PHYSICOCHEMICAL, VOLATILE COMPOUND, AND METAGENOMIC ANALYSIS ON THE QUALITY OF MALAYSIAN BUDU (FISH SAUCE)

By

SHANTI DWITA LESTARI

Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my parents, husband, and children, who poured out countless prayers, loves, and sacrifices to ensure I had the most favourable opportunity to succeed. May Allah bless them abundantly

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Inconsistency in product quality has often been a common problem for *budu* as it relies on spontaneous fermentation. This study aimed on a process improvement by applying a starter culture selected based on metagenomic analysis and hydrolytic activities. The first objective was to assess the process variations and how it affects the quality attributes and compliance with the Malaysia Food Regulation 1985. Chemometric-based analysis clustered *budu* into raw, heated, and non-heated types with total plate count, Enterobacteriaceae, moisture, protein, ash, salt, total free fatty acids, pH, water activity, and viscosity contributed considerably to the grouping. *Budu* whose quality complied with regulation standards were further evaluated for its microbial community and metabolite profiles during fermentation in second objective. *Budu* microbiota altered significantly after 1, 3, 6, and 12 months of fermentation, with Chao1 and Shannon indices indicating a decrease in microbial richness and diversity with time. The canonical correspondence analysis showed that bacterial community explained 84.53% of the variance in volatile metabolites. The high accumulation of 3-methylbutanoic acid with nutty and cheesy notes in the 12-month sample corresponded

to the abundance of *Lentibacillus*, Bacilli, and *Halomonas*. The metagenome function prediction suggested that protein and lipid degradation pathways were responsible for most *budu* flavour compound synthesis. Therefore, salt-tolerant microbes with high proteolytic and lipolytic activity were isolated and characterized in the third objective. Five strains identified as *Bacillus haikouensis* 3M2G, *Bacillus* sp. 3M3A, *Bacillus* sp. 6M2A, *Bacillus haikouensis* 12M1F, and *Bacillus licheniformis* 12N3A with the highest crude enzyme activity ranged from 124.46-164.78 U (protease), 22.00-30.00 U (lipase), 104.45-155.48 U (esterase) and accounted for 902.33-1993.02 µmol/L glutamic acid production were used as co-culture for a laboratory-scale *budu* fermentation. In the fourth objective, *Bacillus*-mixed strains were compared to backslopping and spontaneous fermentation on physicochemical properties, volatiles, and microbial community profiles. Strain-added *budu* displayed significantly lower total volatile basic nitrogen and higher degree of hydrolysis ($p < 0.05$) than backslopping and spontaneous fermentation. These samples also exhibited a closer amino nitrogen content, browning intensity, and degree of hydrolysis to commercial samples fermented for 12 months. The addition of strains increased the formation of 3-methylbutanoic acid, 2,6-dimethylpyrazine, hexanal, 3-methylbutanal, heptanal, N, N-dimethylamine and phenylethyl alcohol. Fermentation with *Bacillus* strains could benefit the industry by improving the flavour profile of *budu* while controlling unwanted volatile bases formation.

Keywords: Chemometrics, Fermentation, Flavour Analysis, Microbial Community, Starter Culture

SDG: GOAL 2: Zero Hunger

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk Doktor Falsafah

ANALISIS FIZIKOKIMIA, SEBATIAN MERUAP, DAN METAGENOMIK TERHADAP KUALITI BUDU (SOS IKAN) MALAYSIA

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Kualiti produk yang tidak konsisten sering menyebabkan masalah bagi budu kerana ia bergantung kepada penapaian spontan. Kajian ini mensasarkan penambahbaikan proses dengan menggunakan kultur pemula yang terpilih berdasarkan analisis metagenomik dan aktiviti hidrolitik. Objektif pertama kajian ini menilai variasi proses dan bagaimana ia mempengaruhi sifat kualiti dan pematuhan dengan Peraturan Makanan Malaysia 1985. Analisis berasaskan kemometrik mengelompokkan budu kepada jenis mentah, dipanaskan dan tidak dipanaskan dengan jumlah kiraan plat, Enterobacteriaceae, lembapan, protein, abu, garam, jumlah asid lemak bebas, pH, aktiviti air, dan kelikatan menyumbang dengan ketara kepada pengelompokan. Budu yang kualitinya mematuhi piawaian peraturan dinilai selanjutnya untuk komuniti mikrob dan profil metabolitnya semasa penapaian dalam objektif kedua. Mikrobiota budu berubah dengan ketara selepas 1, 3, 6, dan 12 bulan penapaian, dengan indeks Chao1 dan Shannon menunjukkan penurunan kekayaan dan kepelbagaian mikrob mengikut masa. Analisis korespondensi kanonik menunjukkan bahawa komuniti bakteria menjelaskan 84.53% daripada varians dalam metabolit meruap. Pengumpulan

