

**OPTIMIZING PRODUCTION CONDITIONS, SENSORY
ATTRIBUTES, AND CONSUMER ACCEPTANCE
OF GABA SOY SAUCE USING *Aspergillus* sp.,
Bacillus sp., AND *Tetragenococcus* sp.,
AND SUSTAINABLE PRODUCTION
STRATEGIES**

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**FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR**

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Aspergillus sp., *Bacillus* sp., AND *Tetragenococcus* sp.,
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STRATEGIES**

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CONSUMER ACCEPTANCE OF GABA SOY SAUCE USING *Aspergillus* sp.,
Bacillus sp., AND *Tetragenococcus* sp., AND SUSTAINABLE PRODUCTION
STRATEGIES.**

ABSTRACT

The rising demand for functional foods has sparked an interest in boosting the nutritional value of conventional condiments such as soy sauce. Recent soy sauce research has focused on enhancing functional gamma-aminobutyric acid (GABA) by microorganisms since the key inhibitory neurotransmitter provides various health benefits, including anti-anxiety and anti-stress effects. However, optimizing fermentation conditions to increase GABA production with microorganisms remains challenging, specifically, difficulty in balancing multiple fermentation variables such as pH, inoculum percentage, and agitation speed, which interact to impact GABA production levels. These complexities make it hard to achieve optimal conditions for producing a high-quality GABA soy sauce. Additionally, the existing scholarly literature on the sustainability of soy sauce production is relatively scattered. The primary goals of the present study were to determine the ideal fermentation conditions to maximize GABA concentration in soy sauce production using Response Surface Methodology (RSM), assess consumers' acceptance of GABA soy sauce through sensory analysis, and design a sustainable soy sauce production via a systematic review. The fermentation conditions, including the percentage of bacterial inoculum (10, 15, and 20%), initial pH (3, 5, and 7), and agitation speed (100, 150, and 200 rpm), were optimized using RSM with a mixed culture containing *Aspergillus oryzae* strain NSK, *Bacillus cereus* strain KBC and *Tetragenococcus halophilus* strain KBC. The mixed cultures generated 128.69 g/L of GABA after 7 days of fermentation. Remarkably, 239.08 g/L of GABA was produced after 4 weeks of fermentation with 20% of inoculum, initial pH 7, and 100 rpm, which was

36% higher than non-optimized conditions (153.48 g/L). Meanwhile, the sensory analysis revealed that fermented GABA soy sauce received higher consumer acceptance (7.46) than the control (5.90) and commercial soy sauces (4.47). Consumer preference indicated that the starter culture offered a better umami taste while reducing the presence of bitter, sour, and salty tastes compared to the commercial products. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) analysis was performed to facilitate sustainable soy sauce production, applying inclusion criteria to the Scopus and Web of Science databases. The systematic review identified existing gaps in soy sauce fermentation research and proposed future research possibilities, with a particular emphasis on sustainable soy sauce technologies and techniques that could contribute to advancements in soy sauce production. This study proves that under optimal fermentation conditions determined by RSM statistical analysis, the mixed culture demonstrates a remarkable ability to generate high levels of GABA and greatly enhances consumer acceptability toward this GABA-enriched soy sauce. This study provides valuable insights for industry stakeholders interested in sustainable food production, highlighting the potential for health benefits and increased market viability.

Keywords: GABA, mixed cultures, PRISMA model, Response Surface Methodology (RSM), sensory analysis, sustainable soy sauce production.

**MENGOPTIMALKAN KEADAAN PENGELUARAN, Atribut DERIA, DAN
PENERIMAAN PENGGUNA TERHADAP Kicap GABA MENGGUNAKAN
Aspergillus sp., *Bacillus sp.*, DAN *Tetragenococcus sp.*, DAN STRATEGI
PENGELUARAN LESTARI.**

ABSTRAK

Permintaan yang meningkat untuk makanan berfungsi telah mencetuskan minat untuk meningkatkan nilai pemakanan perasa konvensional seperti kicap. Penyelidikan kicap baru-baru ini telah memberi tumpuan kepada meningkatkan asid gamma-aminobutirik (GABA) berfungsi oleh mikroorganisma memandangkan neurotransmitter yang menghalang utama menyediakan pelbagai manfaat kesihatan, termasuk kesan anti-keimbangan dan anti-tekanan. Walau bagaimanapun, mengoptimalkan keadaan penapaian untuk meningkatkan pengeluaran GABA dengan mikroorganisma kekal mencabar, khususnya, kesukaran untuk mengimbangi berbilang pemboleh ubah penapaian seperti pH, peratusan inokulum dan kelajuan pengadukan, yang berinteraksi untuk memberi kesan kepada tahap pengeluaran GABA. Kerumitan ini menyukarkan untuk mencapai keadaan optimum untuk menghasilkan kicap GABA berkualiti tinggi. Selain itu, literatur ilmiah sedia ada mengenai kelestarian pengeluaran kicap agak tersebar. Matlamat utama kajian ini adalah untuk menentukan keadaan penapaian yang ideal untuk memaksimumkan kepekatan GABA dalam pengeluaran kicap menggunakan Metodologi Permukaan Tindak Balas (RSM), menilai penerimaan pengguna kicap GABA melalui analisis deria, dan mereka bentuk pengeluaran kicap yang mampan melalui tinjauan yang sistematik. Keadaan penapaian, termasuk peratusan inokulum bakteria (10, 15, dan 20%), pH (3, 5, dan 7), dan kelajuan pengadukan (100, 150, dan 200 rpm), telah dioptimumkan menggunakan RSM dengan kultur campuran yang mengandungi

Aspergillus oryzae strain NSK, *Bacillus cereus* strain KBC dan *Tetragenococcus halophilus* strain KBC. Kultur campuran menghasilkan 128.69 g/L GABA selepas 7 hari penapaian. Hebatnya, 239.08 g/L GABA dihasilkan selepas 4 minggu penapaian dengan 20% inokulum, pH 7, dan 100 rpm, iaitu 36% lebih tinggi daripada keadaan tidak dioptimumkan (153.48 g/L). Sementara itu, analisis deria mendedahkan bahawa kicap GABA yang ditapai mendapat penerimaan pengguna yang lebih tinggi (7.46) berbanding kawalan (5.90) dan kicap komersial (4.47). Keutamaan pengguna menunjukkan bahawa budaya pemula menawarkan rasa umami yang lebih baik sambil mengurangkan kehadiran rasa pahit, masam dan masin berbanding produk komersial. Analisis Item Pelaporan Pilihan untuk Kajian Sistemik dan Analisis Meta (PRISMA) telah dilakukan untuk memudahkan pengeluaran kicap yang mampan, menggunakan kriteria kemasukan ke pangkalan data Scopus dan Web of Science. Semakan sistematik mengenal pasti jurang sedia ada dalam penyelidikan penapaian kicap dan mencadangkan kemungkinan penyelidikan masa hadapan, dengan penekanan khusus pada teknologi dan teknik kicap mampan yang boleh menyumbang kepada kemajuan dalam pengeluaran kicap. Kajian ini membuktikan bahawa di bawah keadaan penapaian optimum yang ditentukan oleh analisis statistik RSM, campuran kultur menunjukkan keupayaan yang luar biasa untuk menjana tahap GABA yang tinggi dan sangat meningkatkan penerimaan pengguna terhadap kicap yang diperkaya GABA ini. Kajian ini memberikan pandangan berharga untuk pihak berkepentingan industri yang berminat dalam pengeluaran makanan yang mampan, menonjolkan potensi untuk manfaat kesihatan dan meningkatkan daya maju pasaran.

Kata Kunci: GABA, campuran kultur, model PRISMA, Kaedah Permukaan Tindakan Balas (RSM), analisis deria, pengeluaran kicap yang mampan.

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TABLE OF CONTENTS

ABSTRACT	iii
ABSTRAK.....	v
ACKNOWLEDGEMENTS.....	vii
TABLE OF CONTENTS.....	viii
LIST OF FIGURES	xi
LIST OF TABLES	xiii
LIST OF SYMBOLS AND ABBREVIATIONS	xiv
LIST OF APPENDICES.....	xvii
CHAPTER 1: INTRODUCTION	1
1.1 Research Background	1
1.2 Statement of Problems	4
1.3 Research Questions.....	5
1.4 Research Objectives.....	5
CHAPTER 2: LITERATURE REVIEW	7
2.1 Soy Sauce.....	7
2.1.1 Introduction to Soy Sauce.....	7
2.1.2 The History of Soy Sauce and Its Microbial Pioneers.....	11
2.2 Microorganisms in Soy Sauce.....	15
2.2.1 Microbial Communities in Soy Sauce Production.....	15
2.2.2 Microbial Control During Soy Sauce Production.....	24
2.3 Asian Traditional Soy Varieties and Microbes Involved	27
2.4 Advances in Soy Sauce Fermentation.....	36

2.4.1	Advances in Soy Sauce <i>Moromi</i> Maturation	37
2.4.2	Advances in Removing Soy Sauce Precipitation	38
2.4.3	Advances on Safety of Soy Sauce.....	39
2.5	Soy Sauce Microbial Fermentation: Progress and Advances.....	42
2.5.1	Identification of Microbe During Soy Sauce Fermentation.....	42
2.5.2	Enhancement of Soy Sauce Flavor by Genome Shuffling Starter Culture	44
2.5.3	Enhancement of Soy Sauce Flavor by Mixed Starter Culture	45
2.5.4	Enhancement of GABA and Other Beneficial Compound in Soy Sauce Fermentation by Microbes	46
CHAPTER 3: MATERIALS AND METHODOLOGY		49
3.1	Chemical	49
3.2	Microorganisms Isolation and Bacteria Culture	49
3.2.1	<i>Aspergillus oryzae</i> strain NSK (AO)	49
3.2.2	<i>Bacillus creuse</i> strain KBC (BC).....	51
3.2.2.1	Isolation and identification of BC.....	51
3.2.2.2	Suspension Mother Broth of BC.....	52
3.2.2.3	Serial Dilution of BCSKBC.....	53
3.2.3	<i>Tetragenococcus halophilus</i> KBC (TH)	53
3.2.3.1	Isolation and Identification of TH	53
3.2.3.2	Suspension Mother Broth of TH.....	55
3.2.3.3	Serial Dilution of TH	55
3.3	<i>Koji</i> Production.....	56
3.4	<i>Moromi</i> Fermentation	57
3.5	Refining and Pasteurise	59

3.6	GABA Detection and Quantification.....	60
3.7	Optimization of <i>Moromi</i> Fermentation Condition Using Response Surface Methodology (RSM).....	60
3.8	Sensory Evaluation.....	63
3.9	Statistical Analysis	67
3.10	Systematic Review	67
3.10.1	Search Strategy.....	67
CHAPTER 4: RESULTS AND DISCUSSIONS		71
4.1	Optimisation of <i>Moromi</i> Fermentation Conditions for GABA Production	71
4.1.1	Verification of Optimized Condition	79
4.2	GABA Production by The Trio of AO, BC, and TH.....	79
4.3	Sensory Analysis.....	81
4.4	Systematic Review.....	86
4.4.1	Initial Identification and Search Strategy	86
4.4.2	Eligibility Assessment	88
4.4.3	Quantitative and Qualitative Analyses	88
CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS		94
5.1	Conclusions.....	94
5.2	Recommendations.....	95
REFERENCES		97
APPENDICES		132

LIST OF FIGURES

Figure 2.1	: Traditional soy sauce brewing process.....	10
Figure 2.2	: Three main microorganisms involved in soy sauce brewing.....	15
Figure 2.3	: Improvement in soy sauce fermentation.....	44
Figure 3.1	: Ultra zoom of fermented soybean covered with green colour <i>A. oryzae</i> spore strain NSK. (Author's personal collection)	50
Figure 3.2	: (a) <i>A. oryzae</i> strain NSK under x10 magnification. (b) <i>A. oryzae</i> strain NSK with methyl blue under x400 magnification (Author's personal collection).....	50
Figure 3.3	: (a) Bacteria colonies form on the MRS agar plates after 3 d of incubation at 30°C. (b) Gram-stained <i>B. cereus</i> strain KBC. (c) <i>B. cereus</i> strain KBC under 1000x magnification by Scanning Electron Microscope (Bar= 2 µm) (Author's personal collection)	52
Figure 3.4	: (a) Bacteria colonies form on the MRS agar plates after 3 d of incubation at 30°C. (b) Gram-stained <i>T. halophilus</i> strain KBC. (c) <i>T. halophilus</i> strain KBC under 1000x magnification by Scanning Electron Microscope (Bar= 2 µm) (Author's personal collection)...	54
Figure 3.5	: (a) Day one of 3-4 cm thickness koji with regular intervals of 2 inches valleys. (b) <i>Koji</i> after 7 d of incubation.....	57
Figure 3.6	: (a) <i>B. cereus</i> strain KBC (b) <i>T. halophilus</i> strain KBC (Author's personal collection)	58
Figure 3.7	: (a) Pressing process of the soy sauce moromi in a nylon sheet. (b) Filtering process of the raw soy sauce. (c) Pasteurize the raw soy sauce at 90-95°C for 30 min. (d) The pasteurize liquid is now ready to be served.....	59
Figure 3.8	: Panellists from Universiti Malaya.....	64
Figure 3.9	: Sensory analysis setup.....	65
Figure 3.10	: Soy sauce samples used for sensory analysis: Control (525), trio (815) and Commercial (701)	66

Figure 3.11	: The systematic review of literature search follows a general flow that includes identification, screening, eligibility assessment and inclusion based on the PRISMA flow diagram. The flow was reproduced from (Oláh et al., 2020), MDPI, 2020.	68
Figure 4.1	: Response surface curve (3D plot) of GABA production from the trio of AO, BC and TH displaying the interaction between (a) pH and inoculum percentage, (b) inoculum percentage and agitation speed and (c) pH and agitation speed.	78
Figure 4.2	: The comparison of GABA production yield by the trio (under optimized condition: 20% bacteria inoculum percentage, pH 7, 100 rpm) and control during 28 d of fermentation.....	80
Figure 4.3	: The spider diagram illustrates a descriptive sensory analysis of six taste (sweet, umami, salty, bitter, sour, and astringent) on three different soy sauce samples.....	82
Figure 4.4	: Mean scores for overall acceptability for all the soy sauce samples for 300 panellists. Notes: Different alphabets indicate significant difference ($P < 0.01$)	85
Figure 4.5	: Number of articles (n) at each level of PRISMA analysis.....	87
Figure 4.6	: The majority focus area of research articles obtained.....	89
Figure 4.7	: Categorization of selected articles focusing on different area on sustainable soy sauce production by year.....	91
Figure 4.8	: Potential approaches for sustainable soy sauce production.....	93

LIST OF TABLES

Table 2.1	:	The main microorganisms involved in soy sauce fermentation.....	16
Table 2.2	:	Microbial diversity and predominance in <i>koji</i> and <i>moromi</i> stage...	20
Table 2.3	:	Soy-fermented food in history in the Orient.....	28
Table 3.1	:	The fermentation time for each analysis.....	58
Table 3.2	:	The selected variables range, and levels inputted for optimization study.....	61
Table 3.3	:	20 experimental runs of three variables of inoculum percentage (%), Initial pH, and agitation speed (rpm) according to CCD.....	62
Table 3.4	:	Eligibility criteria for study selection.....	69
Table 4.1	:	Experimental runs of three variables according to CCD. Experimental and predicted values of GABA production (mg L ⁻¹) obtained using the trio consisting of <i>A. oryzae</i> strain NSK, <i>B. cereus</i> strain KBC and <i>T. halophilus</i> strain KBC.....	73
Table 4.2	:	Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for GABA production using the trio.....	74
Table 4.3	:	Validation of model using verified condition on GABA response.....	79
Table 4.4	:	10-cm line scale for the six tastes (sweet, umami, salty, bitter, sour and astringent) and overall acceptability of soy sauce samples (0= none, 1= extremely weak, 2 = very weak, 3 = weak, 4 = moderately weak, 5= moderately, 6 = moderately strong, 7= strong, 8 = very strong and 9 = extremely strong).....	83

LIST OF SYMBOLS AND ABBREVIATIONS

<i>%</i>	:	Percentage
<i>+ve</i>	:	Positive
<i>-ve</i>	:	Negative
<i>°C</i>	:	Degree Celsius
<i>cfu/mL</i>	:	Colony-forming unit per millilitre
<i>cm</i>	:	Centimetre
<i>g</i>	:	Gram
<i>g/L</i>	:	Gram per litre
<i>hr</i>	:	Hour
<i>kb</i>	:	Kilobit
<i>kHz</i>	:	Kilohertz
<i>kg</i>	:	Kilogram
<i>L</i>	:	Liter
<i>mg/L</i>	:	Milligram per litre
<i>mg/kg</i>	:	Milligram per kilogram
<i>mg/g</i>	:	Milligram per gram
<i>min</i>	:	Minute
<i>mL</i>	:	Millilitre
<i>mL/min</i>	:	Millilitre per minute
<i>mm</i>	:	Millimetre
<i>nm</i>	:	Nanometre
<i>Rpm</i>	:	Revolution per minute
<i>w/v</i>	:	Weight per volume

W/cm^2	:	Watt per square metre
μL	:	Microliter
μm	:	Micrometer
3-MCPD	:	3-Chloropropane-1,2 –diol
AFB1	:	Aflatoxin B1
ANOVA	:	Analysis of variance
AO	:	<i>Aspergillus oryzae</i> strain NSK
APE	:	Prolyl endopeptidase
BAC	:	Bacteria
BAs	:	Biogenic amines
BCE	:	Before Common Era
BCSKBC	:	<i>Bacillus cereus</i> strain KBC
$CaCO_3$	:	Calcium carbonate
CCD	:	Central composite design
CE	:	Common Era
CO_2	:	Carbon dioxide
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
EC	:	Ethyl carbamate
et al.	:	Et alia (and others)
FAO	:	Food and Agriculture Organization
FDA	:	Food and Drug Administration
GA	:	Glutamic acid
GABA	:	Gamma aminobutyric acid
GAD	:	Glutamate decarboxylase

GRAS	:	Generally Recognized as Safe
HDMF	:	Isobutyl alcohol, isoamyl alcohol, 2-phenylethanol)4- hydroxy-2,5-dimethyl-3-(2H)- furanone
HEMF	:	4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone
HPLC	:	High Performance Liquid Chromatography
HSDL	:	High-salt dilute-state
HVP	:	Hydrolysed vegetable proteins
IARC	:	International Agency for Research on Cancer
JAS	:	Japanese Agriculture Standards
LAB	:	Lactic acid bacteria
MRS	:	de Man, Rogosa, Sharpe OXOID CM0361
MSG	:	Monosodium glutamate
NaCl	:	Sodium chloride
Omics Lab	:	Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya
PCR-DGGE	:	Polymerase chain reaction/denaturing gradient gel electrophoresis
PRISMA	:	Preferred Reporting Items for Systematic Reviews And Meta-Analysis
Ret Time	:	Retention Time
RSM	:	Response surface methodology
SEM	:	Scanning Electron Microscope
SMF	:	Submerged Fermentation
SSF	:	Solid State Fermentation
TH	:	<i>Tetragenococcus halophilus</i> strain KBC

UM	:	Universiti Malaya
UV	:	Ultraviolet
VFCs	:	Volatile flavor compounds
WGS	:	Whole-genome shotgun
W/O	:	Without
YE	:	Yeast Extract

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LIST OF APPENDICES

Appendix A	: First Page of Yee et al. (2021).....	132
Appendix B	: First Page of Sassi et al. (2022).....	133
Appendix C	: First Page of Chong et al. (2023).....	134
Appendix D	: First Page of Chong Shin Yee et al. (2023).....	135
Appendix E	: First Page of Yee et al. (2024).....	136
Appendix F	: Collaboration between Universiti Malaya with Kilang Kicap Kwong Bee Chun (KBC) Sdn Bhd soy sauce factory	137
Appendix G	: GABA standard and HPLC chromatograms.....	138

CHAPTER 1: INTRODUCTION

1.1 Research Background

Soy sauce, a liquid condiment with its origins traced back to China, is well acknowledged for its dark colour and distinct umami taste (Fukushima, 2004). Serving as both a dipping sauce and a cooking sauce, soy sauce holds a significant position in the culinary traditions of East and Southeast Asia. As the birthplace of soy sauce, China has established a significant impact in various nations, including Japan, Thailand, Vietnam, Indonesia, and Malaysia (Luh, 1995). The quality and flavor of soy sauce vary across different nations due to various factors such as the use of distinct raw materials, varying salt concentrations, diverse starting cultures, microbial strains, fermentation conditions, and the employment of distinct processing procedures throughout the soy sauce brewing process (Gao et al., 2017). Soy sauce is traditionally produced from soybeans, roasted wheat grains, starting moulds (such as *Aspergillus oryzae* or *Aspergillus sojae*), and brine solution (Devanthi & Gkatzionis, 2019).

The classification of soy sauce into Japanese-style and Chinese-style can be attributed to the compositional difference between soybeans and wheat (Diez-Simon et al., 2020a). The production of Japanese-style soy sauce, which is mostly consumed in Japan and Western nations, is produced with the equal use of soybeans and wheat. In contrast, Chinese-style soy sauce is produced with a higher proportion of soybeans and less wheat. China, Indonesia, the Philippines, Thailand, Singapore, and Malaysia are the primary consumers of Chinese-style soy sauce (Huang & Teng, 2004).

To the best of our knowledge, research into the sensory attributes of Malaysian soy sauce is limited, in comparison to other prime soy sauce producers like Thai, Japanese-, Korean-, and Chinese-style soy sauces (Lee et al., 2013; Sikora et al., 2008; Wang et al., 2018b; Zhang et al., 2023a). The processing method, raw ingredients, and microorganism strains all affect how soy sauce develops its unique aroma and distinctive

flavor (Sassi et al., 2021). Soy sauce consists of six basic flavors or tastes: sweet, salty, sour, bitter, astringent and umami. The harmonious blend and the delicate balance of its taste help to explain why it is used as an all-purpose condiment (Ito & Matsuyama, 2021). Sugar (such as glucose) provides sweetness, salt provides saltiness, and organic acids (such as lactic and acetic acids) provide sourness. However, amino acids and peptides derived from protein-based raw materials also contribute to sweetness, bitterness, and umami flavors (Maya et al., 2023).

There are two ways to produce soy sauce: natural brewing or chemical hydrolysis of the soybeans. Traditional soy sauce fermentation involves two main stages, including *koji*-stage fermentation (solid-state) and *moromi*-stage fermentation (liquid-state) (Feng et al., 2013). For the first stage of making the soy sauce, the cooked soybean will be mixed with roasted and crushed wheat grain and the starter moulds for short-term solid-state fermentation. The mixture will then be soaked in a high salt concentration solution for long-term liquid-state fermentation (Devanthi & Gkatzionis, 2019). Alternatively, soy sauce can be manufactured using chemical hydrolysis of soybean, which involves adding hydrochloric acid (HCl) to the soybean protein to hydrolyze it and reduce the soy fermentation time from many months to just few days (Diez-Simon et al., 2020b).

During the soy sauce fermentation process, a diverse microbial population that includes fungus, bacteria, and yeast emerges (O'toole, 2019). The primary microbe involved during the *koji* stage fermentation is a type of mould known as *koji* moulds (*A. oryzae* sp., or *A. sojae* sp.). These *koji* moulds will help in the synthesis of extracellular enzymes such as proteolytic and saccharolytic enzymes in order to break down the soybean and wheat into micro molecules that will then be consumed by microbes such as Lactic acid bacteria LAB and yeast from the second stage of fermentation (Liu, 2012). For the *moromi* fermentation stage, the dominant microbes are thought to be bacteria, mainly LAB, followed by yeast and moulds (Wei et al., 2013b). All these microbes will

grow and break down soybeans and produce bioactive compounds, including γ -aminobutyric acid (GABA), during the fermentation process. Studies have shown that soy sauce increases gastric juice production while also having antioxidant, anticarcinogenic, antibacterial, and hypotensive properties (Baruah et al., 2019).

