clinical examination and confirmed by qualified healthcare professionals.

Keywords: Artificial intelligence, Deep learning, Oral disease, Image classification

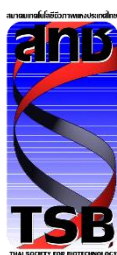


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