tinggi asid 3-metilbutanoik dengan nota kacang dan keju dalam sampel 12 bulan sepadan dengan banyakan *Lentibacillus*, Bacilli, dan *Halomonas*. Ramalan fungsi metagenom mengesahkan bahawa laluan degradasi protein dan lipid bertanggungjawab untuk kebanyakan sintesis sebatian perisa dalam budu. Oleh itu, mikrob tahan garam dengan aktiviti proteolitik dan lipolitik yang tinggi telah diasingkan dan dicirikan dalam objektif ketiga. Lima strain dikenal pasti sebagai *Bacillus haikouensis* 3M2G, *Bacillus* sp. 3M3A, *Bacillus* sp. 6M2A, *Bacillus haikouensis* 12M1F, dan *Bacillus licheniformis* 12N3A dengan aktiviti enzim kasar tertinggi berjulat antara 124.46-164.78 U (protease), 22.00-30.00 U (lipase), 104.45-155.48 U (ed 9.3-155.48) dan 104.45-155.48 mol/L pengeluaran glutamik asid digunakan sebagai kultur campuran untuk penapaian budu skala makmal. Dalam objektif keempat, kesan strain campuran *Bacillus* dibandingkan dengan backslopping dan penapaian spontan pada sifat fizikokimia, metabolit meruap, dan profil komuniti mikrob. Budu yang ditambah kultur menunjukkan jumlah nitrogen bes meruap yang jauh lebih rendah dan tahap hidrolisis yang lebih tinggi ($p < 0.05$) berbanding backslopping dan penapaian spontan. Sampel-sampel ini juga mempamerkan kandungan nitrogen amino, keamatan keperangan, dan tahap hidrolisis yang hampir menyamai sampel komersial yang ditapai selama 12 bulan. Penambahan strain meningkatkan pembentukan asid 3-metilbutanoik, 2,6-dimetilpirazin, heksanal, 3-metilbutanal, heptanal, N, N-dimethylamine dan phenylethyl alcohol. Penapaian dengan strain *Bacillus* boleh memberi manfaat kepada industri dengan menambah baik profil rasa budu sambil mengawal pembentukan bes meruap yang tidak diingini.

Kata Kunci: Analisis Rasa, Chemometrics, Fermentasi, Komuniti Mikrob, Kultur Pemula

SDG: MATLAMAT 2: Kelaparan Sifar

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LIST OF ABREVIATIONS

µg	microgram
µl	microlitre
µm	micrometre
$AgNO_3$	Silver nitrate
AMU	Atomic Mass Unit
AN	Amino nitrogen
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
ASV	Amplicon Sequence Variant
a_w	Water activity
B	Backslopped *budu*
BCAA	Branched-chain amino acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Control (*budu* made with spontaneous fermentation)
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$CaCO_3$	Calcium carbonate
CCA	Canonical Correspondence Analysis
CCR	Carbon Catabolite Repression
CFS	Cell-free supernatant
CFU	Colony Forming Unit
CH_3COOH	Acetic Acid
cm	Centimetre

ddH_2O	Double-Distilled Water
DGGE	Denaturing gradient gel electrophoresis
DH	Degree of hydrolysis
dH_2O	Distilled Water
DMA	Dimethylamine
DNA	Deoxyribonucleic acid
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
EDTA	Ethylenediaminetetraacetic acid
FAA	Free amino acids
FFA	Free fatty acids
FN	Formol nitrogen
g	gram
GC-MS	Gas Chromatography-Mass Spectrometry
GDA	Glutamate dehydrogenase
gDNA	Genomic DNA
GS-GOGAT	Glutamine synthetase-glutamate synthetase
H	Heated *budu*
h	hour
H_2O	Water
H_2SO_4	Sulphuric acid
HCA	Hierarchical Cluster Analysis
HCl	Hydrochloric acid
HPL	Hydroperoxide lyase
HPLC	High Performance Liquid Chromatography
HSD	Honest Significant Difference
K_2CrO_4	Potassium chromate
K_2HPO_4	Dipotassium hydrogen phosphate