GABA is one of the main neurotransmitters reported to have a calming effect on the brain and nervous system. It can be found naturally in the brain and synthesized in vivo with LAB (Wiley et al., 2021). GABA is produced when L-glutamic acid undergoes α -decarboxylation, which is catalyzed by glutamate decarboxylase. The genes that encode for this enzyme are known as the *gad* genes, which can be found in many microbes such as LAB (Cui et al., 2020a). Studies have shown that some microbes, including *A. oryzae* strain NSK (Ab Kadir et al., 2016), *Bacillus cereus* strain KBC (Wan-Mohtar et al., 2020), and *Tetragenococcus halophilus* strain KBC (Yee et al., 2021), can produce GABA during soy sauce brewing. Due to its health benefits, GABA has recently become a functional food ingredient in a variety of food (Sun et al., 2022). Fermented food products, such as GABA-enriched soy sauce (Yee et al., 2021) and GABA-enriched sourdough bread (Bhanwar et al., 2013), have been shown to contain increased levels of GABA.

Different regions and manufacturers typically produce a variety of soy sauces. As a result, each has its own unique combination of non-volatile compounds. The taste threshold for each component is crucial in determining the sensory impact and concentration. Thus, the non-volatile compounds synthesised during soy sauce fermentation have a significant impact of its flavor (Kong et al., 2018a). For this reason, taste-active non-volatile compounds in Malaysian soy sauce should be identified in order to standardise the quality and address production optimisation. The present study sought to address these research gaps, focusing on the initial step of optimising GABA production in soy sauce. This was achieved by employing a combination of three

microbes: *A. oryzae* strain NSK, *B. cereus* strain KBC, and *T. halophilus* strain KBC using the Response Surface Methodology (RSM). Additionally, this study used sensory analysis to assess consumer acceptance of this GABA-enriched soy sauce, comparing it to both a control sample (soy sauce without any treatment and additional bacteria) and commercially available soy sauce. Finally, the study conducted a systematic review to identify current gaps in soy sauce fermentation research and propose future research directions, emphasising sustainable technologies and techniques to advance soy sauce production. These approaches intend to enhance nutritional benefits, ensure high consumer satisfaction, and expand market potential while promoting sustainable production practices.

1.2 Problem Statement

Several challenges must be addressed to ensure the sustainable development and market acceptance of GABA-enriched soy sauce. First and foremost, optimising the fermentation conditions is essential for improving productivity while reducing production costs (Vassileva et al., 2021). More understanding and effective communication regarding the health benefits of GABA soy sauce is required to enhance consumer acceptance. Moreover, it is crucial to understand customer preferences GABA soy sauce flavors to achieve effective market penetration (Byeon et al., 2023). In Malaysia, my research focuses on these challenges, emphasizing the local market's unique preferences and conditions, which are vital for the successful introduction and acceptance of GABA-enriched soy sauce.

Soy sauce is a popular condiment in East Asian cuisine; nonetheless, increasing amounts of salt-fermented soy sauce might potentially jeopardize food safety and raise health-related issues. Furthermore, the globalization of culinary influences has led to the introduction of novel or unfamiliar soy sauce flavors into local and regional cuisines,

including Western counterparts. Nevertheless, there is a lack of comprehensive understanding about the processes that facilitate the effective incorporation of these flavors into food products (Ushiama et al., 2021). Therefore, it is crucial to review existing knowledge on soy sauce fermentation, identify research gaps and key areas for enhancing soy sauce yields and quality, all while emphasising the use of sustainable technologies and approaches to advance soy sauce production.

1.3 Research Questions

1. What are the best conditions (initial pH, agitation, and inoculum percentage) for the trio of *A. oryzae* strain NSK, *B. cereus* strain KBC, and *T. halophilus* strain KBC in order to produce the highest GABA production yield in submerged liquid fermentation using Response Surface Methodology (RSM)?
2. How does the acceptance of consumers vary when evaluating GABA soy sauce, the control (without treatment and additional bacteria culture), and commercial soy sauce based on sensory analysis?
3. What are the current gaps in soy sauce fermentation research, and how can future research directions focus on the integration of sustainable technologies and approaches to increase soy sauce production?

1.4 Research Objectives

The aim of this study was to optimise the production of GABA soy sauce, analyse consumer acceptance of this soy sauce, and provide insights into the current state and

future directions of sustainable soy sauce fermentation research. The objectives of the project are listed below.

1. To identify the optimal conditions, including initial pH, agitation, and bacteria inoculum percentage, for submerged liquid fermentation using Response Surface Methodology (RSM) to maximize GABA production yield by the trio of *A. oryzae* strain NSK, *B. cereus* strain KBC, and *T. halophilus* strain KBC.
2. To assess and compare the acceptance of consumers towards GABA soy sauce, the control (without treatment and additional bacteria culture), and commercial soy sauce through sensory analysis, focusing on taste and overall acceptability.
3. To identify current gaps in soy sauce fermentation research through a systematic review and recommend future research directions, with a particular emphasis on sustainable technologies and approaches that can help enhance soy sauce production.

CHAPTER 2: LITERATURE REVIEW

2.1 Soy Sauce

2.1.1 Introduction to Soy Sauce

Soy sauce is a dark brown liquid condiment or flavouring derived from soybean fermentation, mostly attributed to its umami taste and salty flavor, making it a popular choice for flavoring (Lioe et al., 2010). In Japan, this condiment is often referred to as *shoyu* or *tamari shoyu*, whereas in China, it is known as *chiang-yu* or *-yi*; in Indonesia, *kecap*; in Korea, *kanjang*; in the Philippines, *toyo*; in Thailand, *see-ieu*; and in Malaysia, *kicap* (Sassi et al., 2021). China and Japan are widely recognized as the world's leading producers and consumers of soy sauce. According to Liujun et al. (2008), the annual production of soy sauce in China exceeds 5 million tons, representing over 55% of the global output. On the other hand, Kataoka (2005) estimated the annual production of soy sauce in Japan at approximately 1.2 million tons. The average per capita consumption of soy sauce in Japan is estimated to be around 10 L per year. Japanese people use an average of around 34.1 g of soy sauce per day, which gives 2.4 g of protein, 0.2 g of fat, 5.8 g of salt, and 14 kcal (Chong et al., 2023). A recent market research study projected that the worldwide soy sauce industry would rise by USD 6.39 billion between 2020 to 2024 (Technavio, 2020).

Due to its low water activity and high salt content, soy sauce is stable at room temperature and does not require refrigeration for storage. The high concentration of brine solution used to ferment the soy sauce will subsequently help control bacterial propagation during soy sauce brewing and act as a preservative (Liu et al., 2020b). Salt is crucial in soy sauce brewing since it is not only helps in preserving the soy sauce but also provides minerals such as sodium and potassium to consumers. Because of its high potassium level, bamboo salt is a better choice as compared to table salt, coarse salt,

French sea salt, or Himalayan pink salt (Tan et al., 2016). Bamboo salt is high in potassium and low in sodium. Thus, it is good in reducing the sodium intake by consumers and indirectly reduces the blood pressure or hypertension in the community (Henry & Appel, 2021). Soy sauce contains phytochemicals, such as isoflavones, phenolic acid, flavonoids, saponins, phytosterol, and sphingolipids, which provide both short-term and long-term health benefits (Alghamdi et al., 2018). Studies showed that soy sauce can help boost gastric juice production while also having hypotensive, antimicrobial, anticarcinogenic, and antioxidant effects (Dini & Laneri, 2021).

Food allergy is an unusual immunological reaction to protein-rich foods such as eggs, milk, wheat, soybeans, tree nuts, and peanuts (Muthukumar et al., 2020). However, some studies showed that fermented foods such as soy sauce do not contain allergens (hypoallergenic) even though soy sauce is primarily made up of soybeans and wheat (Kobayashi et al., 2004). Biogenic amines (BAs) are light-molecular weight nitrogenous organic compounds produced in foods by different kinds of microbes such as LAB, Enterobacteriaceae, and yeasts capable of amino acid decarboxylation (Önal, 2007). Several surveys, particularly those conducted in Asian countries such as China, Korea, Thailand, and Malaysia, have investigated the presence of BAs in fermented soy sauce and soybean paste. Although they found that most of the soy sauce and soybean paste contained BAs, the levels were lower than those considered detrimental to consumers' health (Stute et al., 2002). Headache, diarrhoea, nausea, rashes, elevated blood pressure, heart palpitation, and mortality are the physiological consequences when BAs is consumed above the permissible limits (Omer et al., 2021).

Soy sauce contains over 300 flavor compounds, resulting in a well-balanced mix of the five primary flavors: umami, sweet, bitter, salty, and sour (Zhao et al., 2021). The flavor-aroma creation in soy sauce is determined by its manufacturing processes, raw

ingredients, and starter cultures. Hence, soy sauce from diverse origins is dictated by their variation and complexity (Yan et al., 2013).

According to the Japanese Agriculture Standards (JAS), Japanese-type soy sauce is classified into five categories: *koikuchi*, *usukuchi*, *tamari*, *shiro*, and *saishikomi* (Asanuma et al., 2024). *Koikuchi* is prepared by using soybean and wheat at a ratio of 1:1 (Hajeb & Jinap, 2015). *Usukuchi* has a similar soybean and wheat ratio as *koikuchi*. However, it is lighter, thinner, and has a more assertive and saltier flavor than *koikuchi* (Kaneko et al., 2012). *Tamari*, also known as Chinese-style soy sauce, is made of soybean with a small amount of wheat, originated from China (Fukushima, 1979; Kaneko et al., 2012; Lite, 2005). *Shiro* is the opposite of *tamari* as it is made of wheat with a small amount of soybean (Ehara, 2019). For *saishikomi*, instead of the usual saltwater, it is made from enzymatically degraded soybeans and wheat. Both *shiro* and *saishikomi* account for less than 1% of Japanese soy sauce production (Reddy, 2018).

Traditional soy sauce fermentation consists of two steps: *koji* fermentation and *moromi* fermentation, as illustrated in **Figure 2.1**. In the first stage, the soybeans are soaked overnight to soften and are steamed to cook. The wheat is roasted and crushed lightly to open it up. Then, the cooked soybeans, roasted wheat grains, and starter mould (*A. oryzae*) are mixed in a wooden tray to make the *koji*. The *koji* is incubated at room temperature for one week (Devanthi & Gkatzionis, 2019). Following this, the matured *koji* is added to a brine solution to make a mash mixture which is known as *moromi* (Liu, 2017). The *moromi* mash is kept in a wooden barrel and fermented for at least four months, after which the *moromi* is poured onto pressed cheesecloths, and the raw soy sauce is squeezed out by pressing the layers of *moromi*. The raw soy sauce is then pasteurised, refined, inspected, and bottled up for consumption or shipment (Luh, 1995).

SOY SAUCE IS MADE OUT FROM



Soy Beans



Wheat



Salt



Water

THE BREWING PROCESS



Soybeans are soaked for overnight and are steamed to cook.



The wheat is roasted and crushed.



The cooked bean, wheat grain and starter mold are mixed together to make koji.



Koji mold is spread out on the wooden tray and fermented for 1 week.



The soy sauce is then being inspected and bottled up before shipment.



The raw soy sauce is pasteurized and refined.



The moromi is poured onto pressed cloths and the raw soy sauce is squeezed out by pressing layers of moromi.



Salt, water and koji is added into a fermentation tank to make moromi.

Figure 2.1: Traditional soy sauce brewing process.

Koji fermentation is known as solid-state fermentation (SSF), while *moromi* fermentation is known as submerged fermentation (SmF) (Barzkar et al., 2021). The difference between these two methods is the substrate used during the fermentation (Subramaniam & Vimala, 2012). For SmF, free-flowing media such as media broth and molasses are used as the substrates. Microbes involved in SmF require high water activity to grow like bacteria (Kumar et al., 2021). Since the microbes will rapidly use the nutrients in the broth, nutrients should be continuously supplied to the broth to ensure microbial growth and survival. When there is a continuous nutrient supply, abundant bioactive compounds will be secreted into the nutrient broth. Overall, SmF requires less fermentation time, and the end products are easier to purify than SSF (Subramaniam & Vimala, 2012).

For SSF, solid substrates such as soybeans, wheat, and grains are used in fermentation. These substrates are nutrient-rich and could support long fermentation as the microbes use the nutrients in these substrates gradually (Wang & Yang, 2007). Microbes such as moulds, yeasts, and bacteria, which require low water activity, will flourish in SSF (Obi, 2019). Due to the variety of microbes involved in SSF and the long fermentation period, a vast quantity of bioactive compounds can be found in SSF compared to SmF (Subramaniam & Vimala, 2012).

2.1.2 The History of Soy Sauce and Its Microbial Pioneers

Soy sauce originated from '*jiang*', a fermented soybean paste used to preserve food or as a condiment and was initially found in the late Zhou dynasty (1046 - 256 BC) in China to preserve meat, fish, and grains. During the Han dynasty (206 BC - 220), soybeans became much more accessible and could easily be cultivated even on poor land (Gao et al., 2010; Shurtleff & Aoyagi, 2012b). Even though it was founded in China, soybean pastes evolved into soy sauce in its neighbouring countries, Japan and Korea.

Buddhist monks from China introduced soybean paste to Japan and Korea. After a series of trials and modifications, they accidentally discovered soy sauce while collecting the by-product of soybean paste in the fermentation tanks (Fukushima, 2004; Gao et al., 2010; Shurtleff & Aoyagi, 2012b; Yue et al., 2021b). Soybean paste is known as '*doujiang*' or '*dajiang*' in China, '*doenjang*' or '*cheonggukjang*', in Korea, and '*miso*' in Japan. Soybean pastes from different countries and regions have distinctive tastes and flavors. The raw ingredients used, surrounding environments, and processing methods are the main factors that vary the types of soybeans paste (Kim et al., 2010; Liu et al., 2022).

Traditional soy sauce making consists of *koji* fermentation and *moromi* fermentation. *Koji* making technique has been a distinctive skill of the Orient for over 3,000 years (Machida et al., 2008). This *koji*-making process was introduced into Japan around the Yayoi period (300 BC - 300) (Machida et al., 2008), and filamentous fungal inoculation for fermentation has been commercially available as *koji* since the 13th to 15th centuries. During that period, people cultivated *koji* without realising it was made up of microbes (*koji* mould). Thus, the term *koji* refers to both the substance fermented by *A. oryzae* and the *A. oryzae* mould itself (Machida et al., 2008). *Koji* had different designations depending on the country: *kōji* in Japanese, *qu* in Chinese, and *nurukgyun* in Korean. It is a culture made up by inoculating different filamentous moulds (*Aspergillus* spp., *Rhizopus* spp., *Monascus* spp., *Mucor* spp., and *Absidia* spp.) on cooked grains or legumes in a humid and warm place (Chen et al., 2009; Murooka & Yamshita, 2008; Shurtleff & Aoyagi, 2012b). *Koji* can typically be divided into two types: inoculated with *A. oryzae* and red rice *koji* inoculated with *Monascus purpureus*. Red rice *koji* is also known as *beni* in Japanese and *hong qu* in Mandarin (Samsudin & Abdullah, 2013). It is typically used as a natural food colouring and preservative (Shurtleff & Aoyagi, 2012b). Despite the various forms of *koji*, the *koji* mould appears to be the key to synthesis of

hydrolytic enzymes, which are responsible for the breakdown of macromolecules into micromolecules in traditional fermented foods (Machida et al., 2008).

A. oryzae is the most common mould used to make *koji* (Shurtleff & Aoyagi, 2012b). In 1876, when H. Ahlburg was invited to the Japanese Medical College, he isolated *A. oryzae* from *koji* for the first time. Later, F. Cohn renamed it *A. oryzae* from its previous name, *Eurotium oryzae*, due to its inability to reproduce sexually (Machida et al., 2008). In 2005, the genome of *A. oryzae* RIB40 (ATCC-42149) was entirely sequenced, and it was found that the sequenced strain is a wild-type strain that most akin to those used in *sake* brewing but still possessed one of the essential features in soy sauce brewing which is producing proteases (Machida et al., 2005). The *koji* mould is critical ingredient in creating a spectacular umami flavor in soy sauce (Machida et al., 2008). It plays a vital role in fermenting the elements and producing many enzymes such as amylases, proteases, lipases, and speciality chemicals (Yu et al., 2004). *A. oryzae* has been extensively used in fermented foods such as *sake* (rice wine), *miso* (soybean paste), and *shoyu* (Okuzawa) (Abe et al., 2006; Okuzawa, 2003). Furthermore, *A. oryzae* has received the “Generally Recognized as Safe (GRAS)” status from the United States Food and Drug Administration (USFDA) for safe use in foods (Machida et al., 2008; Taylor & Richardson, 1979).

In the *moromi* stage, the main microbes present in the mash are LAB and yeasts. Studies since the early 1900s revealed that *T. halophilus*, a halo-tolerant LAB, was present in the *moromi* mash (Tochikura et al., 2001). At present, there are four identified species in the *Tetragenococcus* genus which is *T. halophilus* (Collins et al., 1990), *Tetragenococcus muriaticus* (Satomi et al., 1997), *Tetragenococcus solitarius* (Ennahar & Cai, 2005), and *Tetragenococcus koreensis* (Lee et al., 2005). In the manufacturing of soy sauce, soy paste, fish sauce, shrimp paste, and Taiwanese fermented mustard '*suan-*

tsai', both *T. halophilus* and *T. muriaticus* play a crucial role in halophilic fermentation (Justé et al., 2008; Yee et al., 2021).

Halo-tolerant yeasts were also found during the early (*Zygosaccharomyces rouxii*), middle (*Candida etchellsii* and *Candida versatilis*), and late (*C. etchellsii*) fermentation stages (Tanaka et al., 2012). *Z. rouxii* (family *Saccharomycetaceae*) is a notable yeast for its high osmotic stress tolerance (Pribylova et al., 2007). Originally named *S. rouxii*, Barnett et al. (1990) reclassified it to the present name, *Z. rouxii*, in 1983. In the latest edition of '*The Yeast, a Taxonomic Study*' by Kurtzman et al. (2011), they have accepted six species that belong to genus *Zygosaccharomyces* which are *Zygosaccharomyces bailii* (Linder) (Guilliermond, 1912), *Zygosaccharomyces bisporus* (Naganishi, 1917), *Zygosaccharomyces kombuxhaensis* (Kurtzman et al., 2001), *Zygosaccharomyces lentus* (Steels et al., 1999), *Zygosaccharomyces mellis* (Fabian & Quinet, 1928), and *Z. rouxii* (Boutroux) (von Arx et al., 1977). Numerous species in this genus are substantially resistant to many standard food preservation techniques. This yeast is known as a spoilage yeast in the food industry, such as fruit juices, fruit concentrates, syrups, alcoholic beverages, honey, and jams (Guilliermond, 1912; Naganishi, 1917; Steels et al., 1999; von Arx et al., 1977). However, in soy sauce making, the LAB *T. halophilus* and the yeast *Z. rouxii* yeast are the main microbes that drive the *moromi* fermentation (Singracha et al., 2017). *Z. rouxii* will undergo alcoholic fermentation and produce abundant secondary metabolites that give flavor and aroma to the soy sauce (Tanaka et al., 2012).

Several reports investigated the interaction between these species (Kusumegi, 2001); Tochikura et al. (2001) suggested that *koji* moulds and yeasts may aid in the development of LAB by providing essential nutrients to them, while yeasts and LAB can survive using the glucose resulting from the enzyme activity of the *koji* moulds. Furthermore, when *T. halophilus* grows in high salt concentration brine, it will release

lactic acid, lowering the pH, and providing conducive environment for the salt-tolerant *Z. rouxii* to grow. As a result, *Z. rouxii* will take over, generating major soy sauce flavors such as alcohols, esters, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF), and (isobutyl alcohol, isoamyl alcohol, 2-phenylethanol) 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) (Wang et al., 2023).

2.2 Microbes in Soy Sauce

2.2.1 Microbial Communities in Soy Sauce Production

Soy sauce fermentation is often made in a non-disinfected environment for a lengthy period and usually takes four months to four years. During this period and under this condition, a wide range of microbes, mainly moulds, yeasts, and LAB such as *Aspergillus*, *Weissella*, *Tetragenococcus*, *Staphylococcus*, *Bacillus*, *Pichia*, *Zygosaccharomyces*, and *Candida* may be introduced and flourish (Tanaka et al., 2012; Wei et al., 2013a; Yang et al., 2017). The microbial communities involved in soy sauce production are highlighted in **Figure 2.2** and **Table 2.1**.

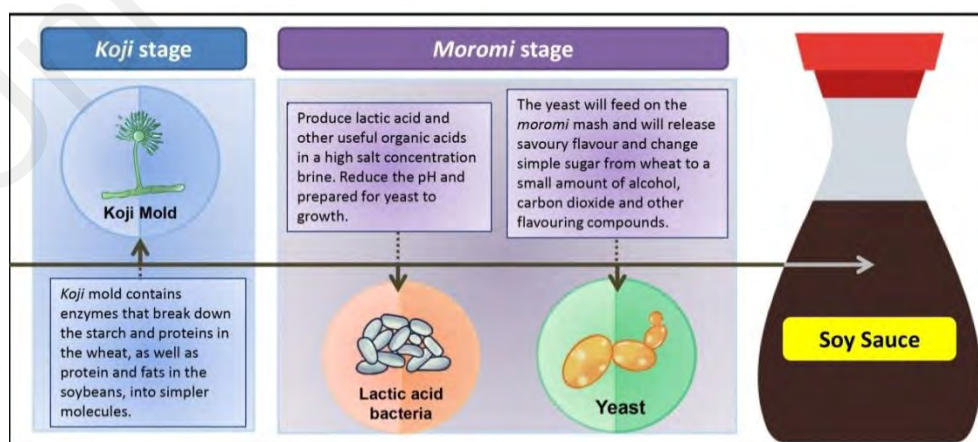


Figure 2.2: Three mains microbes involved in soy sauce brewing.

Table 2.1: The main microbes involved in soy sauce fermentation.

Class	Microorganism	Role / Remark	Reference
Mould	• <i>A. oryzae</i> • <i>A. sojae</i>	• Do not produce the common <i>Aspergillus</i> spp. mycotoxins such as aflatoxins, ochratoxin A, or sterigmatocystin under any conditions • Possess high proteolytic, amylolytic, and macerating enzyme levels • Digest proteins and starches in the raw materials	(Sugiyama, 1984)
	• <i>Aspergillus tamarii</i>	• <i>Tamari</i> brewing, a variety of soy sauce	(Lioe et al., 2010)
LAB	• <i>T. halophilus</i> KBC • <i>Weissella</i> spp. (<i>Weissella confusa</i>, <i>Weissella paramesenteroides</i>, and <i>Weissella cibaria</i>) • <i>Enterococcus</i> spp. • <i>Lactococcus</i> spp. • <i>Leuconostoc</i> spp. • <i>Lactobacillus fermentum</i>	• Produce lactic acid and other useful organic acids in a high concentration of salt brine • Reduce the pH and allow for yeast growth such as <i>S. rouxii</i> • Do not possess tyrosine decarboxylase and histidine decarboxylase	(Sugiyama, 1984; Wan-Mohtar et al., 2020; Yee et al., 2021)
	• <i>Pediococcus soyae</i> • <i>Pediococcus soyae</i> nov. sp.	• Produce lactic acid and other useful organic acids in a high concentration of salt brine • Remove undesirable flavors • Create essential aromas and flavors for soy sauce	(Sakaguchi, 1958, 1959)
	• <i>Bacillus</i> spp. • <i>B. cereus</i> KBC	• Generate odours and ammonia • A specific drug-like odour of soy sauce brewed with <i>A. sojae</i> could be diminished by the addition of bran culture of <i>B. subtilis</i> • The polypeptide-like turbidity of soy sauce could also be removed	(Sugiyama, 1984; Wan-Mohtar et al., 2020)

Table 2.1, continued

	• <i>Staphylococcus gallinarum</i> • <i>Staphylococcus kloosii</i> • <i>Staphylococcus arlette</i>	• Create colour and flavor	(Wei et al., 2013a)
Osmophilic yeast	• <i>S. cerevisiae</i>	• Convert some of the sugars to ethanol to create other flavor compounds	(Sugiyama, 1984)
	• <i>Z. rouxii</i>	• Produce alcohol and excellent flavor substances in a high salt concentration brine	(Sugiyama, 1984)
	• <i>C. versatilis</i> • <i>C. etchellsii</i>	• Produce excellent flavor substances in a high salt concentration brine • Over 270 flavor substances have been isolated	(Sugiyama, 1984)

Each stage of soy sauce fermentation contains a different amount of microbial community. Microbial diversity in the *koji* stage was greater than that in the *moromi* stage (Gao et al., 2023). This is due to some of the bacterial groups in the *koji* stage (*Staphylococcus*, *Enterobacter* and *Bacillus*) are also present in the *moromi* stage (Wei et al., 2013b). Even though some bacteria (*Corynebacterium*, *Klebsiella*, *Kurthia*, and *Paenibacillus*) and fungi (*A. sojae*, *A. parasiticus*, *Trichosporon ovoides*, and *Trichosporon asahii*) present in the *koji* stage may not be preserved in the *moromi* stage, the high salt concentration in the *moromi* stage makes it unfavourable for non-halotolerant microbes to develop and grow, notably in the middle to late stage of *moromi* fermentation. As a result, the microbial diversity in the *moromi* stage decreases throughout the *moromi* fermentation period (Wei et al., 2013a; Wei et al., 2013b; Zhang et al., 2016).

Yang et al. (2017) discovered microbial isolates belonging to the bacterial genera of *Bacillus*, *Shimwellia*, *Weissella*, *Pantoea*, *Enterobacter*, *Scopulibacillus*, *Lactococcus*, and *Klebsiella*, and the fungal genera of *Aspergillus*, *Cladosporium*, *Absidia*, *Lichtheimia*, and *Sterigmatomyces*, after studying the microbial diversity and community dynamics in natural *moromi* of Xianshi soy sauce for four years using Polymerase chain reaction/denaturing gradient gel electrophoresis (PCR-DGGE) analysis. The study conducted by Wei et al., (2013) also revealed that bacterial species such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *B. subtilis*, *Brachybacterium rhamnosum*, *Delftia tsuruhatensis*, *Enterobacter pulveris*, *Kurthia gibsonii*, *Pantoea dispersa*, *Staphylococcus cohnii*, *Staphylococcus condimenti*, *Staphylococcus gallinarum*, and *S. kloosii* were found during the *inyu* (Taiwanese soy sauce) fermentation. By applying the PCR-DGGE method, they could discover more bacterial species such as *Citrobacter farmeri*, *Pantoea agglomerans*, *Salmonella enterica*, *Serratia marcescens*, *Enterococcus faecium*, and *Weissella confuse*.

The microbial community dynamics vary between the *koji* and *moromi* stages. *Koji* fermentation starts by soaking the soybeans in water overnight to soften them, and then the beans' hulls are removed to eliminate fungal inhibitors in the soybeans. This is essential for the fungi to flourish during the *koji* fermentation. Additionally, during the soaking stage, the soybeans will start to ferment, which indirectly lowers the pH. The low pH encourages fungal growth while inhibiting the growth of spoilage microbes (Santhirasegaram et al., 2016). Several bacterial and yeast species were found when the soybeans were soaking in the tap water. Bacterial species included *Lactobacillus casei*, *Streptococcus faecium*, *Staphylococcus epidermidis*, *Streptococcus dysgalactiae*, *Klebsiella ozaenae*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Citrobacter diversus*, and *Bacillus brevis*. On the other hand, yeast species included *Pichia burtonii*, *Candida diddensiae*, and *Rhodotorula rubra* (Devanthi & Gkatzionis, 2019). After soaking the beans overnight, they will be cooked at high temperatures, eventually reducing the number of bacteria, yeasts, and moulds in the soybeans (Santhirasegaram et al., 2016).

Table 2.2: Microbial diversity and predominance in *koji* and *moromi* stages.

Microbial Group	Koji stage	Predominant Species in <i>koji</i>	Moromi stage	Predominant Species in <i>moromi</i>	Reference
Bacteria	<i>Staphylococcus</i> , <i>Enterobacter</i> , <i>Bacillus</i> , <i>Lactobacillus</i> , <i>Weissella</i> , <i>Corynebacterium</i> , <i>Klebsiella</i> , <i>Kurthia</i> , and <i>Paenibacillus</i>	<i>Lactobacillus</i> spp. (e.g., <i>L. fermentum</i> , <i>L. iners</i> and <i>L. plantarum</i>), <i>Staphylococcus</i> spp. (e.g., <i>S. arlettae</i> , <i>S.</i> <i>caprae</i> , <i>S. cohnii</i> , <i>S.</i> <i>gallinarum</i> , <i>S. kloosii</i> , <i>S.</i> <i>saprophyticus</i> , <i>S.</i> <i>succinus</i> , and <i>S.</i> <i>xylosus</i>), <i>Weissella</i> spp. (e.g., <i>W.</i> <i>cibaria</i> , <i>W. confuse</i> , <i>W.</i> <i>kimchii</i> , and <i>W.</i> <i>salipiscis</i>) <i>Streptococcus</i> <i>thermophilus</i> , <i>Lactococcus</i> <i>raffinolactis</i> , <i>Leuconostoc</i> <i>mesenteroides</i>	<i>Staphylococcus</i> , <i>Enterobacter</i> , <i>Bacillus</i> , <i>Lactobacillus</i> , <i>Weissella</i> , <i>Tetragenococcus</i> , <i>Escherichia coli</i> and <i>Klebsiella</i>	<i>Tetragenococcus</i> spp. (e.g. <i>T.</i> <i>halophilus</i>), <i>Weissella</i> spp. (e.g., <i>W.</i> <i>cibaria</i> , <i>W. confuse</i> , <i>W.</i> <i>kimchii</i> , and <i>W. salipiscis</i>), <i>Lactobacillus</i> spp. (e.g., <i>L.</i> <i>fermentum</i> , <i>L. iners</i> and <i>L.</i> <i>plantarum</i>), <i>Staphylococcus</i> spp. (e.g., <i>S.</i> <i>arlettae</i> , <i>S. caprae</i> , <i>S.</i> <i>cohnii</i> , <i>S. gallinarum</i> , <i>S.</i> <i>kloosii</i> , <i>S. saprophyticus</i> , <i>S.</i> <i>succinus</i> , and <i>S. xylosus</i>), <i>Bacillus</i> spp. (e.g., <i>B.</i> <i>amyloliquefaciens</i> , <i>B.</i> <i>subtilis</i> , <i>B. lincheniformis</i> , and <i>B. methylotrophicus</i> , <i>B.</i> <i>cereus</i>)	(Lee et al., 2005; Song et al., 2015; Tanaka et al., 2012; Tanasupawat et al., 2002; Wei et al., 2013b; Yang et al., 2017; Yee et al., 2021; Zhang et al., 2016)

Table 2.2, continued

				<i>Pediococcus</i> spp. (e.g., <i>Pediococcus pentosaceus</i> , <i>Pediococcus acidilactici</i>) <i>S. thermophilus</i> , <i>L. raffinolactis</i> , <i>L. mesenteroides</i> , <i>E. coli</i> , <i>Klebsiella pneumonia</i>	
Fungi	<i>Aspergillus</i> and <i>Trichosporon</i>	<i>Aspergillus</i> spp. (e.g., <i>A. oryzae</i> , <i>A. sojae</i> , <i>A.</i> <i>parasiticus</i>)	<i>Aspergillus</i>	<i>Aspergillus</i> spp. (e.g., <i>A.</i> <i>oryzae</i>)	(Liu, 2012)
Yeast	<i>Candida</i> , <i>Geotrichum</i> , <i>Kluyveromyces</i> , <i>Pichia</i> and <i>Trichosporon</i> •	<i>Candida</i> spp. (e.g., <i>C.</i> <i>catenulata</i> , <i>C.</i> <i>glabrata</i>)	<i>Zygosaccharomyces</i> , and <i>Candida</i>	<i>Z. rouxii</i> , <i>Candida</i> spp. (e.g., <i>C.</i> <i>etchellsii</i> , <i>C. versatilis</i>)	(Huang & Teng, 2004; Tanaka et al., 2012; Wei et al., 2013b)

The next stage in soy sauce making is *koji* inoculation. According to Wei et al. (2013b), bacteria dominated the *koji*, notably LAB, followed by yeasts and moulds. *Lactobacillus* spp., *Staphylococcus* spp., and *Weissella* spp. are the most predominant bacteria in the *koji* compared to the other species. *Lactobacillus* spp. includes *L. fermentum*, *L. iners*, and *L. plantarum*. *Staphylococcus* spp. include *S. arlettae*, *S. caprae*, *S. cohnii*, *S. gallinarum*, *S. kloosii*, *S. saprophyticus*, *S. succinus*, and *S. xylosus*. *Weissella* spp. include *W. cibaria*, *W. confusa*, *W. kimchii*, and *W. salipiscis*. Additionally, *S. thermophilus*, *L. raffinolactis*, and *L. mesenteroides* have also been found during the *koji* stage (Table 2.2).

During the *moromi* fermentation, the microbial diversity varies over time. The microbial diversity reduces as the fermentation progresses, notably in the middle to late *moromi* fermentation (Wei et al., 2013b; Yang et al., 2017; Zhang et al., 2016). This phenomenon occurs mainly due to high salt concentrations in the *moromi*, which eventually renders the *moromi* environment unfavourable to non-halotolerant bacteria and inhibits their growth. Wei et al. (2013b) suggested that the *moromi* stage is dominated by bacteria, especially LAB, followed by yeasts and moulds. *T. halophilus* has been identified as the most common and predominant LAB during the *moromi* fermentation. This is because *T. halophilus* is a salt-tolerant LAB that can survive in high salinity (Tanaka et al., 2012; Tanasupawat et al., 2002). Another study revealed that in high-salt dilute-state (HSDL) soy sauce, *Weissella* was found to be dominant. As the fermentation continued, the prevalent bacterial species evolved into *Weissella* and *Tetragenococcus* (Zhang et al., 2016). Other bacterial species found in the *moromi* include *Lactobacillus* spp. (*L. fermentum*, *L. iners*, and *L. plantarum*), *Staphylococcus* spp. (*S. arlettae*, *S. caprae*, *S. cohnii*, *S. gallinarum*, *S. kloosii*, *S. saprophyticus*, *S. succinus*, and *S. xylosus*), *Weissella* spp. (*W. cibaria*, *W. confusa*, *W. kimchii*, and *W. salipiscis*), *S. thermophilus*, *L. raffinolactis*, *L. mesenteroides*, *T. halophilus* strain KBC, *E. coli*, *K. pneumonia*,

Bacillus spp. (*B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. methylotrophicus*, *B. cereus* strain KBC), *P. pentosaceus*, and *P. acidilactici* (Tanaka et al., 2012; Wan-Mohtar et al., 2020; Yan et al., 2013; Yang et al., 2017; Yee et al., 2021). Among these bacterial species, *Bacillus* and *Staphylococcus* have been identified in both the *koji* and *moromi* stages (Song et al., 2015; Wei et al., 2013a).

The second predominant microorganism present during soy sauce making, in both *koji* and *moromi* stages, is yeast (Wei et al., 2013b). In the *koji* stage, *C. catenulata*, *C. glabrata*, *Candida rugosa*, *Candida tropicalis*, *Geotrichum silvicola*, *Kluyveromyces marxianus*, *Pichia anomala*, *Pichia fabianii*, *T. asahii*, *Trichosporon faecale*, *Trichosporon inkin*, *Trichosporon insectorum*, *Trichosporon japonicum*, *Trichosporon jirovecii*, and *T. ovoides* were detected (Tanaka et al., 2012). However, these yeasts are only present in the *koji* stage and are not found in the *moromi* stage. This implies that these yeasts have *koji*-specific roles in soy sauce making. For example, these yeasts may regulate the enzymatic activity of the *koji* moulds or control their growth. Therefore, they are absent in the *moromi* stage. Besides, according to Tanaka et al. (2012), *K. marxianus*, *C. rugosa*, *P. fabianii*, *C. glabrata*, and *C. tropicalis* are the yeast species that are commonly found in plant fermentation and have been related to flavor development when combined with LAB.

A dramatic fungal transition was observed in the *moromi* mash yeast analysis. The organic acid produced by LAB increased the mash's acidity, thus allowing acid-tolerant yeasts to flourish throughout *moromi* fermentation. *Z. rouxii*, a salt-tolerant and acid-tolerant yeast, was reported to appear in the early stage (Tanaka et al., 2012) and proliferate in the middle to late stage of *moromi* fermentation when *A. sojae* started to diminish (Huang & Teng, 2004). The study showed that *Z. rouxii* is the most dominant yeast present in *moromi* mash. It is capable of alcoholic fermentation, hydrolysing different kinds of amino acids into their respective alcohols and producing important

aroma compounds such as alcohol, ester, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) and (isobutyl alcohol, isoamyl alcohol, 2-phenylethanol) 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) (Huang & Teng, 2004). After the alcohol fermentation is done by the *Z. rouxii*, *C. etchellsii* and *C. versatilis* were detected in the middle stage of *moromi* fermentation. In contrast, *C. etchellsii* and *Torulopsis versatilis* were found in the late stage or maturation stage of the *moromi* fermentation (Tanaka et al., 2012). Nunomura et al. (1980) identified over 270 flavor compounds from the soy sauce. Many studies have shown that *Candida* spp. (or *Torulopsis* spp.) contributes to some excellent aroma and flavor compounds in high salt concentration brine (Devanthi, 2018; Liu et al., 2021a; Nout & Aidoo, 2011). *Candida* spp. produce several aroma compounds such as 4-ethyl-guaiacyl phenol, which gives the soy sauce a clove flavor, 4-ethylguaiacol (4-EG) and 4-ethyl phenol (4-EP), which gives a smoky flavor, and lastly, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) which gives a caramel aroma (Tanaka et al., 2012; Yang et al., 2017). Among all identified yeast species in both the *koji* and *moromi* stages, *Z. rouxii* was identified as the predominant yeast throughout soy sauce fermentation (Wei et al., 2013b).

According to Wei et al. (2013b), mould is the third predominant microorganism present during soy sauce making, in both *koji* and *moromi* stages, of which *A. oryzae* and *A. sojae* were found to be the most prevalent, producing extracellular enzymes such as proteolytic enzymes and saccharolytic enzymes. These enzymes will hydrolyse the soybean proteins into peptides and amino acids and carbohydrates into simple sugars, respectively (Liu, 2012).

2.2.2 Microbial Control During Soy Sauce Fermentation

Soy sauce, in general, has a high salt concentration, making it an unfavourable medium for microbial development (Yan et al., 2013). Nevertheless, the *koji* fermentation

is usually carried out in a non-sterile environment and under aerobic solid-state conditions, which can often lead to microbial contamination. Therefore, preventing the *koji* stage from being contaminated to produce high-quality soy sauce is critical. According to Jiang et al. (2019), *Lactobacillus pobuzihii*, *Aspergillus flavus*, and *A. parasiticus* will naturally contaminate the *koji* during *koji* fermentation. Several *Bacillus* spp. have also been found to cause spoilage of the *koji* and, therefore, the resulting soy sauce. Sumague et al. (2008) reported that *B. subtilis* contaminated the *koji* and competed with other *koji* moulds at high temperatures. Other researchers revealed that in both the *koji* and *moromi* stages, *B. subtilis* and *B. pumilus* were found in sour soy sauce (Devanthi & Gkatzionis, 2019). Researchers isolated *Bacillus circulans*, *Bacillus maquariensis*, and *Bacillus pantothenicus* from sour soy sauce (Sumague et al., 2008).

Considering the conditions of handling, incubation, and open-air storage, bacteria would simply infect the *koji* from air, dirt, hands, or even insects. Natural contamination is difficult to regulate and may cause quality and safety risks. Traditional soy sauce brewing takes time, and due to this low efficiency in production, such contamination risks become intense (Jiang et al., 2019). Previous studies highlighted the impact of microbes accidentally introduced into the *koji* stage during soy sauce production. When bacteria contaminate the *koji*, the *koji*'s pH will rise due to the ammonia released by the bacteria. This bacterial growth will inhibit the necessary *koji* mould growth and subsequently suppress the *koji*'s enzyme activity (Takada, 2004).

After 20-24 hr of *koji* fermentation, the number of bacteria reaches its peak, and after the *koji* moulds are spread, the bacterial number steadily declines. The decrease in the number of bacteria could also be attributed to the reduction in the moisture content of the *koji*, as suggested by Sano et al. (2007). On the other hand, Nishimura et al. (2018) suggested that the bacterial reduction could be due to the antibacterial compounds produced by the *koji* moulds. Therefore, using *koji* moulds that produce antibacterial

compounds properly can help achieve microbial control during *kōji* production. The *kōji* mould selection will enhance the quality of soy sauce brewing (Liu et al., 2024).

During *moromi* fermentation, the *moromi* mash can quickly become contaminated or spoiled due to microbial contamination by raw ingredients or the surrounding environment (Sumague et al., 2008). In order to mitigate this, the *moromi* mash will usually be submerged in 18-20% brine solution; the high salt content in the brine will create a high salinity environment, which will then inhibit the growth of non-halotolerant microbes (Luh, 1995; Wei et al., 2013b; Zhang et al., 2023a). However, since the *moromi* surface is not submerged in the brine solution and is exposed to air, ambient microbes could still contaminate it. The bacterial contamination could occur primarily while making low-salt soy sauce, as reducing the salt content allows more bacteria to grow and proliferate (Feng et al., 2020).

Among the potential contaminants, *Enterococcus* spp. are of particular concern. Nam et al. (2012); Yoon et al. (2008) reported that these opportunistic pathogens also comprise a substantial portion of the autochthonous microflora found in various foods including traditional *moju* and other fermented soy products (Kim et al., 2009; Nam et al., 2012). Despite their pathogenic potential, some strains of *Enterococcus* spp. contribute to the unique properties of soy products through their strong glycolytic, lipolytic, and proteolytic activities (Nam et al., 2012). Notably, a strain known as *E. faecium*, isolated from soy products, did not contain virulence genes (Yoon et al., 2008). Stute et al. (2002); Yongmei et al. (2009) reported that the synthesis of BAs in soy sauce is assumed to be mainly attributable to the proteolytic *E. faecium*.

Staphylococci exist in various food items and environmental sources (Even et al., 2010). *Staphylococcus* is the dominant microorganism in fermented seafood containing high salt content, thus demonstrating its high resistance to salt stress (Guan et al., 2011). *Staphylococcus* has also emerged spontaneously during soy sauce manufacturing,

especially in Japanese and Chinese soy sauce. However, in Korean soy products, it is rarely being detected (Kim et al., 2009). *Staphylococcus* species such as *S. epidermis* and *S. xylosus* frequently possess enterotoxin-encoding genes and could generate BAs (Even et al., 2010). The emergence of enterococci and staphylococci by reducing salt might pose significant safety issues due to their potential contamination risk (Song et al., 2015). To overcome this, Ferng et al. (2020) suggested that ultraviolet (UV) treatment for fermenting low-salt soy sauce can effectively prevent microbial contamination. Meanwhile, Song et al. (2015) reported that adding *Torulaspora delbrueckii* produces a lot of ethanol and effectively suppresses the proliferation of putrefactive microbes in the *moromi*. By combining both *T. delbrueckii* with *Pichia guilliermondii*, a high-producing fuel alcohol strain, the *moromi* production resulted in a balance of more complex and richer taste with a flavor profile pattern similar to that of high-salt soy sauce.

2.3 Asian Traditional Soy Varieties and Microbes Involved

Soybeans have been grown as a commercial crop for thousands of years. It was first cultivated in Northern Asia and then slowly expanded to North America (Hyten et al., 2006). It is an essential protein source for millions of people globally. The bioactive compounds in soybeans provide various health benefits to humans, such as anti-hypercholesterolemic, chemo-preventive, anti-hypertensive, anti-diabetes, and immunomodulatory activities. As a result, the soybean market has been diversified to various soybean-based products worldwide (Naresh et al., 2019). Generally, traditional soy-based foods are divided into fermented and non-fermented products (Kim et al., 2011). This section will discuss the fermented soybean products in the Orient, and the overview of all the fermented soy products is highlighted in **Table 2.3**.

Table 2.3: Soy-fermented food history in the Orient.

Product	Country / Region	Period	Substrate	Product form	Microbes involved	Consumption / Role	Reference
<i>Chee-fan</i> 	China	Han dynasty (179 – 122 BC)	Soybean and whey curd	Solid	<i>Mucor</i> spp., <i>Aspergillus glaucus</i>	Cheese-like food, eaten fresh	(Padmaja & George, 1999)
<i>Dou-chi</i> 	China	165 BC	Black soybean	Solid	<i>Aspergillus</i> sp.	Seasoning agent	(Chen et al., 2005)
<i>Hama natto</i> (salted <i>natto</i>) 	Japan	Heian period (794 – 1185)	Soybean and roasted wheat <i>koi</i> or barley <i>koi</i>	Soft / raisin-like	<i>A. oryzae</i> , <i>Streptococcus</i> sp., <i>Pediococcus</i> sp.	Eaten with rice, as topping for sushi, salad, and toast, and served as breakfast food	(Blandino et al., 2003; Kiuchi et al., 1976; Kon & Ito, 1974; Shurtleff & Aoyagi, 2012a)

Table 2.3, continued.

<i>Itohiki</i>							
<i>natto</i> (non-salted <i>natto</i>)	Japan	Heian period (794 – 1185)	Soybean	Soft / raisin-like	<i>B. subtilis</i> var. <i>natto</i>	Eaten with rice, as topping for sushi, salad, and toast, and also served as breakfast food	(Blandino et al., 2003; Kiuchi et al., 1976; Kon & Ito, 1974; Shurtleff & Aoyagi, 2012a)
							
<i>Kecap</i>							
	Indonesia	Mid-19 th century (1830 – 1860)	Soybean and wheat	Liquid	<i>A. oryzae</i> , <i>Lactobacillus</i> sp.	Liquid condiment and flavoring agent	(Blandino et al., 2003; Lockwood & Smith, 1950)
<i>Ketjap</i>							
	Indonesia	Mid-19 th century (1830 – 1860)	Black soybean	Syrup	<i>A. oryzae</i>	Liquid condiment and flavoring agent	(Blandino et al., 2003; Lockwood & Smith, 1950)

Table 2.3, continued.

	China, Taiwan	1937	Soybean cake	Solid	<i>Actinomucor elegans</i>	Fried in oil or cooked with vegetables	(Kronenberg & Hang, 1984; O'Toole, 1999; Shurtleff & Aoyagi, 1985)
	Korea	680	Soybean	Paste	<i>A. oryzae, Rhizopus sp.</i>	Flavoring agent	(Padmaja & George, 1999; Shurtleff & Aoyagi, 2009)
	Japan	800 – 900	Soybean and rice	Paste	<i>Aspergillus sp., Torulopsis etchellsii, Lactobacillus sp., S. rouxii</i>	Paste, soup base	(Lockwood & Smith, 1950; Shurtleff & Aoyagi, 2012b)

Table 2.3, continued.

Cultured							
soybean milk 	China, Japan	Han dynasty (179 – 122 BC)	Soybean	Liquid	LAB	Beverage	(Blandino et al., 2003; Shurtleff & Aoyagi, 2004; Wang, 1979)
Japan,							
Soy sauce/ Shoyu 	China, the Philippines , and other oriental countries	Han dynasty (206 BC – 220 AD)	Soybean and wheat	Liquid	<i>A. oryzae</i> , <i>A. sojae</i> , <i>Lactobacillus</i> sp., <i>S.</i> <i>rouxii</i>	Liquid condiment and flavoring agent	(Shurtleff & Aoyagi, 2012b)

Table 2.3, continued.

	<i>Sufu / furu</i> China, Taiwan	Wei dynasty (220 – 265)	Soybean whey curd	Solid	<i>Aspergillus elegans</i>, <i>Mucor hiemalis</i>, <i>Mucor silvaticus</i>, <i>Mucor subtilissimus</i>, <i>Mucor sufu</i>	Soybean cake and as a condiment	(Han et al., 2001, 2004; Wai, 1929)
	<i>Tao-si</i> The Philippines	200 BC	Soybean and wheat	Solid	<i>A. oryzae</i>	Flavoring agent	(Lockwood & Smith, 1950; Shurtleff & Aoyagi, 2011)
	<i>Taotjo</i> East India, Indonesia	-	Soybean and roasted meal or glutinous rice	Solid	<i>A. oryzae</i>	Condiment and flavoring agent	(Lockwood & Smith, 1950; Shurtleff & Aoyagi, 2009)

Table 2.3, continued.