KCl	Potassium chloride
kDa	kiloDalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kg	kilogram
KMO	Kaiser-Meyer-Olkin
KO	KEGG orthologs
KOH	Potassium hydroxide
L	litre
L^*	Lightness
LAB	Lactic acid bacteria
LCh	Lightness, chroma, hue
LEfSe	Linear discriminant analysis effect size
LOX	Lipoxygenase
M	Molar
M1	Month 1
M12	Month 12
M3	Month 3
M6	Month 6
MB	Marine Broth
MgO	Magnesium oxide
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
min	minute
mL	millilitre
mM	millimolar
$MnSO_4.7H_2O$	Manganese sulphate heptahydrate
MRS	De Man–Rogosa–Sharpe
MSA	Mannitol Salt Agar

MW	Molecular weight
N	Normal (normality unit)
NA	Nutrient Agar
Na_2HPO_4	Disodium hydrogen phosphate
$Na_3C_6H_5O$	Trisodium citrate
NaCl	Natrium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Natrium hydroxide
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
NH	Non-heated *budu*
NH_3	Ammonia
nm	nano meter
NPN	Non-protein nitrogen
OAV	Odour activity value
OD	Optical density
PBS	Phosphate buffered saline
PC	Principal component
PCA	Plate count agar
PCA	Principal Component Analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PLSR	Partial Least Squares Regression
p-NP	p-nitrophenol

PUFA	Polyunsaturated Fatty Acids
R	Raw *budu*
RI	Retention Index
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RuMP	Ribulose monophosphate
s	second
S	Strain-added *budu*
SCFA	Short-chain fatty acid
SD	Standard deviation
SMA	Skim milk agar
SPME	Solid phase microextraction
SPRI	Solid phase reversible immobilisation
STAMP	Statistical analysis of metagenomic profiles
TCA	Trichloroacetic acid
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TMP	2,4,6-trimethylpyridine
TN	Total nitrogen
TPC	Total Plate Count
TSS	Total soluble solid
TVBN	Total volatile basic nitrogen
U	Unit
UPLC	Ultra performance liquid chromatography
v	volume
VFA	Volatile fatty acid
VOC	Volatile organic compound

VRBG	Violet red bile glucose
w	weight
γ-PGA	poly-γ-glutamic acid

CHAPTER 1

INTRODUCTION

Budu is a fermented anchovy sauce accounted as the second most significant fermented fish product in Malaysia (Huda, 2012) and has been endorsed as a National Food Heritage under the gravies and accompaniments category (National Heritage Department, 2012). Similar to other fermented fish product, the popularity of *budu* among consumers is due to their appetite-stimulating flavour contributed by the volatile and non-volatile taste-active components. The formation of volatile nitrogenous compounds and volatile fatty acids as contributory factors in the taste and odour of fish sauce is determined by the fish, ingredients, and fermentation procedure (Nakano *et al.*, 2017). Current *budu* making practices are characterized by low technological input, dependence on spontaneous fermentation, and considerable variation in quality across batches and producers (Adesulu-Dahunsi *et al.*, 2020; Sim *et al.*, 2015). Moreover, the process of quality control including the fermentation duration is always determined by the subjective perception of producers based on their past experiences, not by scientific evidence (Shoid *et al.*, 2022). Although the inconsistent product quality due to unstandardized production process has always been associated with Malaysian *budu*, the extent of varieties in quality attributes and the factors contribute to the variance have never been thoroughly assessed and studied. An in-depth study of the actual practice of *budu* fermentation and processing, including field observation, interviews, and characterization of the resulting products is required to provide useful information regarding the extent of process variation and quality inconsistency in Malaysian *budu*. By using chemometric tools, the quality variables attributed to separation between *budu* products can be determined. In addition, protocols can be developed based on the production process that result in high quality *budu*. Such protocol is important for maintaining a repeatable and controlled fermentation process that ensures the quality consistency of the end product (Gao *et al.*, 2022).