<i>Taoco</i> 	West Java, Indonesia	-	Soybean and cereals	Solid	<i>Rhizopus oligosporus, A. oryzae</i>	Flavoring agent	(Lockwood & Smith, 1950; Shurtleff & Aoyagi, 2009)
<i>Tempeh</i> 	Indonesia and nearby regions	400	Soybean	Solid	<i>Rhizopus</i> sp.	Fried in oil or roasted; as a meat substitute	(Djurtoft & Jensen, 1977; Shurtleff & Aoyagi, 1979)
<i>Yukiwari natto</i> (cracked <i>natto</i>) 	Japan	Middle of the Edo period (1603 – 1867)	Fermented soybean, <i>itohiki natto</i> with rice <i>koji</i>	Soft / raisin-like	<i>A. oryzae, B. subtilis</i> var. <i>natto</i>	Served with rice	(Blandino et al., 2003; Kiuchi et al., 1976; Kon & Ito, 1974; Shurtleff & Aoyagi, 2012b)

China has a wide range of fermented soybean products such as *chee-fan*, *dou-chi*, *meitauza*, soybean milk, soy sauce, and *sufu*. *Chee-fan* is a soft cheese made from soybean and whey curd is inoculated with *Mucor* spp. and *A. glaucus* for fermentation. It is traditionally consumed like cheese by the Chinese (Padmaja & George, 1999). *Dou-chi* is salted and fermented black soybean inoculated with *Aspergillus* sp. for about a month. It is also known as one of the most popular seasoning agents worldwide among the Chinese (Chen et al., 2005). *Meitauza* is a soybean cake inoculated with *A. elegans*, and people usually fry it in oil or cook it with vegetables. It is a popular dish or cooking ingredient in Taiwan and China (Nout et al., 2007).

Soybean milk or soya milk or soy milk is a beverage made from soybean extraction, which resembles milk. It is first soaked and ground, then filtered through cheesecloth and boiled. Several varieties of soy milk are present in the market, such as plain soy milk, dairy-like soy milk, soy milk soft drinks, cultured soy milk, soymilk infant formula, and soymilk blends. Cultured soy milk typically undergoes lactic acid fermentation by LAB (Shurtleff & Aoyagi, 2004).

Sufu or *furu* is a flavorful creamy bean paste made by fermenting the soybean curd with moulds followed by ageing or curing in saltwater or rice wine mixture. *A. elegans*, *M. hiemalis*, *M. silvaticus*, *M. subtilissimus*, *M. sufu* are the moulds involved in *sufu* fermentation. *Sufu* can be classified into four types: red *sufu*, white *sufu*, grey *sufu* and sauce *sufu*, based on the different dressing ingredients used during *sufu* production. *Sufu* is usually consumed as an appetiser and a condiment (Han et al., 2001, 2004).

The three most famous soybean-fermented products in Japan are *natto*, *miso*, and *shoyu* (Japanese-style soy sauce). *Natto* is a traditional Japanese food consisting of fermented soybeans. There are three main types of *natto* available in the market, which include *itohiki natto* (non-salted natto), *hama natto* (salted natto) and *yukiwari natto* (cracked natto). *Itohiki natto* is made from soybeans inoculated with *Bacillus natto*. *Hama*

natto is made from soybeans with roasted wheat *koji* or barley *koji* inoculated with *A. oryzae*, *Streptococcus* sp. and *Pediococcus* sp.. *Yukiwari natto* is made from fermented soybeans (*itohiki natto*) with rice *koji* inoculated with *A. oryzae* and *B. natto*. They are usually eaten with rice or served as breakfast in Japan. Additionally, *natto* can be used as a topping for sushi, salad, or toast, especially *itohiki natto* and *yukiwari natto* (Shurtleff & Aoyagi, 2012a).

Miso is a traditional Japanese seasoning paste or soup base that is used in various Japanese cooking. It is made from soybeans, fermented rice *koji* or barley *koji* and salt inoculated with *Aspergillus* sp., *T. etchellsii*, *Lactobacillus* sp. and *S. rouxii*. The length of *miso* fermentation is similar to *shoyu*, which takes up six months to five years (Shurtleff & Aoyagi, 2012a).

In Indonesia, examples of fermented soybean products are *kecap*, *ketjap*, *taotjo*, *taoco*, and *tempeh*. Even though *kecap* and *ketjap* are both liquid condiments and flavoring agents, *kecap* is made from soybeans and wheat inoculated with *A. oryzae* and *Lactobacillus* sp.. In contrast, *ketjap* is made from black soybeans and inoculated with *A. oryzae*. Furthermore, *kecap* is in the form of liquid, while *ketjap* is in the form of syrup (Blandino et al., 2003). *Taotjo* and *taoco* are both flavoring agents, and solid in form. *Taotjo* is made from soybeans and roasted meal or glutinous rice, while *taoco* is made from soybeans and cereals. The microbes involved during the fermentation of *taotjo* are *A. oryzae*, and for *taoco*, *R. oligosporus* and *A. oryzae*. *Tempeh* is a traditional Indonesian fermented soy-based food. It is in cake form and was very popular. It is cooked whole soybeans inoculated with tempeh starter *Rhizopus* sp. and fermented for 24 to 36 hr to allow the spores to germinate and grow. The easiest way to cook *tempeh* is by deep-frying or stir-frying it in hot cooking oil. *Tempeh* is also known as a meat substitution due to its high soy protein content (Djurtoft & Jensen, 1977; Shurtleff & Aoyagi, 1979).

There are several other soy-fermented products in other countries, such as *meju* from Korea, and *tao-si* from the Philippines. *Meju* is a brick dried soybean made from soybeans inoculated with *A. oryzae* and *Rhizopus* sp. It is usually not consumed directly but used as the base for Korean condiments or flavoring agents such as *doenjang* (soybean paste), *ganjiang* (soy sauce) and *gochujang* (chilli paste) (Padmaja & George, 1999; Shurtleff & Aoyagi, 2009). *Tao-si* is also known as salted black soybeans or soy nuggets. It is resembling *dou-chi* from China, *hamma-natto* from Japan, and *taotjo* from Indonesia. The whole soybeans are first inoculated with *A. oryzae* and then added into the brine solution to ferment and mature for about six months. *Tao-si* is not eaten alone but is typically used as a flavoring agent or condiment (Lockwood & Smith, 1950; Shurtleff & Aoyagi, 2011).

2.4 Advances in Soy Sauce Fermentation

Soy sauce is commonly produced using traditional fermentation or brewing, chemical-hydrolysis or non-brewing, or a combination of these methods (Diez-Simon et al., 2020a). Traditional brewing involves microbes to ferment, while chemical-hydrolysed soy sauce is produced by hydrolysing the soy protein with acid and heat (Luo et al., 2017). Chemical-hydrolysed soy sauce, also known as hydrolysed vegetable proteins (HVP) soy sauce, is made from soy protein that has been acid-hydrolysed by HCl into amino acids and combined with sugars and colouring and flavoring agents to create a sauce that resembles the natural fermented soy sauce. This method is claimed to be cheaper and faster due to the lack of naturally fermentation steps (Lee & Khor, 2015). It can shorten the processing time from 4 months to 3 days on average (Wong et al., 2020). However, research have shown that several unwanted compounds like 3-chloropropane-1,2-diol (3-MCPD) and 1,3-dichloropropanol (1,3-DCP) not produced during natural fermentation are produced during the acid-hydrolysed reaction (Kataoka, 2005).

2.4.1 Advances in Soy Sauce *Moromi* Maturation

It is crucial to enhance production efficiency by expediting the maturation of soy sauce aroma and reducing the fermentation period of soy sauce. Researchers have recently attempted to utilise ultrasonic technologies in the production of soy sauce (Yu et al., 2024). Ultrasound has been found to be an effective tool for enhancing microbial growth, metabolic performance, and enzyme activity in the process of fermenting foods (Yu et al., 2021). Not only that, but these techniques are also found to be useful in facilitates chemical reactions, giving rise to a significant rise in yield and enabling the occurrence of reactions that are challenging or not feasible under typical conditions (Zhang et al., 2015).

Researchers conducted an experiment where they subjected soy sauce *moromi* to ultrasonic treatment for 10 min each day at a frequency of 24 kHz and an amplitude of 100%. On the fourth day of fermentation, the glutamic acid and total free amino acid levels in the sonicated group reached 343.0 mg/100 g and 1720.0 mg/100 g, respectively. The levels of glutamic indicates that the process of sonication greatly expedited the flavor development of acid and total free amino acids in the fermentation group exceeded those in the control group by the seventh day, reaching 295.0 mg/100 g and 1614.5 mg/100 g, respectively. This indicates that the process of sonication greatly expedited the flavor development of soy sauce, as reported by Goh et al. (2017).

Besides, another researcher found that implementing high-intensity ultrasound in soy sauce *moromi* fermentation could not improve product quality but also shortens the maturation duration and reduces costs for the soy sauce industry (Yu et al., 2024). The ideal parameters for ultrasound treatment were determined to be a temperature of 90°C and an ultrasound intensity of 39.48 W/cm² for a duration of 60 min. This treatment markedly enhanced the concentration of overall reducing sugars and soluble salt-free solids. Nevertheless, the concentration of unbound amino acids declined, mostly as a

result of the Maillard reaction. The improved Millard reaction generated a range of flavor compounds, including esters, pyrazines, and ketones, that played a role in creating a delightful scent in the aged soy sauce (Chen & Pan, 2023). The percentage of peptides played a crucial role in the umami flavor, was substantially greater in the soy sauce that underwent ultrasound maturation (10.19%) compared to both freshly made soy sauce (8.34%) and samples that were thermally processed (8.89%) (Yu et al., 2024). In short, ultrasound-assisted maturation offers a potential alternative method for enhancing the maturing of soy sauce

2.4.2 Advances in Removing Soy Sauce Precipitation

Fermented soy sauce is made via a series of procedures that include the *koji* fermentation, brine fermentation, and key refining steps such as pressing, filtering, centrifugation, pasteurization, and packing (Liu et al., 2020c). Two forms of precipitate, primary and secondary, can occur during the manufacture process of soy sauce. The primary precipitate forms during the precipitation and sterilization phases, mostly consisting of organic substances such as proteins, polysaccharides, and microbiological waste that can be readily eliminated through filtration. Upon packing, the secondary precipitate becomes apparent at the base of the bottle throughout storage, transportation, and sales, creating a stratum that resembles yellowish sediment. The presence of the secondary precipitate had a significant impact on the overall appearance of the final product and customer's buying intentions (Gao et al., 2013).

Currently, the primary methods used by soy sauce companies to reduce soy sauce secondary precipitate are membrane or diatomite filtering, as well as cold and hot alternating pre-precipitation (Guo et al., 2019). Nevertheless, all these methods have several deficiencies, including expensive production, maintenance expenses, high

nutrient loss, low efficacy, and environmental contamination from waste diatomite (Zhang et al., 2023b).

The use of enzymatic hydrolysis and ultrasound technologies have been explored to mitigate secondary precipitate formation in soy sauce, aiming for cost-effective and environmentally sustainable solutions (Zhang et al., 2023b). Previous study revealed that the B₃ subunit constitutes 75% of the protein in soy sauce secondary precipitate and encounters approximately 20% of all soybean protein. The main cause of the formation of soy sauce secondary precipitate is due to the proteases produced by *A. oryzae*'s incapacity to digest the B₃ subunit (Gao et al., 2013). To address this, Shan et al. (2022) identified an acidic protease, prolyl endopeptidase (APE) from *Aspergillus niger*, which capable of degrade approximately 50% of the B₃ subunit, leading to a 61% reduction in the soy sauce secondary precipitate.

In order to catalyze the protease activity, researchers found that appropriate sonication can greatly increase 33-106% of the protease-catalyzed substrate breakdown rate (Li et al., 2020a; Wen et al., 2018). This enhanced activity is attributed to improved accessibility of protease enzymes to their substrates and increased exposure of cleavage sites, ultimately enhancing the efficiency of enzymatic hydrolysis processes (Cui et al., 2020b; Wang et al., 2021).

2.4.3 Advances on Safety of Soy Sauce

The safety concerns associated with soy sauce primarily consist of biological, chemical, and physical aspects (Kang et al., 2016). Currently, soy sauce is most concerned by biological and chemical safety issues, including aflatoxin, biogenic amine, and ethyl carbamate (Gao et al., 2023).

Aflatoxins are the most potent carcinogenic and deadly naturally occurring by-products produced by the fungi *A. flavus* and *Aspergillus parasitica* (Panwar & Gehlot,

2020). The predominant aflatoxins found in nature are aflatoxin B1, B2, G1, and G2. Aflatoxin B1 (AFB1) is classified as a Class I carcinogen by the International Agency for Research on Cancer (IARC) (Li & Liu, 2019). Currently, there are various approaches to mitigate the development of aflatoxins. These strategies can be categorized into physicochemical and biological preventative techniques, with the implementation of excellent agricultural production practices and clean management (Mao et al., 2016). The biological approaches demonstrated notable efficacy and selectivity, while their scalability for large-scale reuse may pose challenges (Singh, 2010). UV irradiation, a non-thermal technology, is extensively used in the food sector for disinfection (Singh et al., 2021). It is also recognized as a feasible and cost-effective method to eliminate aflatoxins because of their photosensitive qualities (Javanmardi et al., 2022).

BAs are small molecules produced by bacteria by the decarboxylation of amino acids. They are also produced by the process of reductive amination and transamination of aldehydes and ketones, or through the metabolic activity of bodily tissues (Wójcik et al., 2021). The fermentation process of soy sauce involves a diverse range of bacteria and is characterized by the presence of abundant amino acids (Qi et al., 2021). Certain microorganisms, such as *Bacillus* and *Lactobacillus*, possess the ability to create amino acid decarboxylase enzymes, which facilitate the removal of carboxyl groups from amino acids, resulting in the production of BAs (Li et al., 2020b). Presently, BAs have been identified at different levels of concentration in nearly all soy sauce products, and many soy sauce have BAs levels that surpass the FDA's standards of 1000 mg/kg (Li et al., 2019; Santos, 1996). Thus, studies found several ways such as maintaining a lower fermentation temperature and increasing the salt content, it is possible to hinder the growth and metabolism of microorganisms that produce amino acid decarboxylase (Jaguey-Hernandez et al., 2021; Liu et al., 2020a). Besides, recent studies also demonstrate that certain substances or plant-derived natural products are preferred for

reducing BAs. This is because they offer advantages like simplicity, affordability, and safety, and their performance surpasses that of starting cultures (Houicher et al., 2021; Lee et al., 2020).

Ethyl carbamate (EC) was identified as a significant toxic compound in food, with a maximum allowable concentration of fewer than 20 µg/L, as determined by the Food and Agriculture Organization (FAO) in 2002 (Weber & Sharypov, 2009). EC synthesis in food systems mostly occurs through various pathways, particularly one that consists of the reaction of certain chemicals such as carbamyl compounds (citrulline, urea, and carbamoyl phosphate) or cyanide react with ethanol, resulting in the generation of EC (Fang et al., 2018). Factors such as heating treatment especially in the refining (extraction of raw soy sauce), pasteurization and storage stage, pH, and UV radiation might speed up the production of EC (Riachi et al., 2014). Various methods have been employed to restrict the formation of EC in the food supply, and ongoing research is enhancing the most effective strategies for EC mitigation (Abt et al., 2021). The existing research on EC reduction predominantly focuses on controlling precursors. This has been mainly achieved either by screening bacteria that secrete low levels of precursors or by introducing bacteria (or enzymes) with a high ability to consume (or decompose) precursors (Liu et al., 2018). Besides, some researchers suggested the addition of acidic urease to remove the urea precursor in order to decrease the concentration of EC (Cerreti et al., 2016). Despite the fact that some of these methods can reduce EC, their practical implementations are still restricted due to the impact on soy sauce flavor, complexities, and relatively low EC reduction rate (Jiao et al., 2014). Thus, new approaches should be established to effectively and securely reduce EC. Several studies also showed that the EC content in soy sauce can be reduced by 50% through the direct inclusion of an EC-degrading enzyme which known as urethanase (Jia et al., 2020; Liu et al., 2017). Additionally, simple addition of phenolic chemicals, such as gallic acid and

protocatechuic acid prior to heat treatment during soy sauce production, was reported to have the potential to hinder the transformation of urea/citrulline into EC (Zhou et al., 2021).

2.5 Soy Sauce Microbial Fermentation: Progress and Advances

Microbes play an essential role in fermenting soy sauce. Diagnostics and monitoring technologies have emerged to isolate, identify, and classify microbes. For decades, both culture-dependent and culture-independent techniques have been widely used to isolate the microbes present in the *moromi* stage (Tanaka et al., 2012). However, besides being laborious and time-consuming, the culture-dependent technique can only detect strains that can grow on a nutrient medium. This method subsequently limits the detection of other microbes that are challenging to culture on a nutrient medium (Tanaka et al., 2012). Several great advanced systematic microbial molecular analysis techniques have evolved significantly in the past 10 years (Gao et al., 2023).

2.5.1 Identification of Microbe During Soy Sauce Fermentation

The PCR-DGGE method has been widely used to determine the microbial community structure in many fields of microbial ecology, such as soil, sea, river, lake water, and wastewater treatment bioreactors without cultivation and to analyse the community dynamics in response to the environmental conditions (Yan et al., 2007; Yu et al., 2008). Of late, food microbiology has also started to implement it. Several studies have discovered and analysed the microbes present in food samples such as soybean paste, *kimchi* and wine by using molecular techniques (Din et al., 2022; Lee et al., 2021; Yue et al., 2021a).

In contrast to PCR-DGGE, high-throughput sequencing such as 16 rRNA gene sequencing has demonstrated precision as well as efficiency in characterizing complex

microbial communities, while also being less labour-intensive and time-consuming (Yue et al., 2021a). A recent study utilized the 16S rRNA gene sequencing method to analyze the bacterial community and shifts occurring during soy sauce fermentation (Liu et al., 2021b). The study revealed that this method is capable of detecting bacteria that cannot be cultured and are present in low abundance. This finding is valuable for identifying and monitoring new potentially infectious bacteria that may arise during soy sauce fermentation (Liu et al., 2021b).

However, the use of PCR-DGGE with 16S rRNA gene sequencing can lead to biased results when assessing microbial abundance (Hong et al., 2009). Thus, microbial fingerprinting, obtained via shotgun such as the whole-genome shotgun (WGS) analysis, has the potential to enhance existing microbiological techniques. This technique can aid in the identification and monitoring of pathogens, verify the authenticity and geographical source of foods, and serve as a reliable tool for studying the spread of antibiotic resistance genes, among other uses (Sabater et al., 2021). This method provides rich data on taxonomic perspectives, as well as the functional and metabolic diversity of microbial populations during fermentation (Simon & Daniel, 2009).

These identification methods help the researchers to better understand the microbes involved in soy sauce production and discover new microbial strains that help improve the soy sauce. Using these methods, researchers can now identify microbes present during the *koji* and *moromi* stages (Chen et al., 2009; Sassi et al., 2021; Tanaka et al., 2012; Yan et al., 2013; Yang et al., 2017; Yee et al., 2021).

The following subsections discuss in depth the advances elicited by the various microbes involved in soy sauce fermentation, which are then summarised in **Figure 2.3**.

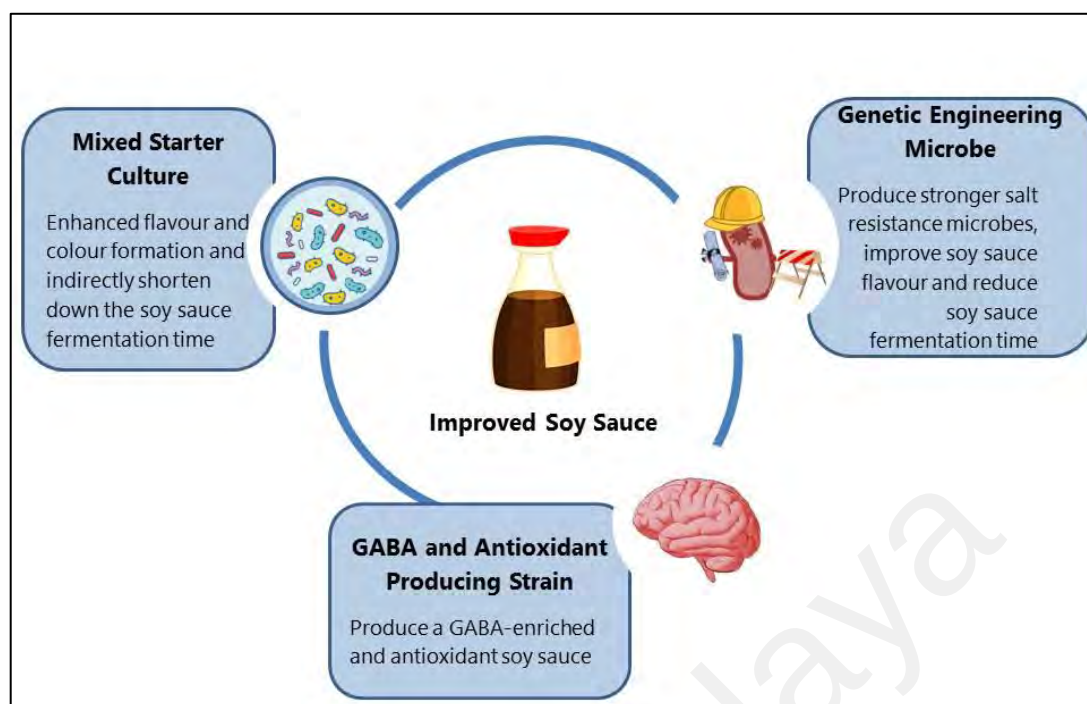


Figure 2.3: Improvement in soy sauce fermentation.

2.5.2 Enhancement of Soy Sauce Flavor by Genome Shuffling Starter Culture

The high salt content in the *moromi* makes it unfavourable for non-salt-tolerant microbes to grow (Ito & Matsuyama, 2021). Therefore, extensive fermentation is required to achieve sufficient flavor formation. Consequently, genetic engineering techniques have to be employed to produce high salt-tolerant microbial strains. Genome shuffling is a technique that shuffles multiple genes and recombines the whole genome to produce a novel and desired strain. This technique has been reported to increase the salt-tolerant properties of *Z. rouxii*, and subsequently increase the flavor formation in soy sauce (Cao et al., 2012; Qi et al., 2016; Wei et al., 2013a).

S3-2 is a *Z. rouxii* mutant strain that has better properties than the original strain. S3-2 could survive in a medium with high salt content, and it showed excellent resistance against potassium chloride and lithium chloride. Besides, S3-2 could also provide a higher level of amino acid nitrogen and a better flavor than the control, producing flavor

compounds such as ethyl acetate, 4-hydroxy-2 (or 5)-ethyl-5 (or 2)- methyl-3(2H) – furanone and 4-ethylguaiacol. This mutant strain can enhance and accelerate the flavor development in soy sauce and shorten the fermentation period (Cao et al., 2012; Wei et al., 2013a).

On the other hand, S3-5 is a mutant strain of *C. versatilis* and is produced using the genome shuffling technique similar to S3-2. S3-5 had higher stress resistance against potassium chloride, lithium chloride, and sodium chloride. S3-5 also improved the soy sauce aroma by producing much higher ethanol and other main aroma compounds such as 4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3 (2H)-furanone (HEMF) as compared to the control (Cao et al., 2010).

Another mutant strain produced by genome shuffling technique is H3-8 of *Hansenula anomala*. Similarly, H3-8 showed better resistance against salts, and had a high survival rate in the YPD medium that contained high sodium chloride content, and within a wide range of pH. H3-8 produced high amounts of hydroxyethylmethylfuranone, ethyl acetate, and 4-ethylguaiacol amounts as compared to the control (Cao et al., 2012).

Although this genetic engineering technique has improved the ability of the strains in terms of salt resistance and flavor formation, there have been raised regarding the safety of the genetically engineered strains, including health and environmental impacts, ethics, and food security (Abdul Aziz et al., 2022; Azadi & Ho, 2010).

2.5.3 Enhancement of Soy Sauce Flavor by Mixed Starter Culture

A mixed-start culture used in soy sauce fermentation comprises of two or more microbes, which is required for flavor development and enhancement when compared to a single-starter culture. The most commonly mixed starter cultures used in *moromi* fermentation are the LAB *T. halophilus* and the yeast *Z. rouxii* (Yao et al., 2020). Salt-tolerant yeasts such as *Z. rouxii*, *C. versatilis*, and *C. etchellsii* have all been implicated

in forming volatile flavor compounds (VFCs) during soy sauce fermentation (Cui et al., 2014). Flavor compounds include ethanol, furanone, esters, aldehydes, acids, pyrone and phenols. The total VFCs in the *moromi* varied depending on whether the starter cultures were single, mixed, or sequential (inoculated one after the other). For example, as previously discussed, the single use of *T. delbrueckii* JBCC-623 in low-salt soy sauce produced high ethanol and inhibited putrefactive microbes' growth. However, when *T. delbrueckii* JBCC-623 was combined with *P. guilliermondii*, more complex flavors were created, much similar to the high-salt soy sauce flavor profile (Song et al., 2015).

In addition, Singracha et al. (2017) indicated that by adding *T. halophilus* TS71, *Z. rouxii* A22, and *Meyerozyma (Pichia) guilliermondii* EM1Y52 into the *moromi*, higher amounts of critical VFCs such as ethanol, 2-methyl-1-propanol, 4-hydroxyl-2,5-dimethyl-3(2H)-furanone (HDMF), and 3-hydroxy-2-methyl-4H-pyran-4-one (maltol) were produced, while lower number of BAs was observed as compared to other treatments. Furthermore, the co-culture of *Z. rouxii* and *P. guilliermondii* in the *moromi* produced more VPCs such as alcohols, furanone, esters, maltol, and benzoic acid as compared to the single culture of only *Z. rouxii* (Wah et al., 2013).

2.5.4 Enhancement of GABA and Other Beneficial Compound in Soy Sauce Fermentation by Microbes

Fermentation has historically produced bioactive compounds that benefit both consumers and industries. Over the years, researchers have found several ways to improve and enhance the functionality of soy sauce, including chemicals that can help lower cholesterol levels, antioxidants, anti-carcinogenic properties, antimicrobial activities, antihypertensive component, an anti-inflammation, anti-allergy, gastric acid secretion, and antiplatelet activities (Zhao et al., 2013).

One such way to enhance the functionality of soy sauce is by enhancing the GABA production during soy sauce fermentation (Sassi et al., 2021). Non-protein GABA is a prominent and influential inhibitory neurotransmitter in mammals' central nervous systems. Additionally, GABA is reported to have anti-hypertensive, anti-cancer, anti-inflammation, anti-diabetes, anti-allergy, and tranquilliser effects, making it an excellent candidate for use in functional foods (Rashmi et al., 2018). GABA can be naturally produced during soy sauce fermentation by breaking down the material proteins by proteases or peptidases or hydrolysing free L-glutamines by glutaminase (Galli et al., 2022; Karabulut et al., 2024).

According to Ab Kadir et al. (2016), four potential *koji* starter strains known as *A. oryzae* NSK, *A. oryzae* NSZ, *A. oryzae* NSJ, and *A. oryzae* NST were found to produce GABA during soy sauce fermentation, with *A. oryzae* NSK producing the highest GABA (194 mg/L). In another study, Hajar-Azhari et al. (2018) found that using 50 g/L of glucose cane molasses as the fermentable substrate instead of native sugar syrup, sugarcane, and nipa, *A. oryzae* NSK produced the highest GABA concentration (354.08 mg/L). Wan-Mohtar et al. (2019) optimised seven key *A. oryzae* NSK culture parameters to produce the highest GABA yield (3,278.31 mg/L) in SmF; initial pH 5, temperature of 30°C, sucrose concentration of 100 g/L, a combination of yeast extract (YE) and glutamic acid (GA), and carbon to nitrogen ratio of C8:N3.

Apart from *koji* moulds, recent studies also found that certain bacterial strains originally found in the soy sauce *moromi* could produce GABA. Wan-Mohtar et al. (2020) successfully isolated, identified, and optimised a novel high GABA-producing bacterial strain known as *B. cereus* strain KBC in SmF. The isolated strain was capable of producing 523.74 mg/L of GABA under unoptimized conditions, while under optimised conditions (initial pH 7, 5 g/L of MSG concentration, at 40°C), the strain was able to produce 6.37-fold higher GABA (3393.02 mg/L). Furthermore, another new bacterial

strain known as *T. halophilus* strain KBC was reported to be a new GABA-producing strain, producing higher GABA (159 mg/L) when compared with *B. cereus* strain KBC (BC)-*moromi* cultured in 20% molasses (118 mg/L) (Yee et al., 2021). Interestingly, the GABA produced with the combination of BC: TH *moromi* in 5%:20% molasses was much lower (83-137 mg/L) when compared with singular 20% TH-*moromi*. Nonetheless, this combination of BC-TH *moromi* shortened the *moromi* fermentation period from 60 d to 30 d (Yee et al., 2021).

It was reported that fermented soybean products are superior and healthier than raw soybeans in terms of antioxidant activity and phenolic content (Zhu et al., 2018). Melanoidin, isoflavones, phenolic acid, pyranones, and furanones are some other chemicals discovered during soy sauce fermentation that have antioxidant effects (Qiao et al., 2022). Researchers discovered that utilising a mixed starting culture of fungus strains (*A. oryzae* J, *Mucor racemosus* 15, and *Mucor racemosus* 42) and bacterium strains (*B. subtilis* TKSP24) increased antioxidant activity in *Doenjang* samples. This mixed culture had excellent antioxidant activity, with a 59.92% scavenging activity for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and a 92.83% inhibitory rate for nitric oxide radicals (Shukla et al., 2016). The starter-grown bacteria also produced a large quantity of bioactive phenolic content, ranging from 25.06 to 29.49 mg/g of gallic acid equivalents (Shukla et al., 2016). Peng et al. (2017) discovered that using a combined starter culture of *A. oryzae* HG-26 and *A. niger* HG-35 during *moromi* fermentation results in increased antioxidant activity, which is 12 % higher on day 120, than using *A. niger* HG-35 alone.

Thus, more studies and research are required to improve the current production process, produce high-value and healthy food products, and overcome the risks and problems.

CHAPTER 3: METHODOLOGY

3.1 Chemical

MRS (de Man, Rogosa, Sharpe, 69964-500G, Sigma-Aldrich, Dorset, UK), glycerol (Chemiz, Selangor, Malaysia), Calcium carbonate, CaCO_3 (Bendosen Laboratory, Bendosen, Norway), Sodium Chloride, NaCl (food grade), *Anaerocult A* (Merck, Kenilworth, USA), GABA Pure Powder (A5835-10g, Sigma-Aldrich, St. Louis, MO, USA), sodium acetate (Chemiz, Selangor, Malaysia), triethylamine (Sigma-Aldrich, St. Louis, MO, USA), acetic acid, and acetonitrile (Fisher Scientific, Hampton, NH, USA).

3.2 Microbes Isolation and Bacteria Culture

3.2.1 *A. oryzae* strain NSK (AO)

A. oryzae strain NSK (AO) were obtained from the Functional Omics and Bioprocess Development Laboratory (Omics Lab), Institute of Biological Sciences, Faculty of Science, Universiti Malaya. The pure culture of *koji* spores (*Aspergillus* sp.) was successfully identified and isolated from a soy sauce company (Kwong Bee Chun Soy Sauce Sdn. Bhd.) located at Kamunting, Perak, Malaysia (4.905858188640449, 100.70816929574252) using the single spore isolation technique with some modifications (Bueno et al., 2004). The fungus on the plate were subjected to the gram staining technique, as described by Coico (2006), to identify and assure the species **Figure 3.2**. The spores were then made into a powder form for future use.



Figure 3.1: Ultra zoom of fermented Soybean covered with green colour *A. oryzae* spore strain NSK (Bar= 1 mm). (Author's personal collection)

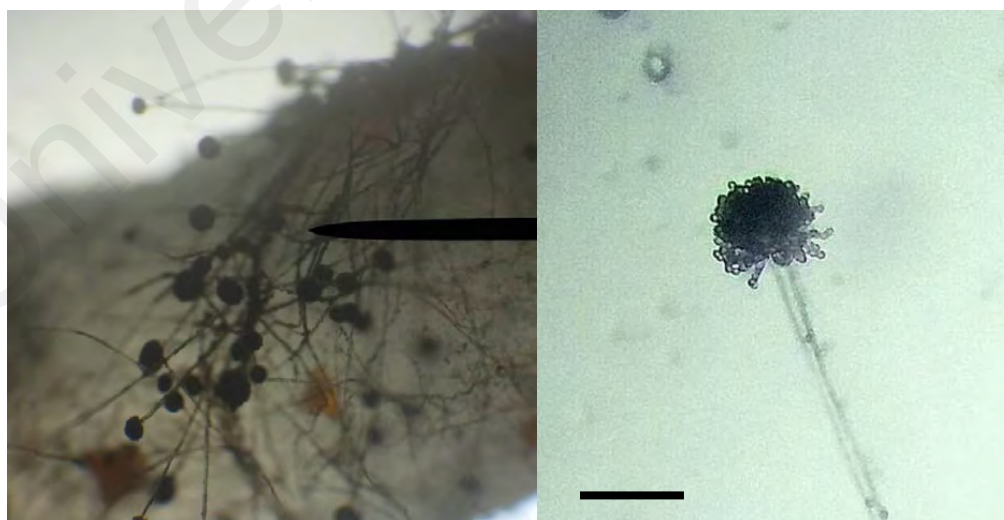


Figure 3.2: (a) *A. oryzae* strain NSK under x10 magnification. (b) *A. oryzae* strain NSK with methyl blue under x400 magnification (Bar= 10 μ m). (Author's personal collection)

3.2.2 *B. cereus* strain KBC (BC)

3.2.2.1 Isolation and Identification of the BC

B. cereus strain KBC (BC) was obtained from the Omics Lab. These bacteria (*Bacillus* sp.) were successfully identified and isolated from a soy sauce company (Kwong Bee Chun Soy Sauce Sdn. Bhd.) located at Kamunting, Perak, Malaysia (4.905858188640449, 100.70816929574252) (Wan-Mohtar et al., 2020; Yee et al., 2021).

Three sets of *moromi* samples at different ages (10 d, 25 d, and 80 d) were collected and transferred back to Omics Lab, Institute of Biological Sciences, Faculty of Science, Universiti Malaya. These samples were stored in a 500 mL Schott bottles at low temperatures, which are in the ice box when travelling back to Universiti Malaya. For the bacteria isolation, the *moromi* samples undergo streaking and spreading on MRS (de Man, Rogosa, Sharpe, 69964-500G, Sigma-Aldrich, Dorset, UK) agar plates at 30°C for 3 d.

Following the 3 d of incubation, single colonies were screened for growth and a single colony was transferred to another MRS agar plate or subculture to achieve a pure culture. The bacteria present on the plate were subjected to the gram staining technique, as described by Coico (2006), in order to identify and assure the species. The pure culture underwent Scanning Electron Microscope (SEM) analysis to obtain a 1000x magnification image of the bacteria **Figure 3.3**. The identified bacteria were then transferred to new MRS agar slants to create culture stocks, with each slant stored at -4°C for future use. For long-term preservation, the identified master strain was initially cultured in MRS broth, and 500 µL of the cultural stock was inoculated into a 2 mL glass vial containing 500 µL of 50% v/v glycerol (Chemiz, Selangor, Malaysia). The master strain was subsequently stored at -80°C for future applications.

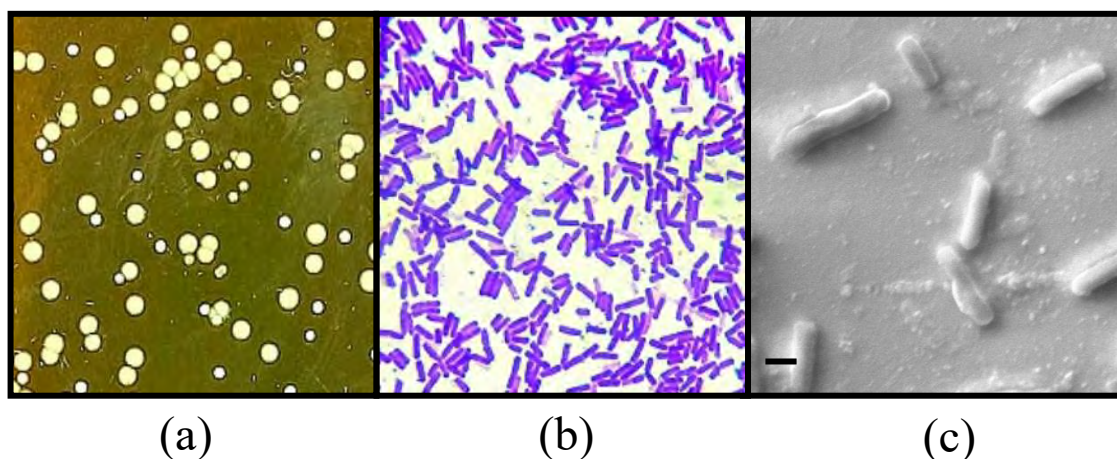


Figure 3.3: (a) Bacteria colonies form on the MRS agar plates after 3 d of incubation at 30°C. (b) Gram-stained *B. cereus* strain KBC. (c) *B. cereus* strain KBC under 1000x magnification by Scanning Electron Microscope (Bar= 2 μm). (Author's personal collection)

3.2.2.3 Suspension Mother Broth of the BC

In order to prepare the mother broth of *B. cereus*, the bacterial culture from the slant was initially cultivated on the MRS plate via the four-streak technique. The plate was incubated at an ambient temperature of 30°C for 3 d to facilitate bacterial growth and proliferation. Upon a three-day incubation period, the bacteria on the plate were subjected to the gram staining technique, as described by Coico (2006), to identify and assure the species. After obtaining a pure culture of bacteria, a loopful bacteria sample was inoculated into a 150 mL volume of MRS broth and then incubated for another 3 d at 30°C until the broth turned into cloudy. A volume of 4 μL of the maternal sample was transferred to a fresh MRS agar plate to let the bacteria grow and reproduce. At the same time, the leftover portion was preserved at a temperature of -4°C for further utilization. After a three-day incubation period, a staining process is employed for the bacteria on the MRS agar plate to ascertain the species of the maternal broth.

3.2.2.3 Serial Dilution of the BC

Serial dilution was employed to achieve an optimal bacteria concentration for incorporation into the *moromi* fermentation process, involving the enumeration of colony numbers from serial dilutions of the sample (Tomasiewicz et al., 1980). To create a 10-fold dilution, 1 mL of the mother broth was combined with 9 mL of pure MRS broth, resulting in a 10 mL mixture. This dilution process was repeated four times, generating dilutions of 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} of the bacteria broth.

Subsequently, 4 μ L of the bacteria broth from each dilution tube was streaked onto MRS agar plates and incubated for 3 d at room temperature. Following the incubation period, the bacterial colonies on each plate were enumerated. Plates containing 30-300 cell colonies were chosen as the appropriate concentration for addition to the *moromi* (Tomasiewicz et al., 1980).

3.2.3 *T. halophilus* strain KBC (TH)

3.2.3.1 Isolation and Identification of the TH

T. halophilus strain KBC (TH) was obtained from the Omics Lab. *Tetragenococcus* sp. shared its origin with *B. cereus* strain KBC which, was isolated from the same soy sauce *moromi* at the commercial soy sauce factory (Kwong Bee Chun Soy Sauce Sdn. Bhd.) in Kamunting, Perak, Malaysia (4.905858188640449, 100.70816929574252) followed a method outlined by Nga et al. (2017) method with some modification. A loopful of *moromi* sample was introduced into a 50 mL media broth containing MRS (de Man, Rogosa, Sharpe, 69964-500G, Sigma-Aldrich, Dorset, UK), 0.5% (w/v) CaCO_3 (Bendosen Laboratory, Bendosen, Norway), and 5% (w/v) NaCl (food grade). This mixture was incubated at room temperature 30°C for 3 d at 100 rpm under anaerobic conditions within an anaerobic jar equipped with *Anaerocult A* (Merck, Kenilworth, USA) (Liu et al., 2012).

After 3 d of incubation, 4 μL of the sample was streaked on an MRS agar plate containing 0.5% (w/v) CaCO_3 and 5% (w/v) NaCl. The plate was then incubated for an additional 3 d to facilitate the development of bacterial colonies. A single colony from the MRS agar plate was transferred to a new 50 mL MRS broth medium, and this process was repeated three times to achieve a pure isolate culture on the observed MRS agar plate. The bacteria on the plate were gram-stained to identify and confirm the species. The pure culture was subsequently sent for SEM analysis to obtain a 1000x magnification image of the bacteria **Figure 3.4**. The identified bacteria were transferred to new MRS agar slants supplemented with 0.5% (w/v) CaCO_3 and 5% NaCl to create culture stocks. Each slant was stored at -4°C for future use. For long-term preservation, the identified master strain was initially cultured in MRS broth, and 500 μL of the cultural stock was inoculated into a 2 mL glass vial containing 500 μL of 50% v/v glycerol. The master strain was subsequently stored at -80°C for future applications.

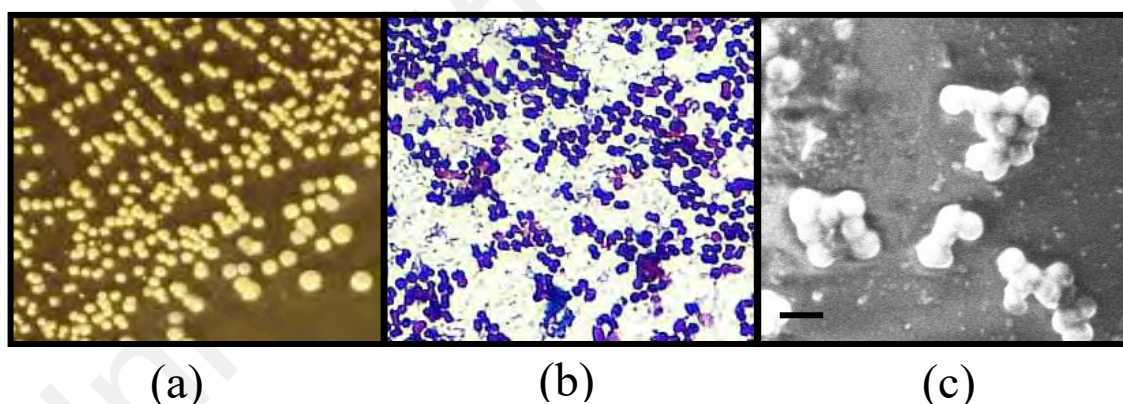


Figure 3.4: (a) Bacteria colonies form on the MRS agar plates after 3 d of incubation at 30°C . (b) Gram-stained *T. halophilus* strain KBC. (c) *T. halophilus* strain KBC under 1000x magnification by Scanning Electron Microscope (Bar= 2 μm). (Author's personal collection)

3.2.3.2 Suspension Mother Broth of TH

To prepare the mother broth for *T. halophilus*, the bacteria from the slant were initially cultured on an MRS agar plate, which was supplemented with 0.5% CaCO₃ and 5% NaCl using the four-streak method. The plate underwent anaerobic incubation inside an anaerobic jar equipped with *Anaerocult A* at room temperature 30°C for 3 d to facilitate bacterial growth. Following the incubation period, a gram stain was performed on the bacteria on the plate to confirm their species.

Once the bacterial species was identified, a loopful of the bacteria was transferred to a 150 mL MRS broth supplemented with 0.5% (w/v) CaCO₃ and 5% (w/v) NaCl. The mixture was incubated under anaerobic conditions at room temperature 30°C for an additional 3 d until a cloudy solution was observed. Subsequently, 4 µL of the mother broth was transferred to a new MRS agar plate supplemented with 0.5% (w/v) CaCO₃ and 5% (w/v) NaCl to stimulate growth while the remaining portion was stored at -4°C for future use. After 3 d incubation period, a gram stain was performed on the bacteria on the MRS agar plate to confirm the species of the mother broth.

3.2.3.3 Serial Dilution of TH

The approach for serial dilution in TH followed the same steps as that for BC. Specifically, 1 mL of the mother broth was mixed with 9 mL of pure MRS broth supplemented with 0.5% CaCO₃ and 5% NaCl, resulting in a 10-fold dilution with a total volume of 10 mL. This dilution process was repeated four times, producing dilutions of 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ of the bacteria broth.

Subsequently, 4 µL of the bacteria broth from each dilution tube was streaked onto MRS agar plates and incubated under anaerobic conditions in an anaerobic jar supplemented with *Anaerocult A* for 3 d at room temperature. Following the incubation

period, the bacterial colonies on each plate were counted. Plates containing 30-300 cell colonies were chosen as the appropriate concentration for incorporation into the *moromi*.

3.3 *Koji* Fermentation

The primary ingredients for *koji* production are soybeans, wheat, and a starting culture of *A. oryzae* (Luh, 1995). The initial stage in *koji* production involved immersing the beans in water for an extended period, typically overnight (8-12 h), to facilitate their softening and absorption of moisture. Following soaking process, the beans were further subjected to boiling at a temperature range of 100-110°C for 3 h until they reached a soft consistency. Wheat is a carbohydrate source that contribute to the pleasant scent and imparts a sweet taste to soy sauce. The organic wheat grain undergoes a process of mild roasting and cracking to facilitate its opening. According to the Kwong Bee Chun Soy Sauce Sdn. Bhd. company recipe, the steamed soybean is then cooled and combined with roasted cracked wheat grain at a ratio of 1 kg of soybean to 500 g of wheat grain, along with a starting mould (*A. oryzae* sp.) dosage of 5 g in order to make the *koji*. The mixture was uniformly distributed across four conventional steel trays, with each tray containing a layer of fermenting *koji* of around 3-4 cm in thickness. In order to prevent the formation of a thick layer resulting in overheating caused by the heat created during the *koji* fermentation process, it is recommended to create little valleys at regular intervals of 2 inches in the mixture **Figure 3.5 (a)**. The *koji* tray was wholly covered with parchment paper and incubated at temperatures ranging from 27-35°C for one week. Following 7 d of incubation inside a controlled environment characterized by adequate humidity and aeration, specifically within an incubation chamber maintained at a temperature range of 25-35°C, the emergence of a greenish-yellow mash can be observed in **Figure 3.5 (b)**. This occurrence may be attributed to the growth and sporulation of the starting mould.



Figure 3.5: (a) Day one of 3-4 cm thickness *koji* with regular intervals of 2 inches valleys. (b) *Koji* after 7 d of incubation (Bar= 1 cm).

3.4 *Moromi* Fermentation

The process of *moromi* fermentation involves the incorporation of the matured *koji* into the brine solution, where the created mash mixture is known as *moromi*. This brine solution facilitates the proliferation of *lactobacillus*, a bacterial species responsible for breaking down carbohydrates or sugars into lactic acid, acting as a natural preservative. This study, the mature *koji* was added to a 20% brine solution to create *moromi* mash. For the trio, bacteria (*Bacillus* sp. (**Figure 3.6 (a)**) and *Teragenococcus* sp. (**Figure 3.6 (b)**) were added to the *moromi* mash to allow lactic acid fermentation. In contrast, for the control, no bacteria were added to the mash during the lactic acid fermentation. BC and TH were cultured in MRS broth to prepare the bacterial inoculum and incubated for 72 h at 37°C. Then, the culture was adjusted to 10^{-3} cfu/mL before being added to the *moromi* mash and incubated aerobically at 37°C.

At the *moromi* fermentation stage, three different types of analysis were done, as shown in **Table 3.1**. Firstly, to obtain the optimized *moromi* fermentation condition (inoculum percentage, pH and agitation speed) for GABA production, the experiment was designed using Response Surface Methodology (RSM) using a 250 mL Erlenmeyer flask

for 7 d in triplicate. Secondly, to evaluate the GABA enhancement by the trio, both unoptimized and optimized *moromi* fermentation conditions were incubated for 28 d. Samples were collected at 7 d intervals. Lastly, to evaluate consumer acceptance, sensory analysis was done using both unoptimized and optimized *moromi* incubated for 30 d. The obtained soy sauce was then compared with the commercial soy sauce for its sensory attribute.

Table 3.1: The fermentation time for each analysis.