As a fermented food, *budu* contains an abundance of microorganisms and metabolites that determine its quality and functionality (Mohamed *et al.*, 2012; Najafian & Babji, 2019; Sim *et al.*, 2015; Zoqratt & Gan, 2021). Microbial activity affects product quality profile through changes in physicochemical properties and metabolites accumulation (Chen *et al.*, 2022a; Zoqratt & Gan, 2021). Despite the substantial impact of microbial community on *budu* quality, limited research has been conducted on the relationship between these two factors during *budu* fermentation. Fish sauce's microbial structure and diversity are impacted by its production methods (Gao *et al.*, 2022). Fermentation conditions, such as ingredients, temperature, pH, oxygen availability, and duration determine the assembly of microbial communities (Gänzle, 2019). Metagenomic study opens the possibility to characterize the microbial community of *budu* sample in a robust way including its structure, interactions and dynamic changes. With the advance of bioinformatic tools, one of which is by using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt), the genomic data can be used to predict the function of the microbe in the community related to its metabolism activities (Douglas *et al.*, 2020) by referring to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. This approach can be used as a guideline for selecting microbe that contribute to development of desirable quality of fish sauce through its metabolism. The selection of key microorganisms for use as starter culture in *budu* has never been conducted based on their role in flavour formation. A better understanding of the microorganisms and their role in volatile and non-volatile metabolites formation is very useful in the improvement and assurance of *budu* quality.

The degradation of fish muscles under high salt concentration and low oxygen levels results on *budu* distinctive sensory characteristics including appearance, texture, taste and aroma (Gowda *et al.*, 2016). The degradation is regulated by the combined action of endogenous proteases and lipases present in fish, as well as those released by microbes during fermentation (Zhu *et al.*, 2021). Large numbers of proteolytic and lipolytic organisms in *budu* may contribute to the overall hydrolysis of fish proteins and lipids, production of flavour precursors and the final volatile metabolite compounds (Han *et al.*, 2022). By exploring the hydrolytic potential of *budu*-derived strains, starter cultures that could speed up fermentation and enhance flavour formation by facilitating hydrolysis could be developed. The selection of bacteria that are part of core microbiome in *budu* may ensure its adaptability when applied in fish sauce fermentation (Kanjan *et al.*, 2021; Udomsil *et al.*, 2022). Despite its significance, there has been a notable lack of research conducted on the exploration of strains expressing proteolytic, lipolytic, esterolytic, and glutamic acid accumulating abilities that originate from *budu*.

Various approaches can be used to modify the fermented fish microbial community to improve its quality, such as using starter cultures and backslopping (Hwanhlem *et al.*, 2011; Li *et al.*, 2022). The application of starter culture is considered a feasible method for controlling fermentation, which may enhance the consistency of *budu*'s quality and favour the development of budu characteristics, including colour and volatile compounds in shorter time (Kanjan et al., 2021). Enhanced proteolysis and lipolysis as indicated by degree of hydrolysis and free fatty acid contents were observed following the inoculation of *Virgibacillus halodenitrificans* PS21 and *Staphylococcus simulans* PMRS35 (Kanjan *et al.*, 2021). However, the application of starter culture and backslopping in *budu* has never been compared to the spontaneous fermentation. The sequential changes in the bacterial communities as a result of different inoculation methods and the extent microbial contribution to the final product quality have never been examined throughout the period of fermentation. The use of metagenomic study can shed light on the interaction of added bacterial inoculum with indigenous *budu* microorganisms, potential changes in organic compound metabolism, and how these factors affect the final product's quality profile. Such valuable and significant information may assist in improving the *budu* fermentation process.

Based on the aforementioned background and identified problems, the objectives of the present study are outlined as follows:

1. To determine the effects of process variations on classification of *budu* based on proximate, physicochemical, and microbiological quality attributes
2. To evaluate the microbial community structure, volatile and non-volatile metabolite profiles of *budu* at different stages of fermentation
3. To isolate and genotypically identify bacterial cultures with high proteolytic, lipolytic, esterolytic, and glutamic acid accumulating activities.
4. To compare the effectiveness of adding bacterial culture, backslopping and spontaneous fermentation in improving quality and accelerating *budu* fermentation

With these outlined objectives, the results of this study would be the first to assess the actual practise of *budu* production in Malaysia and propose production guidelines and quality standards for different types of *budu* products. The profile of the microbial community and its relationship with flavour development will be revealed for the first time, as well as the impact of using starter cultures to improve *budu* quality.

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