Type of Analysis	Duration of <i>moromi</i> fermentation (d)
Response Surface Methodology (RSM)	7
GABA production under optimized and unoptimized condition	28
Sensory Analysis	30

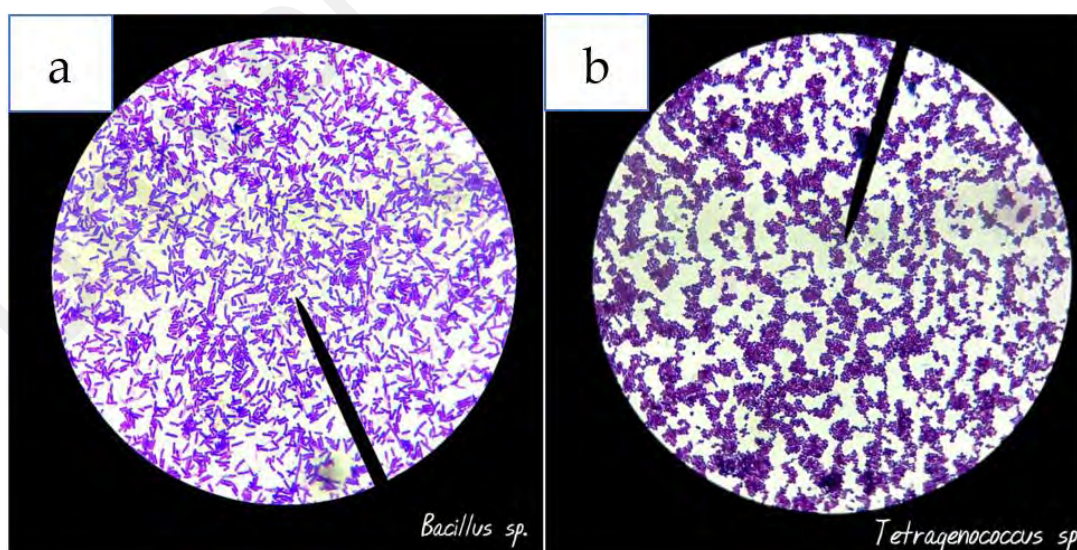


Figure 3.6: (a) *B. cereus* strain KBC (b) *T. halophilus* strain KBC (Author's personal collection)

3.5 Refining and Pasteurise

The last stage of soy sauce fermentation involves the process of refining, which covers pressing, filtration, pasteurization, and bottling **Figure 3.7**. In the refinement process, the *moromi* was wrapped within a nylon sheet and pressed to extract the liquid, resulting in a product called shoyu, or soy sauce. Shoyu has a pronounced flavor profile, with an earthy and slightly sharp taste complemented by a delightful roasted wheat flavor and aroma. The by-product of soy sauce production, called the cake, consists mainly of pure fibre and can be repurposed for animal feed or biodiesel production (Sassi et al., 2021). Following the pressing and extraction, the liquid undergoes pasteurization, maintained at temperatures of 70-80°C for 20 min, before being bottled.

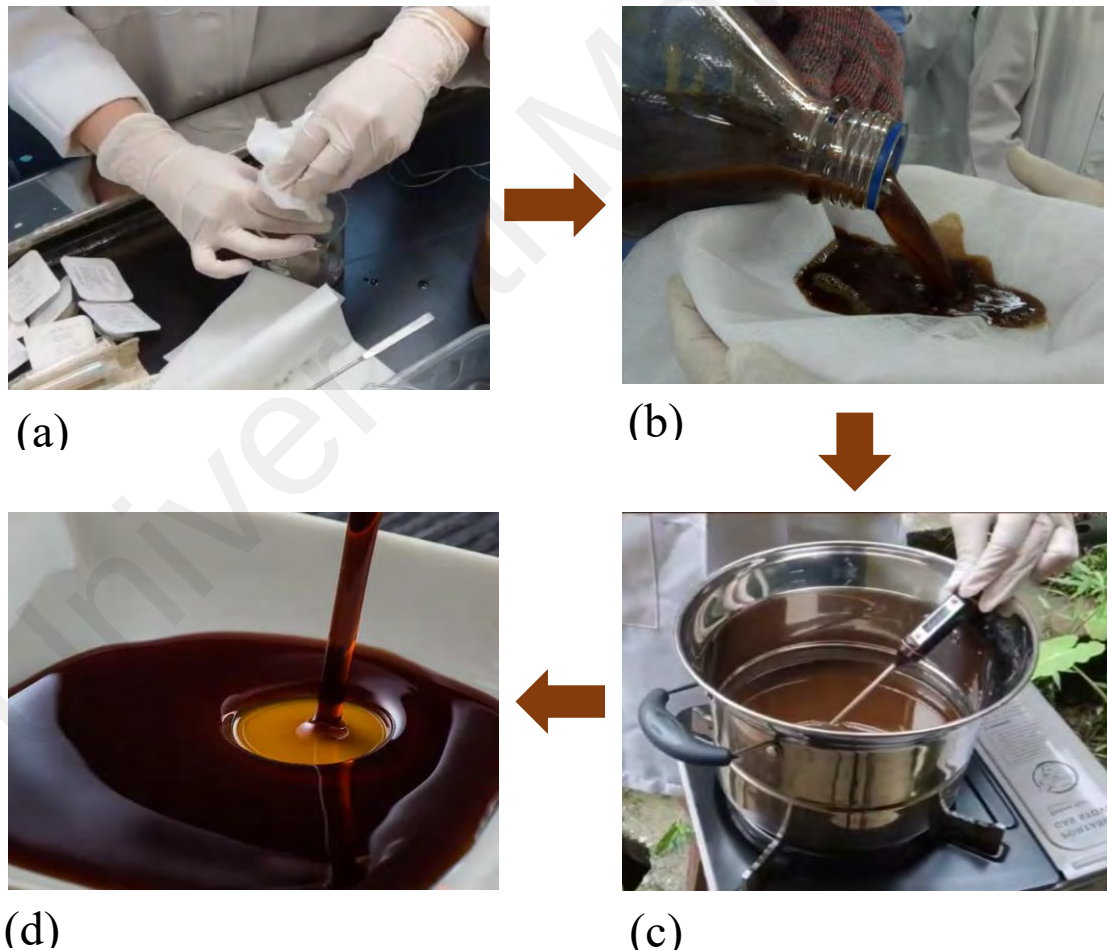


Figure 3.7: (a) Pressing process of the soy sauce *moromi* in a nylon sheet. (b) Filtering process of the raw soy sauce. (c) Pasteurize the raw soy sauce at 90-95°C for 30 min. (d) The pasteurize liquid is now ready to be served.

3.6 GABA Detection and Quantification

The method by Yang et al. (2006) was employed with slight modifications to determine the GABA content of the soy sauce samples. The soy sauce was first filtered through Whatman No.2 filter paper, and the filtrate was then filtered through a 0.22 μm pore-percentage nylon filter (Fisher Scientific Bishop Meadow, UK). A High-Performance Liquid Chromatogram (HPLC) was used to analyze the filtered sample.

For the HPLC analysis, 1 mL of the analyte was placed in a 2 mL HPLC autosampler glass vial. A mobile phase was prepared with 60% solution A (aqueous solution of 8.205g sodium acetate, 0.5mL triethylamine and 0.7 mL acetic acid in 1000 mL) adjusted to pH 5.8, 28% solution B (deionized water) and 12% solution C (acetonitrile). The separation was performed using a Shimadzu LC 20AT apparatus, consisting of a pump system, a CT0-10ASVP model oven with a 20- μL injection loop injector and an SPD-M20A PDA detector in conjunction with a DELL model DELL Optiplex integrator. For separation purposes, a Hypersil Gold C-18 column (250×4.6 mm I.D., particle percentage 5 μm ; Thermo Scientific, Meadow, UK) was used. The mobile phase for gradient elution was pumped at 0.6 mL/min at 25°C, and detection was monitored at 254 nm. The GABA content of the sample was determined by comparing the peak of the graph to the GABA standard curve (Yee et al., 2021). For the construction of the GABA standard curve, four different concentrations (1.0, 0.5, 0.25, and 0.125 mg/L) of pure GABA (A5835-10g, Sigma-Aldrich, St. Louis, MO, USA) were carried out, and the data was plotted to create a standard graph.

3.7 Optimization of *Moromi* Fermentation Condition Using Response Surface Methodology (RSM)

Based on the preliminary studies, inoculum percentage, initial pH and agitation speed affected GABA production in soy sauce. According to Wan-Mohtar et al. (2020), the

selected independent variables can influence the AO, BC, and TH trio for high GABA concentration production. To obtain the optimal GABA production, the independent variables were designed using RSM with a central composite design (CCD) (Design Expert 11.0 software (Version 11, Godward St NE, Suit 6400, Minneapolis, MN, USA). CCD with the α -value set at 2.0 was selected. The range and the levels of the independent variables are given in **Table 3.2**. The lowest minimum values for variables were a culture inoculum percentage of 10%, initial pH 3.0, and 100 rpm, and the maximum values were a culture inoculum percentage of 20%, initial pH 7.0, and 200 rpm. Design Expert 11 software was used to generate a total of 20 runs of experiments based on the CCD design **Table 3.3**. The experimental design runs were prepared in a 250 mL Erlenmeyer flask and fermentation was employed under controlled conditions.

Table 3.2: The selected variables range, and levels input for optimization study.

Variables	Range and Levels		
	-1	0	1
Inoculum Percentage (%)	10	15	20
Initial pH	3	5	7
Agitation (rpm)	100	150	200

Table 3.3: 20 experimental runs of three variables of inoculum percentage (%), Initial pH, and agitation speed (rpm) according to CCD.

Run No.	Variables			Response
	Inoculum	Initial	Agitation (rpm)	GABA (mg/L)
	percentage (%)	pH		Experimental
1	15	5	150	
2	10	7	100	
3	15	5	150	
4	10	5	150	
5	10	3	200	
6	10	3	100	
7	15	7	150	
8	20	3	100	
9	15	5	200	
10	15	5	150	
11	20	3	200	
12	15	3	150	
13	20	7	200	
14	15	5	150	
15	10	7	200	
16	15	5	100	
17	20	5	150	
18	15	5	150	
19	15	5	150	
20	20	7	100	

The experiments for the 20 runs were performed in triplicate using shake flasks (250 mL) under controlled conditions

Experimental results were fitted with a second-order polynomial equation by multiple regression analysis. The quadratic mode for predicting the optimal point was expressed according to Eq. 1, where Y represents the response variable (GABA content), β_i are the regression coefficients, and X_1 , X_2 and X_3 represent the independent variables.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1 X_1^2 + \beta_1 \beta_2 X_1 X_2 + \beta_2 X_2^2 + \beta_1 \beta_2 \beta_3 X_1 X_2 X_3 + \beta_3 X_3^2 \quad (3.1)$$

Design Expert 11.0 software (Version 11, Godward St NE, Suit 6400, Minneapolis, MN, USA) was used for the experimental design and regression analysis of the experimental data. The model evaluated the effects of linear, quadratic and interactive terms of the independent variables on GABA production. The quality of the fitted polynomial model was statistically checked by the magnitude of the coefficient of determination R^2 , and its statistical significance was assessed using the F -test analysis of variance (ANOVA). The coefficients of the response surface were evaluated using the student t -test.

3.8 Sensory Evaluation

Sensory evaluation and acceptability tests were used to define the sensory profiles and the level of pleasantness of the different soy sauce samples. Sensory evaluation involved assessments of taste and overall product acceptance. After fermentation and heat treatment, the soy sauce samples were assessed by 300 untrained consumers (males and females, aged between 18 and 60 years old, students and staff) from Universiti Malaya, Kuala Lumpur, Malaysia. Tests were conducted in triplicate, following a previous method Wan-Mohtar et al. (2020) with some modifications (**Figure 3.8**). The setup for each station is shown in **Figure 3.9**. Each panellist was given three coded samples, 815, 701, and 525, representing the trio, commercial and control, respectively, to be tested with a plain rice ball (**Figure 3.10**). Six common sensory attributes were evaluated: sweet, umami, salty, bitter, sour and astringent, and the overall acceptability of the consumer was evaluated by using a 10-cm line scale with 0 denoting none and 9 denoting extremely strong. First, 10 mL of each soy sauce sample was placed into a plastic sauce plate, and a small rice ball was served to the panellists to taste together with the sauce. After each

evaluation, unsalted crackers and mineral water were served to the consumers as palate cleansers.



Figure 3.8: Panellists from Universiti Malaya.



Figure 3.9: Sensory analysis setup.

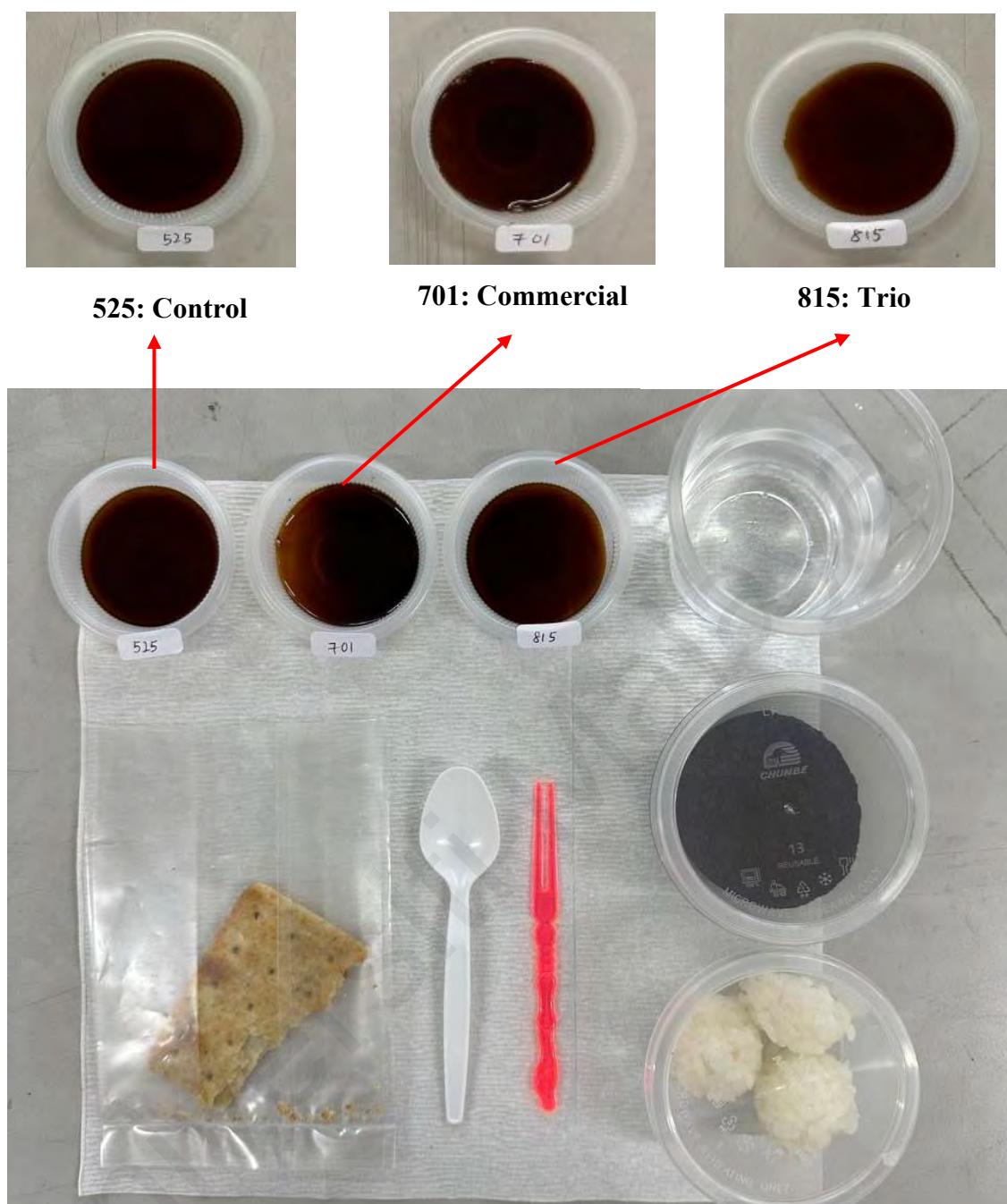


Figure 3.10: Soy sauce samples used for sensory analysis: control (525), trio (815) and commercial (701).

3.9 Statistical Analysis

For the RSM, each experiment was run in triplicate, and the findings are presented as means. Using Design Expert 11.0 software, analysis of variance (ANOVA) was utilized to evaluate significant differences between variables with a *p*-value less than 0.05.

The statistical data from the sensory analysis were analyzed using IBM SPSS Statistics software (version 29; IBM, Chicago, Illinois, USA). Each sample was tested in triplicate. The results are presented as means $\pm$ standard deviation. Turkey's test with a 95% confidence level was performed to determine whether there was a significant difference between samples using one-way analysis of variance (ANOVA) with the Turkey Post Hoc test. The result was considered statistically significant if the *p*-value was less than 0.05.

3.10 Systematic Review

The final part of this study involved a systematic review. The reporting of this review adheres to the guidelines in the Preferred Reporting Items for Systematic Reviews And Meta-Analysis (PRISMA) to systematically review the critical research gaps and propose future directions in sustainability in soy sauce production.

3.10.1 Search Strategy

The databases used to search for peer-reviewed articles included SCOPUS and Web of Science via Universiti Malaya Library (<https://umlibguides.um.edu.my/az/databases>). The search strategy, which excluded grey literature, combined terms describing soy sauce fermentation, emerging technologies, and sustainable food production to determine the past and current soy sauce research. The literature was systematically reviewed following

the guidelines of the PRIMA model for representing the flow of information **Figure 3.11**. The PRISMA methodology was employed because it provides comprehensive documentation of the required data or records. Records included in the study were carefully selected, while those excluded were accompanied by detailed explanations for their exclusion (**Table 3.4**), ensuring transparency and thoroughness in the review process.

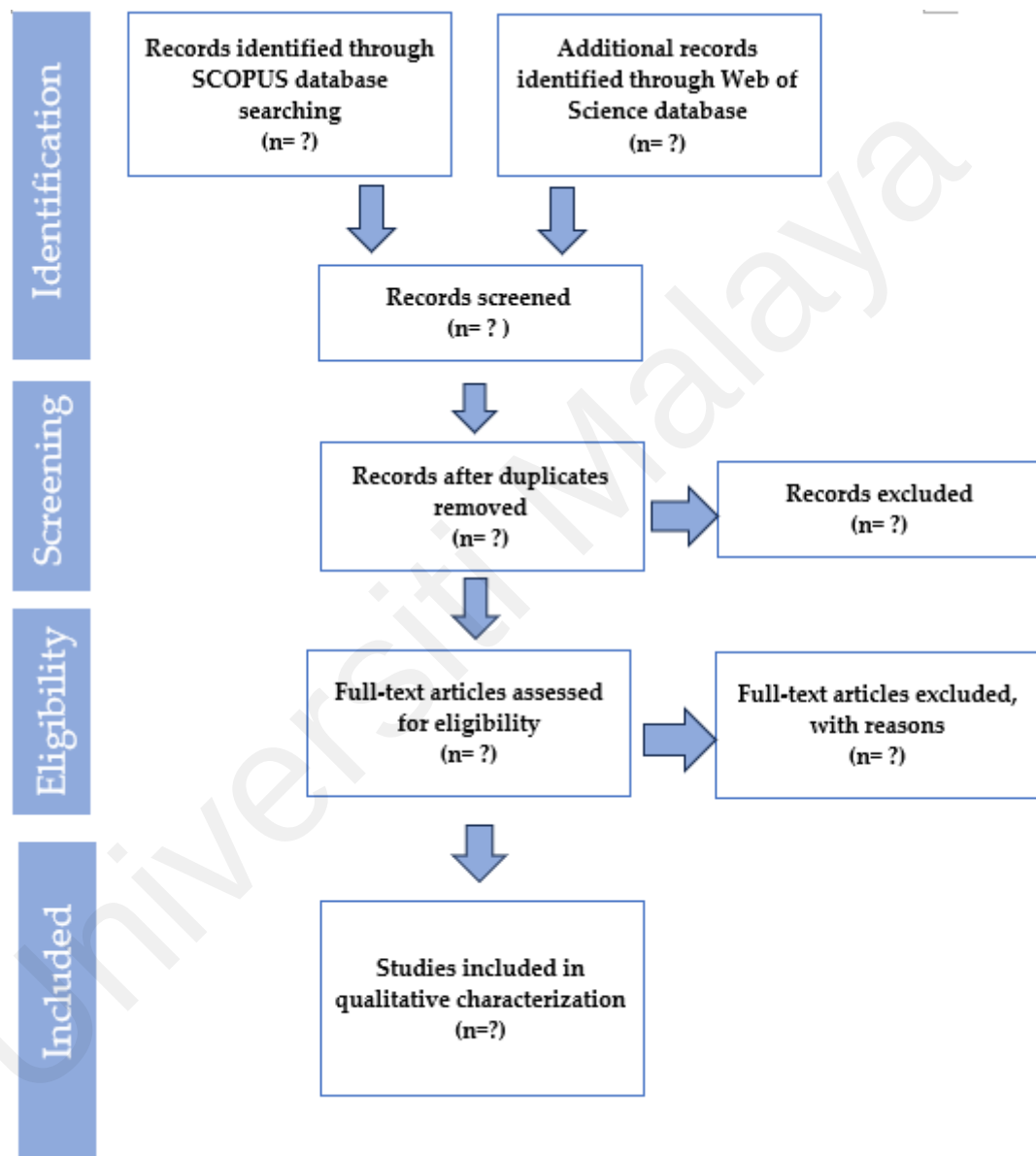


Figure 3.11: The systematic review of literature search follows a general flow that includes identification, screening, eligibility assessment and inclusion based on the PRISMA flow diagram. The flow was reproduced from (Oláh et al., 2020), MDPI, 2020.

All the titles and abstracts were screened in duplicate and excluded studies based on inclusion and exclusion criteria **Table 3.4**. Studies were included to determine if “soy sauce fermentation” or “soy sauce production” was the focus of the research, along with keywords related to soy sauce research such as environment, sustainability, waste management, energy, social responsibility, and technologies. On the other hand, studies were excluded if general or specific fermented foods other than soy sauce (such as miso, fish sauce, and pickles) were documented. Case reports, review articles, short communications (such as editorial and conference abstracts), and articles written in a language other than English were also excluded.

Table 3.4: Eligibility criteria for study selection

Criterion	Inclusion	Exclusion
Product	Soy sauce	Products other than soy sauce (<i>miso</i> , natto, tofu, soymilk, fish sauce and etc)
Intervention or Exposure	Studies that explored both traditional and innovative soy sauce production. Investigations into the impact of specific ingredients, fermentation techniques, or sustainability practices on soy sauce.	Studies unrelated to soy sauce production methods or innovations.
Document Types	Open-access research articles	Non- open access literature (case reports, research article, review articles, and short

		communications such as editorial, book chapter, and conference abstract).
Language	Studies published in English to ensure accessibility.	Studies published in languages other than English.
Publication date	Studies published within the last 12 years to capture recent advancements, unless historical studies provide valuable context	Studies published more than 12 years ago, unless considered historically significant.

SCOPUS and Web of Science database searches were conducted using keywords such as “soy sauce fermentation”, “soy sauce environment”, “soy sauce sustainable”, “soy sauce carbon footprint”, “soy sauce waste management”, “soy sauce water usage”, “energy soy sauce fermentation”, and “packaging soy sauce”. Other terms like “social responsibility soy sauce”, “supply chain soy sauce”, “reduce chemical soy sauce”, “renewable energy soy sauce”, and “technological innovation soy sauce” were also included. By doing so, the comprehensive assessment would bring to light the most recent trends, the knowledge gaps that currently exist, and the opportunities that exist within the creation of sustainable soy sauce.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Optimization of *Moromi* Fermentation Conditions for GABA Production.

RSM is a statistical approach for determining the best combination of input variables to optimize a desired outcome. In this study, bacterial inoculum percentage (10, 15, and 20%), initial pH (3, 5, and 7) and agitation speed (100, 150, and 200 rpm) were chosen as the variables, and the desired outcome or the response was GABA production. **Table 4.1** shows 20 runs of experiments were generated using CCD design in the Design Expert 11.0 software to investigate the production of GABA in a system comprising three microbial strains: *A. oryzae* strain NSK, *B. cereus* strain KBC, and *T. halophilus* strain KBC. The variables manipulated in the experiments were inoculum percentage (%), initial pH, and agitation speed (rpm). The response measured was the concentration of GABA produced (mg/L), with both experimental and predicted values provided for each run.

The inoculum percentage ranged from 10% to 20%. It was observed that a higher inoculum percentage (e.g., 20%) generally resulted in higher GABA production. For example, run 2 with 10% inoculum at pH 7 and 100 rpm yielded 85.32 mg/L of GABA, while run 20 with the 20% inoculum but same at pH 7 and 100 rpm yielded the highest GABA concentration of 128.69 mg/L. This shows that higher inoculum percentage, higher the GABA concentration (Phuengjayaem et al., 2021).

Besides, the pH varied from 3 to 7. Lower pH levels (3 and 5) resulted in higher GABA production. The optimal pH value for achieving the highest GABA synthesis varies depending on the species (Li et al., 2010; Yang et al., 2008). However, in this study, the optimization process using CCD considers the combined effects of all variables (inoculum percentage, initial pH, and agitation speed). The optimal condition is not determined solely by pH but rather by identifying the best combination of all variables (Mohammed et al., 2018). In run 20, the combination of 20% inoculum, pH 7, and 100

rpm was found to be the most effective. This suggests the mixed starter culture may interact differently under varying pH levels. At pH 7, the metabolic activities and interactions among these strains might be more favorable for GABA production. The medium's buffering capacity at pH 7 might better support the stability and activity of the microbes, leading to higher GABA yields.

Furthermore, agitation speeds were set between 100 rpm and 200 rpm. Moderate agitation speeds (e.g., 100 rpm) facilitated better GABA production than higher speeds. For example, runs at 100 rpm showed higher GABA yields than runs at 200 rpm. The agitation speed plays a crucial role in fermentation processes as it directly affects the oxygen transfer rate, the mixing effectiveness, and the shear stress experienced by the culture medium (Amanullah et al., 2003; Mahdinia et al., 2019). High agitation speeds can elevate shear stress, potentially harming microbial cells and reducing metabolic activity and GABA synthesis (Silva-Santisteban & Maugeri Filho, 2005).

The highest GABA production (128.69 mg/L) was achieved in run 20 with 20% inoculum, pH 7, and 100 rpm. This suggests that a combination of higher inoculum percentage, neutral pH, and lower agitation speed is optimal for maximizing GABA production. The results highlight the importance of inoculum percentage, initial pH, and agitation speed in influencing GABA yield. This study provides a valuable reference for scaling up the production process and further enhancing GABA yields in industrial applications.

Table 4.1: Experimental runs of three variables according to CCD. Experimental and predicted values of GABA production (mg L⁻¹) obtained using the trio consisting of *A. oryzae* strain NSK, *B. cereus* strain KBC and *T. halophilus* strain KBC.

Run No.	Variables			Response	
	Inoculum percentage (%)	initial pH	Agitation (rpm)	GABA (mg/L)	
				Experimental	Predicted
1	15	5	150	93.24 ± 1.22	83.20
2	10	7	100	85.32 ± 0.62	87.17
3	15	5	150	79.32 ± 2.07	83.20
4	10	5	150	83.44 ± 3.44	80.64
5	10	3	200	55.55 ± 4.80	59.47
6	10	3	100	83.68 ± 0.54	80.98
7	15	7	150	81.67 ± 2.61	80.78
8	20	3	100	95.67 ± 5.19	96.21
9	15	5	200	67.42 ± 1.27	62.40
10	15	5	150	82.24 ± 3.58	83.20
11	20	3	200	51.41 ± 5.02	49.82
12	15	3	150	68.56 ± 2.18	68.39
13	20	7	200	65.44 ± 2.21	68.40
14	15	5	150	80.74 ± 6.24	83.20
15	10	7	200	55.69 ± 2.65	55.42
16	15	5	100	97.51 ± 2.10	101.47
17	20	5	150	93.00 ± 0.81	94.75
18	15	5	150	82.09 ± 1.64	83.20
19	15	5	150	79.49 ± 2.49	83.20
20	20	7	100	128.69 ± 2.33	125.03

The experiments for the 20 runs were performed in triplicate using shake flasks (250 mL) under controlled conditions

The ANOVA for GABA production using the trio of AO, BC, and TH is presented in **Table 4.2**, where a non-linear regression was used to evaluate the coefficient. ANOVA was performed to measure the model coefficient's significance. $p < 0.05$ served as an indication that each coefficient was significant. The regression coefficient for this model is $R^2 = 0.9586$, which explains 95.86% of the model response's variability. R^2 is a measure used to indicate how well a regression model fits the data, with a higher value indicating a better fit (Menard, 2000).

Table 4.2: Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for GABA production using the trio.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5594.86	9	621.65	25.74	< 0.0001*	Significant
A- Inoculum Percentage	497.45	1	497.45	20.59	0.0011*	Significant
B- initial pH	383.66	1	383.66	15.88	0.0026*	Significant
C- Agitation	3816.55	1	3816.55	158.01	< 0.0001*	Significant
AB	256.17	1	256.17	10.61	0.0086	Significant
AC	309.38	1	309.38	12.81	0.005	Significant
BC	52.48	1	52.48	2.17	0.1713	
A ²	55.43	1	55.43	2.29	0.1608	
B ²	204.12	1	204.12	8.45	0.0156	Significant
C ²	4.4	1	4.4	0.1823	0.6784	
Residual	241.55	10	24.15			
Lack of Fit	104.44	5	20.89	0.7618	0.6137	Not Significant
Pure Error	137.1	5	27.42			
Cor Total	5836.4	19				
Std.Dev= 4.91		R ² = 0.9586		Adeq Precision= 21.6431		
Mean= 80.51		Adjusted R ² = 0.9214				

*Significant value.

For the actual variable GABA concentration, the model is represented by Eq.1 and was regressed using the significant terms.

$$\begin{aligned} \text{GABA} = & 47.6009 - 3.07498 \times \text{Inoculum Percentage} + 19.98939 \times \text{initial pH} \\ & + 0.262322 \times \text{Agitation} + 0.565875 \times \text{Inoculum Percentage} \times \text{initial pH} - \\ & 0.024875 \times \text{Inoculum Percentage} \times \text{Agitation} - 0.025613 \times \text{initial pH} \times \\ & \text{Agitation} + 0.179582 \times \text{Inoculum Percentage}^2 - 2.15386 \times \text{initial pH}^2 - \\ & 0.000506 \times \text{Agitation}^2 \end{aligned} \quad (4.1)$$

In addition, the adjusted coefficient determination value ($\text{Adj.}R^2 = 0.9214$) indicates that the model is significant and that the adjusted R^2 value is within a reasonable range of agreement with the predicted R^2 value. This adjusted R^2 ensures that the additional variables contribute meaningfully to the model's explanatory power, making it a valuable tool for model evaluation, especially in cases involving multiple independent variables (McIntosh et al., 2016). Besides, the p -value < 0.0001 indicates that the model is significant. This model has a non-significant Lack of Fit with a p -value of 0.6137 ($p > 0.05$). With all these values, this model's overall quality and reliability in predicting the GABA production yield is highly acceptable.

According to **Table 4.2**, the effects of the Inoculum Percentage (A), initial pH (B), agitation (C) and quadratic terms (AB, AC, B^2) on the production of GABA are significant ($p < 0.05$).

The effects of the Inoculum Percentage (A) on the production of GABA are significant ($p < 0.05$). This is because the bacteria inoculum percentage has a crucial impact on the production of GABA. This parameter is critical in determining the initial concentration of GABA-producing bacteria, which is added to the soy sauce *moromi* mash. Increasing the inoculum percentage could potentially decrease the lag phase,

accelerating GABA production. However, an extremely high bacteria inoculum percentage leads to intensified competition among bacteria for essential nutrients and can potentially to reduce or halt GABA (Gonzalez & Aranda, 2023). Also, a higher inoculum size ($\sim 10^5$ cfu/mL) could trigger bacterial population growth, resulting in nutrient depletion and the production of toxic metabolites (Pramanda et al., 2023). Consequently, the bacterial population could enter the death phase earlier, resulting in lower GABA production. Hence, finding an appropriate equilibrium by optimizing the bacteria inoculum percentage is necessary to achieve optimal GABA yields and cost-effective production.

Furthermore, the effects of pH regulation on the production of GABA from **Table 4.2** are also significant ($p < 0.05$). This is because pH is one of the critical factors influencing GABA production since it influences the enzymatic activity, specifically the Glutamate Decarboxylase (GAD) enzyme (Komatsuzaki et al., 2005; Tsai et al., 2006; Yang et al., 2008) that plays a pivotal role in the α -decarboxylation reaction of the L-glutamate. This irreversible enzymatic reaction leads to a significant transformation, forming the critical neurotransmitter GABA (Xie et al., 2023). In this reaction, GAD acts as a catalyst, facilitating removing a carboxyl group (COOH) from the L-glutamate molecule. Removing the carboxyl group is a key step in the conversion process, and because of this α -decarboxylation, the L-glutamate molecule is structurally altered, resulting in GABA (Cai et al., 2012). GAD enzyme works well only in a specific pH range (Modi et al., 2015; Wei & Wu, 2008), and consequently, it is crucial to maintain pH levels within this ideal range to facilitate efficient production of GABA. The pH level also considerably impacts on the development and metabolic activities of GABA-producing strains. Variations from the ideal pH level can affect bacterial multiplication, hence GABA production (Li et al., 2010; Yang et al., 2008).

Temperature, pH and initial glutamate concentration have been reported to significantly affect the cell growth of *Lb. brevis* NCL912 and the GABA production (Li et al., 2023). The ideal pH for high GABA production by *B. cereus* strain KBC is pH 7.0 (Wan-Mohtar et al., 2020). The impact of the pH factor on GABA synthesis is strain-specific, and the optimum pH for achieving maximum synthesis of GABA depends upon the species and varies greatly (Milon et al., 2024).

Table 4.2 also shows that the effects of the agitation (C) on the production of GABA were significant ($p < 0.05$). Agitation is a crucial catalyst for promoting optimal production in the fermentation process. The mechanically mixing or swirling the culture media facilitates the uniform dispersion of nutrients, oxygen, and vital components, promoting microbial proliferation and metabolic activities (Mustafa et al., 2019). Nevertheless, it is important to strike a balance, as excessive agitation may damage the bacterial cells, thereby disrupting the fermentation process. These factors are fundamental in attaining optimal GABA yields and guaranteeing the overall effectiveness of the fermentation procedure (Jeon et al., 2018; Suwanmanon & Hsieh, 2014; Yang et al., 2015).

The three-dimensional (3D) graphs illustrate that various quadratic factor combinations had a positive effect on GABA yield (**Figure 4.1**). The effects of initial pH (B) and inoculum percentage (A); agitation (C) and inoculum percentage(A); and initial pH (B), and agitation (C) on the production of GABA are shown in **Figure 4.1 (a), 4.1 (b), and 4.1 (c)**, respectively. The results showed that these factors are established at particular quantities or circumstances, facilitating optimization to maximize GABA production, which is valuable information for process optimization and achieving higher GABA yield production. In short, at 20% bacteria inoculum percentage, initial pH 7, and 100 rpm, the maximal GABA concentration of 128.69 mg/L can be obtained in 7 d.

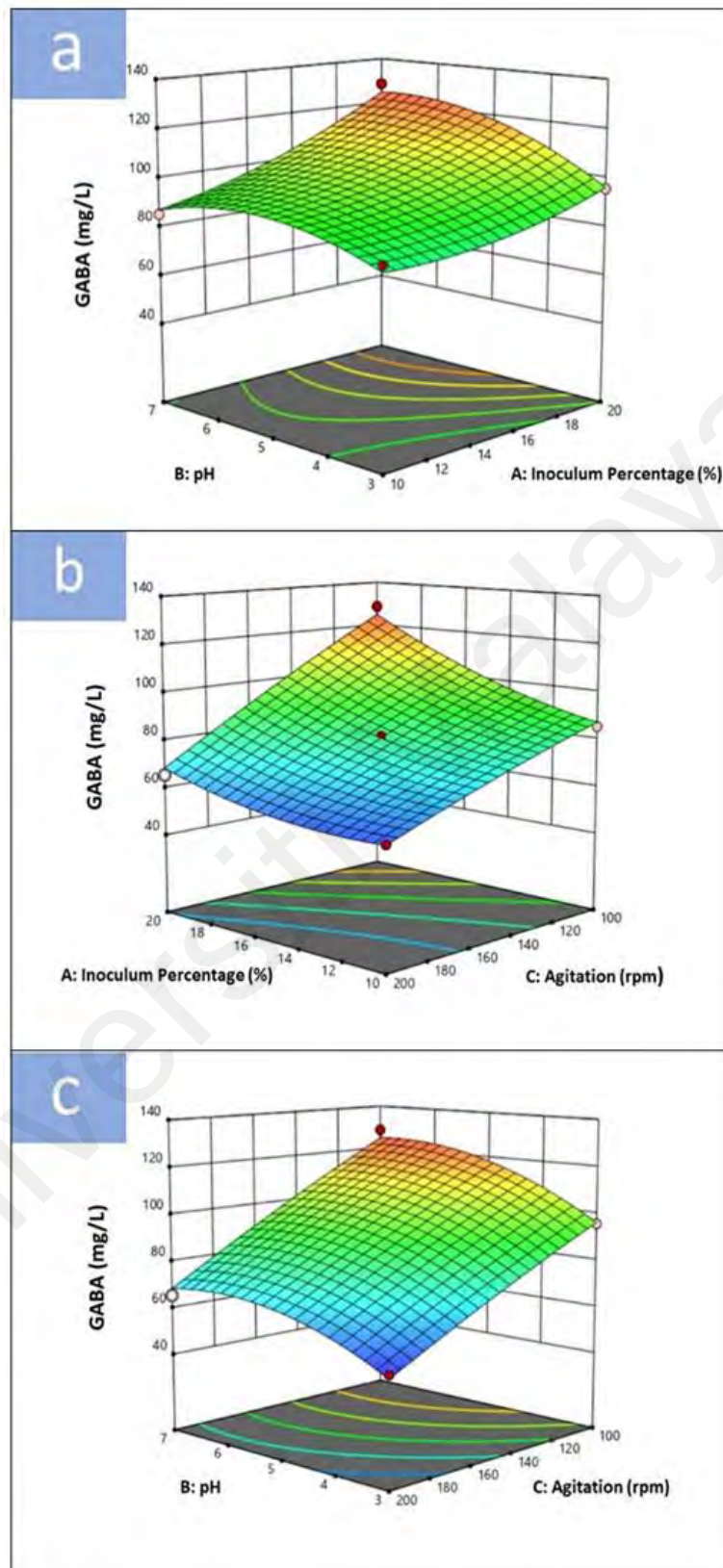


Figure 4.1: Response surface curve (3D plot) of GABA production from the trio of AO, BC and TH displaying the interaction between (a) initial pH and inoculum percentage, (b) inoculum percentage and agitation speed and (c) initial pH and agitation speed.

4.1.1 Verification of Optimized Conditions

The effectiveness of this model was confirmed by comparing and assessing the predicted GABA yield under statistically optimal conditions (**Table 4.3**). A validation test was executed in triplicate using 250 mL shake flasks under the optimal conditions of 20% bacterial inoculum, initial pH 7, and 100 rpm. The model's predictive capability was validated using Eq. 2. The experimental value for GABA production was 128.69 mg/L, whereas the expected value was 125.03 mg/L. The predicted value was 3.66 mg/L lower than the experimental value, and the difference between the values was only 1.03%. Given that the average deviation is below 15%, it can be concluded that this model is valid (Milkey et al., 2014).

Table 4.3: Validation of model using verified condition on GABA response.

Dependent Variable	Response	
	Predicted	Experimental
	GABA (mg/L)	GABA (mg/L)
GABA	125.03	128.69 ± 8.605

4.2 GABA Production by The Trio of AO, BC, and TH

The GABA productions from the mixed culture under optimised conditions (i.e., 20% bacteria inoculum percentage, initial pH 7, and 100 rpm) and unoptimized conditions without any treatment and inoculation (control) were monitored via HPLC analysis at 7-d intervals for 28 d. The GABA concentrations produced over time for both the mixed culture and control are illustrated in **Figure 4.2**.

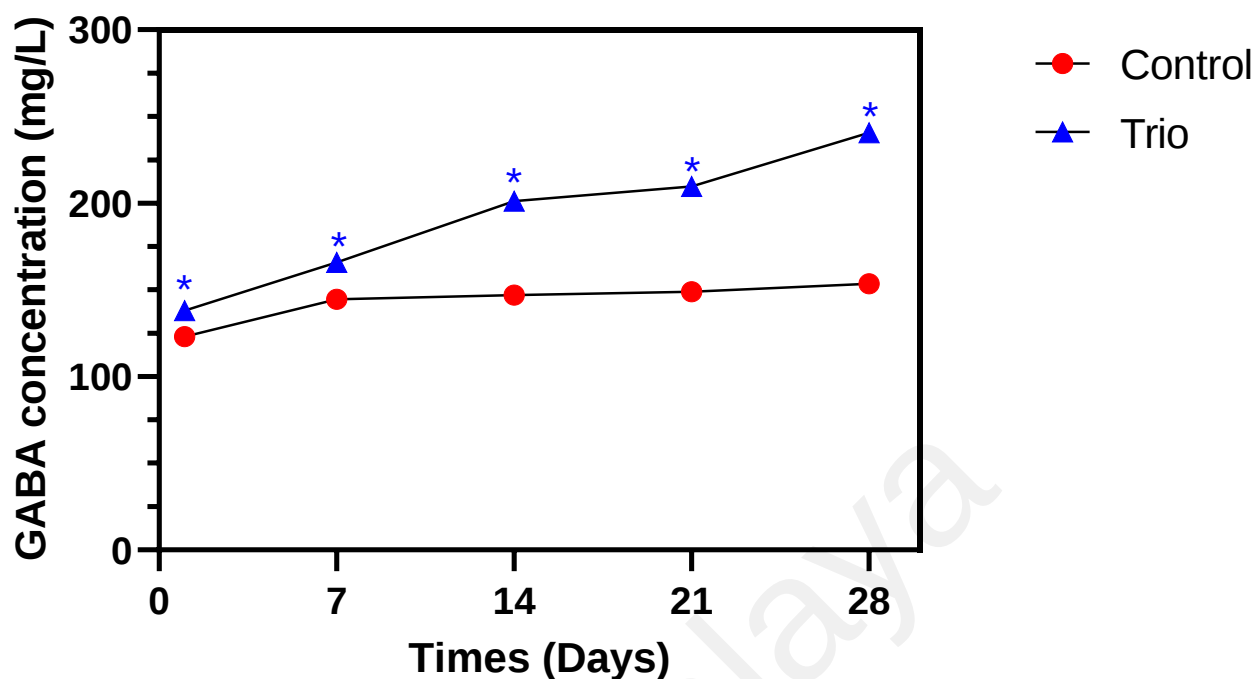


Figure 4.2: The comparison of GABA production yield by the trio (under optimized condition: 20% bacteria inoculum percentage, initial pH 7, 100 rpm) and control during 28 d of fermentation.

Overall, the mixed culture exhibited higher GABA production than the control throughout the monitoring period. This indicates that these additional microbes have a positive and ongoing effect on GABA production, causing it to rise steadily. This is consistent with recent studies which found that the inclusion of microbes does help in boosting GABA production in soy sauce (Ab Kadir et al., 2016; Wan-Mohtar et al., 2020; Yee et al., 2021). On the other hand, the control, which did not receive additional microbes, initially experienced a slight increase but quickly levelled off. As time passed, the values for this sample remained relatively stable with only minor fluctuations. The observed phenomena may not be attributed to the raised levels of *gad* gene expression in response to stress circumstances such as acidic environments and starvation (Arnold et al., 2001; Wei & Wu, 2008).

LAB has extensive applications in various fermented food products, such as *tempeh*, *kimchi*, yoghurt, sourdough and soy sauce, with functional and probiotic properties (Coda et al., 2010; Reale et al., 2019; Thwe et al., 2011). Reports have found that the LAB obtained from fermented food could generate GABA by utilizing glutamic acid as a substrate (Sahab et al., 2020a; Sahab et al., 2020b). Recent studies by Wan-Mohtar et al. (2020) and Yee et al. (2021) suggested that *B. cereus* strain KBC and *T. halophilus* strain KBC are potential GABA-producing strains that can be used in GABA-enriched soy sauce production, validating that the inclusion of supplementary bacteria (*B. cereus* strain KBC and *T. halophilus* strain KBC) can effectively amplify GABA production during the soy sauce fermentation process.

4.3 Sensory Analysis

Sensory analysis is a systematic and scientific method used to analyze and study the sensory attributes of products or materials, specifically in the food and beverage industry and could be an important tool for developing novel food (Elortondo et al., 2007; Reale et al., 2020). Six attributes were selected in the present study to examine soy sauce's sensory profile: sweetness, umami flavor, saltiness, bitterness, sourness, and astringency. **Figure 4.3** presents the average scores for the six attributes across all soy sauce samples. The sensory study included three formulations: control, trio (mixed cultures), and commercial soy sauce (**Table 4.4**). The results showed significant differences among the soy sauce samples for the six attributes evaluated ($p < 0.05$).

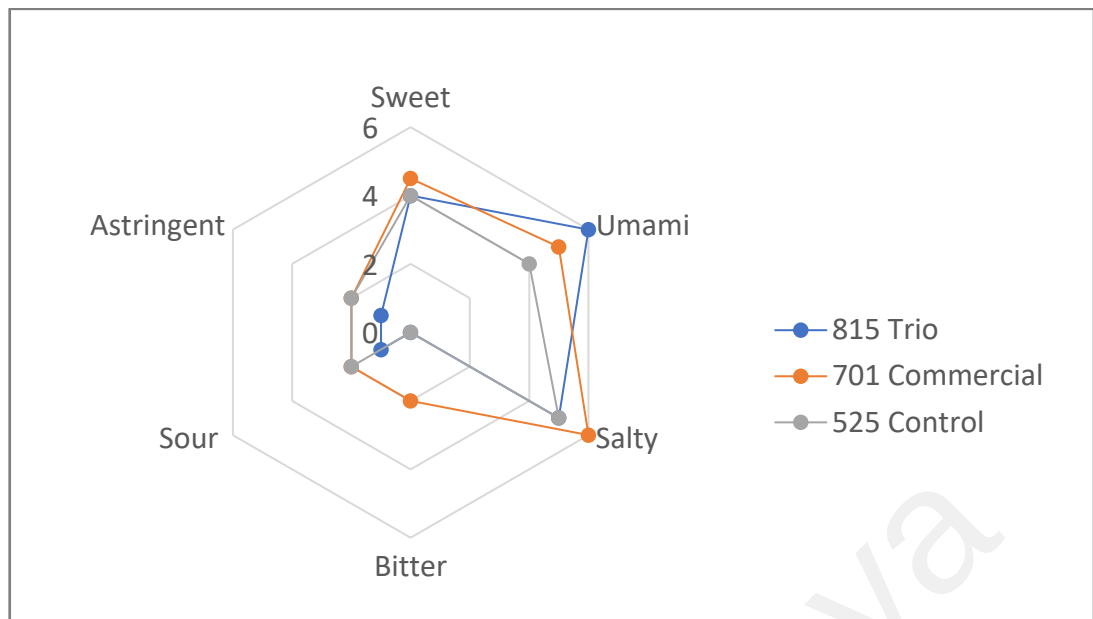


Figure 4.3: The spider diagram illustrates a descriptive sensory analysis of six taste (sweet, umami, salty, bitter, sour, and astringent) on three different soy sauce samples.

Table 4.4: 10-cm line scale for the six tastes (sweet, umami, salty, bitter, sour and astringent) and overall acceptability of soy sauce samples (0= none, 1= extremely weak, 2 = very weak, 3 = weak, 4 = moderately weak, 5= moderately, 6 = moderately strong, 7= strong, 8 = very strong and 9 = extremely strong).

Sample	Preference scores for taste (Mean $\pm$ SD)						
	Sweet	Umami	Salty	Bitter	Sour	Astringent	Overall acceptability
Control 525	3.93 $\pm$ 0.129 ^a	4.42 $\pm$ 2.338 ^c	4.85 $\pm$ 0.118 ^c	1.29 $\pm$ 0.108 ^g	2.32 $\pm$ 0.124 ⁱ	2.31 $\pm$ 0.131 ^k	5.9 $\pm$ 1.51 ^m
Trio 815	4.15 $\pm$ 0.134 ^{ab}	5.59 $\pm$ 0.127 ^{d*}	4.91 $\pm$ 0.125 ^c	1.19 $\pm$ 0.110 ^g	2.01 $\pm$ 0.126 ⁱ	1.98 $\pm$ 0.140 ^k	7.46 $\pm$ 2.21 ^{n*}
Commercial 701	4.52 $\pm$ 0.155 ^{b*}	4.56 $\pm$ 0.142 ^c	5.98 $\pm$ 0.125 ^{f*}	2.29 $\pm$ 0.123 ^{h*}	2.92 $\pm$ 0.156 ^{j*}	3.04 $\pm$ 0.162 ^{l*}	4.47 $\pm$ 1.83 ^o

Note: Values shows are the means $\pm$ standard error (triplicate). The mean values with different superscript alphabets within the same column indicate significant difference ($p < 0.05$). The symbol asterisk (*) shows the highest mean value. Control is the soy sauce sample without any additional bacteria throughout the process, trio is the soy sauce sample obtained using trio, while commercial is the commercial soy sauce sample.

A significant difference in sweetness was observed between the control and commercial samples ($p < 0.05$). The commercial variant had the highest mean score of 4.52, followed by the trio at 4.15 and the control at 3.93 (**Table 4.4**). Notably, no significant difference was found between the trio-commercial and trio-control comparisons, indicating the compatibility of the trio with the commercial soy sauce in terms of sweetness. The perception of sweetness in soy sauce can also be influenced by the interaction of sweet compounds with other taste components (Wilkie & Phillips, 2014). The presence of salt can enhance the perception of sweetness (Keast & Breslin, 2002). The commercial sample has a high salty score (5.98) compared to the trio (4.91), which could contribute to its perceived sweetness.

Regarding umami, the trio showed the highest mean score of 5.59, followed by the commercial at 4.56 and the control at 4.42 (**Table 4.4**). Significant differences emerged between the trio and commercial and control samples ($p < 0.05$). As one of the key flavor profiles, umami is significantly important for soy sauce flavor. The term ‘umami’, originating from the Japanese language, denotes a sensation of deliciousness (Lioe et al., 2010). Thus, the higher the umami tastes, the better the soy sauce will taste.

Furthermore, the commercial sample had the highest mean scores for salty (5.98), bitter (2.29), sour (2.92) and astringent (3.04) attributes. However, no significant difference was found between the trio and control. Intriguingly, a significant difference was observed between the commercial-trio and commercial-control comparisons ($p < 0.05$) (**Table 4.4**). Individual soy sauce possesses distinct qualities. The observed variability can be attributed to the complicated production process or the fermentation time (Diez-Simon et al., 2020a). Additionally, the commercial soy sauce was fermented for three months instead of one month for the trio and control in this study. Thus, this might be why the commercial soy sauce had stronger saltiness, bitterness, sourness and astringency attributes than the trio and control (Zhu et al., 2019).

The trio also showed higher overall acceptability (7.46) compared to the control (5.90) and commercial soy sauces (4.47) (Figure 6). This may be due to the high amount of umami flavor in the trio. Moreover, the trio had a sweetness (4.15) comparable with commercial soy sauce (4.52). In addition, the trio had lower saltiness, bitterness, sourness and astringency attributes than the commercial soy sauce, which was more palatable for consumers.

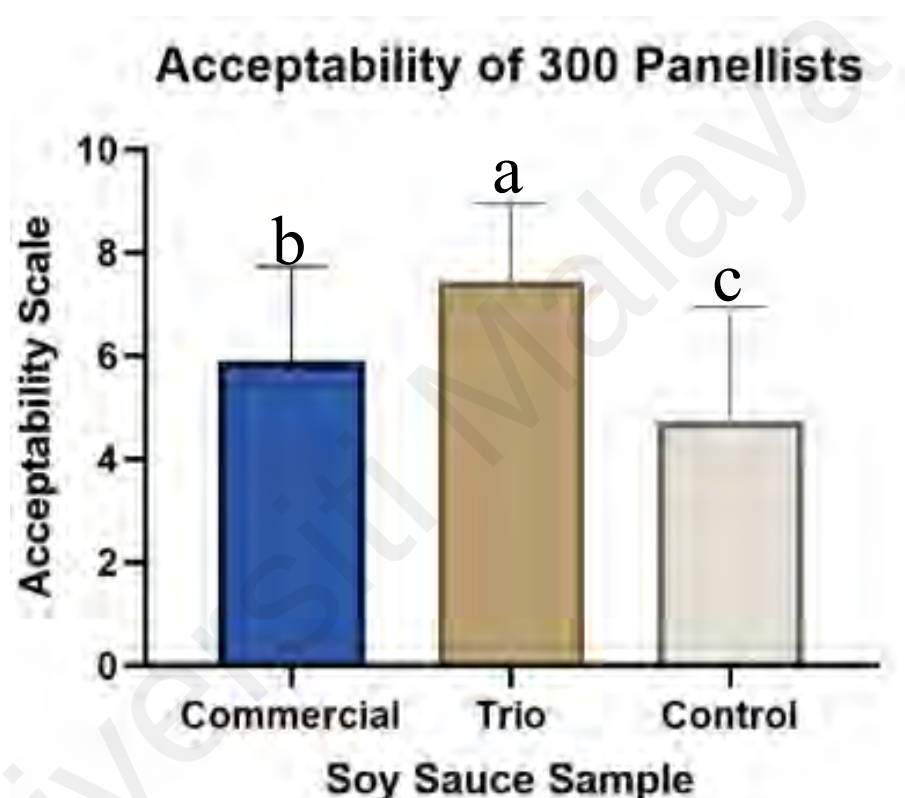


Figure 4.4: Mean scores for overall acceptability for all the soy sauce samples for 300 panellists. Notes: Different alphabets indicate significant difference ($P < 0.01$)

The global market offers a wide array of contrasting soy sauce products due to distinct production methods, such as microbial or chemical synthesis, various microbe cultures introduced during fermentation, and ageing time. It is well known that each soy sauce variety has a distinctive flavor profile (Chong et al., 2023). Soy sauce contains various non-volatile chemicals, mostly free amino acids, 5'-nucleotides, short peptides,

soluble saccharides and polyols. Non-volatile compounds play a significant role as flavor molecules, particularly associated with fundamental tastes of sweetness, saltiness, sourness, bitterness and umami (Kong et al., 2018b).

Based on the overall results, the panellists accepted the mixed culture soy sauce at a higher level as compared to the commercial soy sauce, as it provided higher a umami taste score of 5.59 in the sensory test than commercial soy sauce, which had a score of 4.56 (**Table 4.4**). The trio also had less salt, bitterness, and sourness than the commercial soy sauce. These results indicate that using this mixed starter culture can improve the sensory qualities of soy sauce, rendering it a promising alternative to conventional and commercial production methods. In order to optimize sensory attributes, future research should investigate consumer preferences in a broader range of groups and further refine the trio process.

4.4 Systematic Review

4.4.1 Initial Identification and Search Strategy

The initial literature search was conducted using the SCOPUS and Clarivate Web of Science search engines through the Universiti Malaya Library. The abstracts of the retrieved articles were carefully screened, and those that did not address soy sauce production and soy sauce fermentation were excluded. The abstracts that fulfilled the study's purpose were thoroughly reviewed, and those that lacked relevant information were removed. In addition, articles explicitly produced for conferences, conference proceedings, short communications (such as editorial and conference abstracts), case reports, review articles, and articles written in a language other than English were also removed from consideration. This careful procedure followed the PRISMA guidelines to ensure a comprehensive and systematic approach.

This search across various open-access journals, publishers, and databases initially yielded 1,034 papers, with 583 sourced from the SCOPUS database and 496 from the Clarivate Web of Science database. After removing the 179 duplicate records identified in both databases, 855 unique articles remained. These remaining articles were then subjected to a comprehensive full-text analysis for further evaluation (**Figure 4.5**).

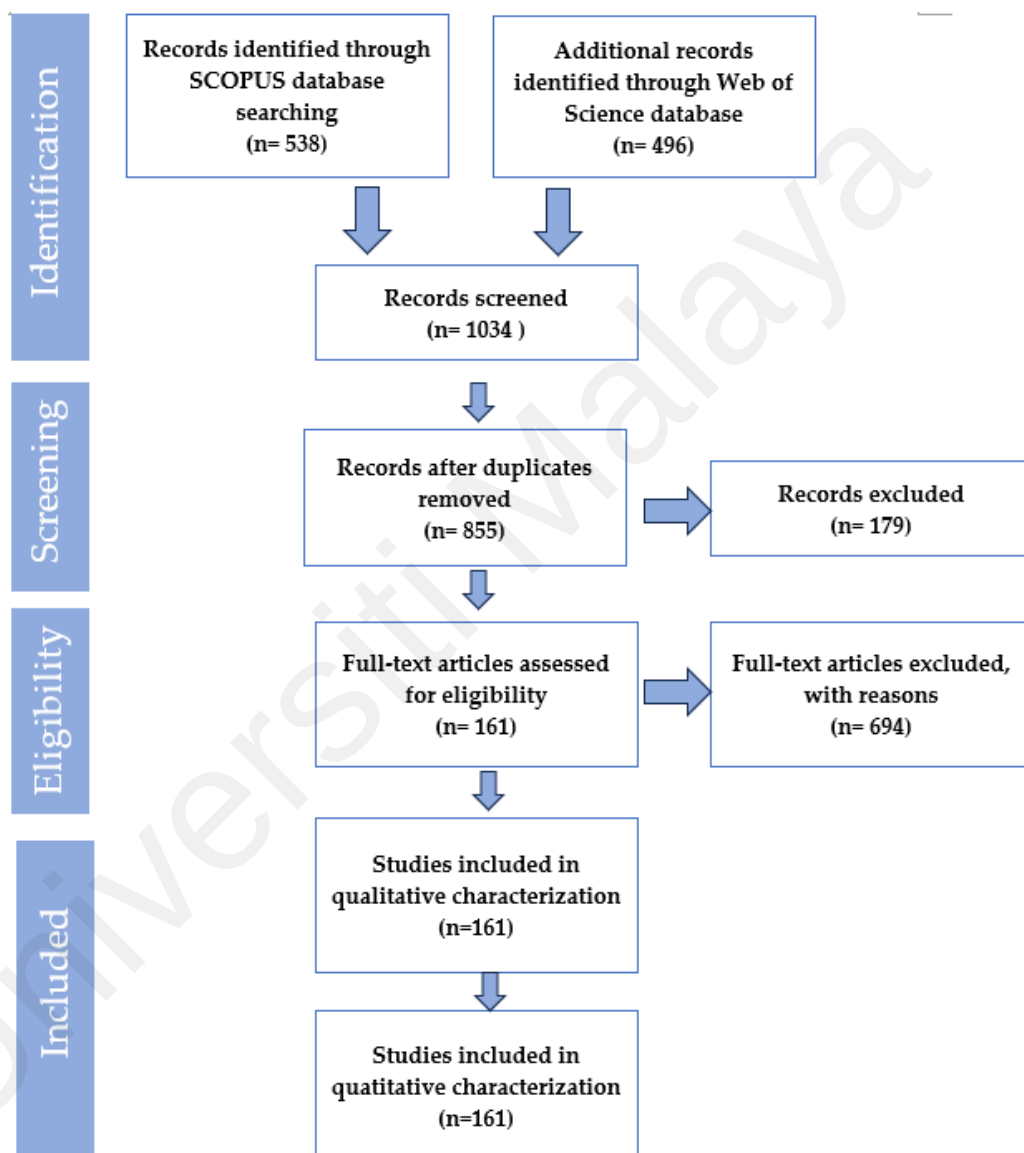


Figure 4.5: Number of articles (n) at each level of PRISMA analysis

The first publication on soy sauce research that can be retrieved from Clarivate Web of Science was published in 2003 by (Nyman et al., 2003). Hence, the data retrieval

timeframe was 12 years (2003 to 2024), with a noticeable increase in research conducted following 2023. This could be attributed to researchers' interest in microbial fermentation, waste management, safety, and the sustainable soy sauce production process; each article included mainly highlighted sustainable soy sauce production, soy sauce fermentation, emerging technologies, and technological advancements in soy sauce production.

4.4.2 Eligibility Assessment

The introduction, result analysis, and discussion sections of the selected articles were carefully reviewed to determine how they correlated with the study's objectives which included identifying the gaps in sustainable soy sauce production, exploring future directions for improving the production process, and investigating the possible utilisation of innovative fermentation techniques and tools. The process of selecting appropriate articles was guided by a set of criteria specified in **Table 3.4**. Of the articles initially reviewed, 160 were considered suitable for further examination in the qualitative and quantitative analysis stages. This decision was based on their relevance to the study objectives and compliance with the set criteria. 695 publications were discarded because they were irrelevant and did not fit earlier criteria.

4.4.3 Quantitative and Qualitative Analyses

Recent papers were categorized into key areas to identify current trends in this field: microbial fermentation, new detection methods, waste management, safety and soy sauce production process (**Figure 4.6**). The pie chart illustrates the distribution of research subjects in the field of sustainable soy sauce production and fermentation.

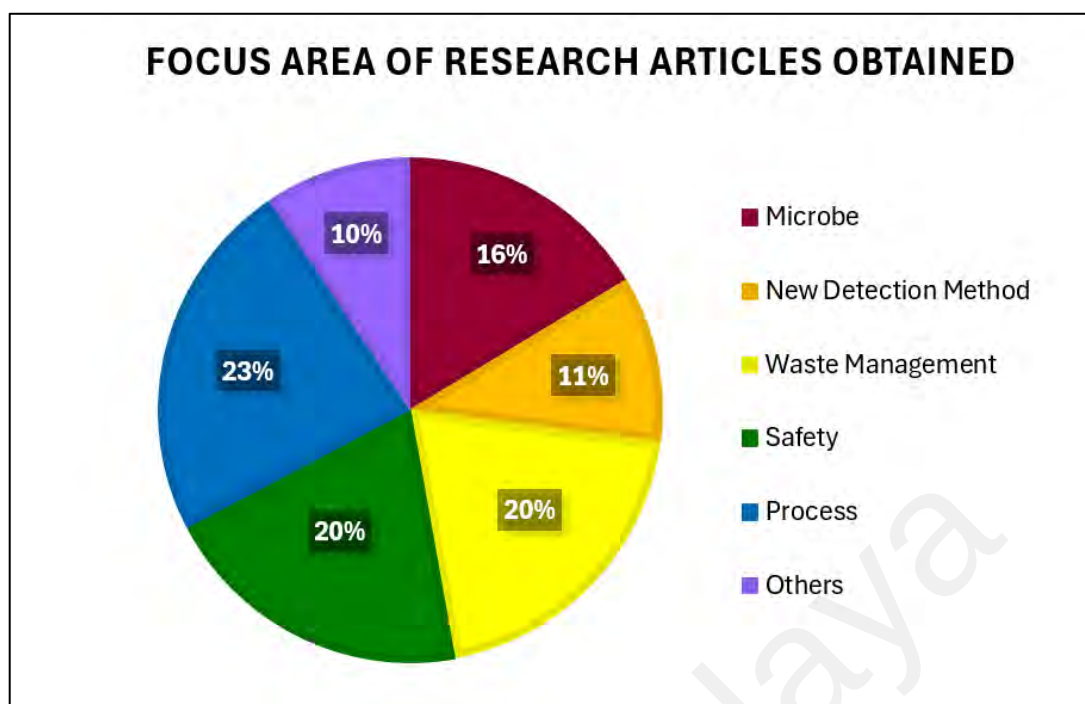


Figure 4.6: The majority focus area of research articles obtained.

The most frequently researched topic was the sustainable production process, which accounts for approximately 23% of the total, likely due to the critical importance of experimentation in production efficiency and sustainability. This includes traditional and modern fermentation techniques, ingredient optimization, and technological advancements aimed at improving efficiency and product quality (Adebo et al., 2018; Gao et al., 2023; Terefe, 2022). Both safety and waste management account for 20% of the focus of the study. Studies classified under safety focus on biological and chemical safety issues, including the detecting and regulating dangerous compounds such as aflatoxin, biogenic amine, and ethyl carbamate (Gao et al., 2023). The research on waste management in soy sauce production focuses on achieving sustainability, which is essential for reducing the environmental effect and improving the sustainability of production processes (Singh & Krishnaswamy, 2022). In addition, researchers exhibit an acute interest in microbial fermentation, which is 16%. The microbe category emphasizes areas and encompasses research on the role of microorganisms in soy sauce fermentation.

Microorganisms such as molds, yeasts, and bacteria play a crucial role in the fermentation process, impacting the end product's taste, smell, and overall excellence (An et al., 2023). Approximately 11% of the research is dedicated to developing new detection methods incorporated with technologies to identify and analyze various components in soy sauce, underscoring their significance in driving future research efforts. These techniques are crucial for guaranteeing safety and maintaining quality control (Edith & Ochubiojo, 2012; Tang et al., 2023). The remaining 10% is classified as "others," which includes a diverse range of issues that do not fit into the previously specified categories. These studies may encompass research on consumer preferences, market trends, and nutritional evaluations (Chong et al., 2023). In short, these findings indicate that sustainable production processes are at the forefront of reporting progress, with microbial fermentation and new detection methods playing a vital role in future studies

A total of 160 studies were selected to explore the previous and current research patterns on sustainable soy sauce production, specifically from 2013 to 2024, due to more studies focusing more on sustainable practices in soy sauce production (**Figure 4.7**). The overall increase in articles addressing sustainable soy sauce production topics over the last 12 years may result from several factors, including improvements in fermentation techniques, advancements in microbial fermentation and safety standards, and increased attention to environmental research based on the data shown in **Figure 4.7**.

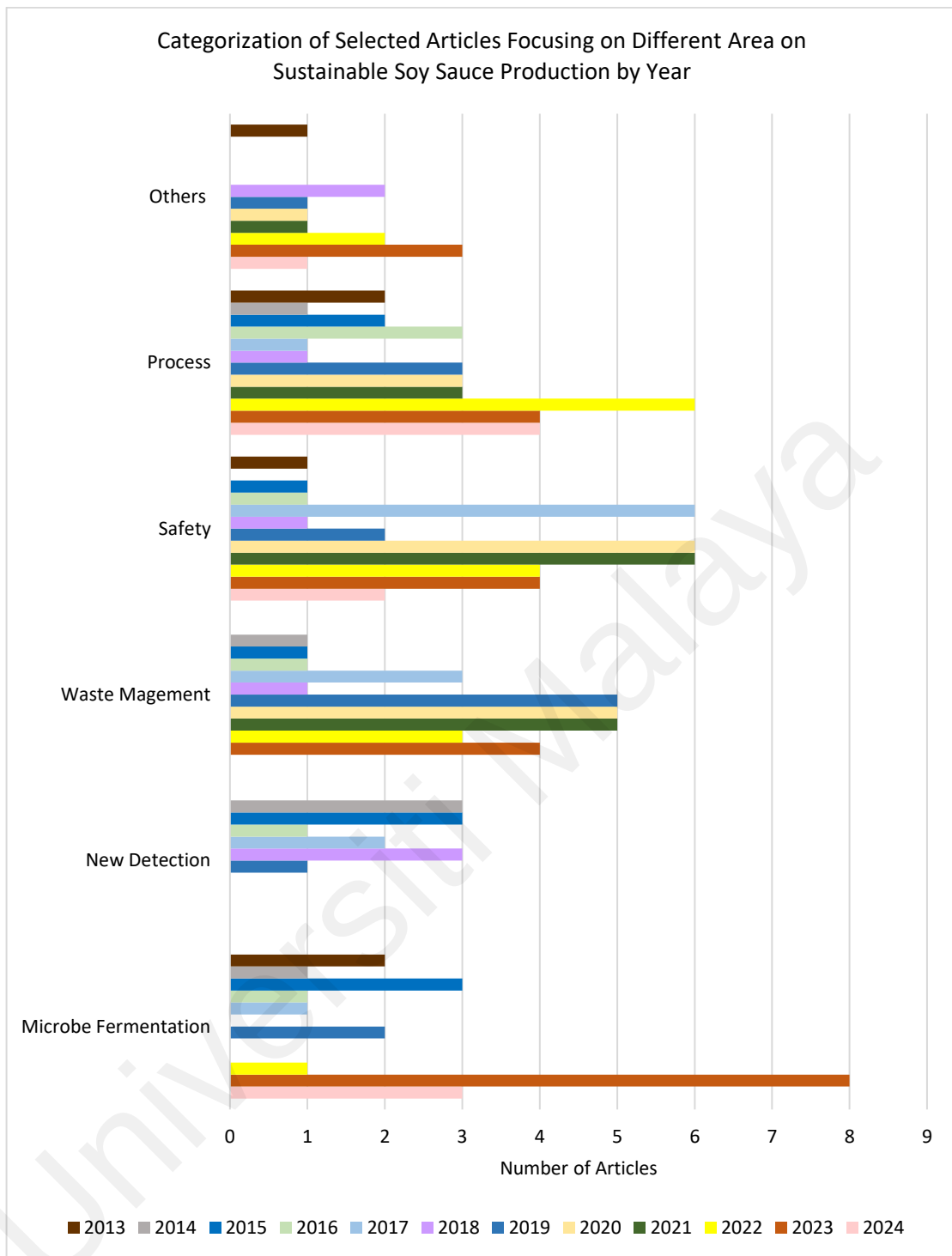


Figure 4.7: Categorization of selected articles focusing on different area on sustainable soy sauce production by year.

Nevertheless, while the preceding statistics present a generally positive picture of the changes in sustainable research over the last 12 years in the soy sauce research area, there is an exception to these trends. Researchers' interest in new detection technologies

is rapidly declining (**Figure 4.7**). This suggests that there might be a delay in acknowledging the important role of innovative detection methods in improving product safety and quality, potentially due to challenges in adopting and implementing new detection methods in research and promotion (Ferone et al., 2020). These methods are crucial for comprehensively advancing sustainable soy sauce production (Diez-Simon et al., 2020a).

Rather than strictly focusing on themes or results, the strength of this study resides in the systematic review process employed to aggregate studies analyzing contents across different publications. However, this study has several drawbacks, including the initial emphasis on the production method, which was motivated by the need to produce soy sauce in a sustainable manner and its practical viability. Even though the articles reviewed discussed selected themes in detail, future research should investigate temporal trends and other aspects that affect the study of other pertinent topics. For example, analyzing the accessibility and functioning rather than purity of raw materials could yield significant knowledge on the soy sauce production in a sustainable manner (van der Goot et al., 2016). Additionally, understanding consumer preferences and market trends can assist in customizing sustainable strategies to align with changing demands (White et al., 2019).

The search strategy was also intentionally broad, and consequently, there is a possibility that research that focuses on unconventional topics has been overlooked. Moreover, the inclusion criteria were limited to research published in English, which may have excluded pertinent studies published in other languages (Walpole, 2019).

Through this systematic review, several potential areas of sustainable soy sauce research have been identified, as illustrated in **Figure 4.8**. Simplifying processes, using environmentally friendly materials, and incorporating cutting-edge technologies all contribute to increased resource efficiency and waste reduction, eventually advancing soy

sauce production (Bruin & Jongen, 2003). Besides, optimising microbial strains and managing fermentation conditions enhance output and product quality in sustainable soy sauce production while reducing resource consumption and waste (Graham & Ledesma-Amaro, 2023). Furthermore, strict quality control and real-time monitoring are ensured by sophisticated detection systems, which encourage the creation of safe, premium soy sauce with little waste and contamination (Mei et al., 2022). Repurposing byproducts, energy recovery, and wastewater treatment are effective waste management techniques that considerably lessen their negative environmental impact (Khan et al., 2022). Strict attention to regulations, hygienic practices, and safe working conditions reduce the danger of contamination and operational disruptions, promoting a productive and safe production process (Trienekens & Zuurbier, 2008).



Figure 4.8: Potential approaches for sustainable soy sauce production.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study successfully optimized soy sauce fermentation conditions using response surface methodology (RSM) to enhance gamma-aminobutyric acid (GABA) production. The incubation parameters, including bacteria inoculum percentage (5-20%), initial pH (3-7), and agitation (100-200 rpm), were systematically varied using the Trio of *A. oryzae* strain KBC (AO), *B. cereus* strain KBC (BC), and *T. halophilus* strain KBC (TH). The results revealed that all the factors, such as bacteria inoculum percentage (A), initial pH (B), and agitation speed (C), significantly influenced GABA production (p -value < 0.0001). Under the optimal fermentation conditions determined by RSM, such as 20% bacterial inoculum percentage, initial pH 7 and 100 rpm, the GABA production can increase from 137.98 mg/L to 239.08 mg/L compared to the control from 122.91 mg/L to 153.48 mg/L after 4 weeks of incubation.

Additionally, the soy sauce obtained using the trio received greater acceptability (7.46) than both control (5.90) and commercial variants (4.47). Based on these findings, the mixed culture used for producing soy sauce can produce higher GABA than the control and is comparable, if not superior, to commercial soy sauce in terms of taste and sensory attributes. Considering the importance of microbial fermentation to increase GABA content, optimization of stater culture conditions is of great interest because it may offer good opportunities for the design of new functional foods for consumers seeking natural ways to improve their daily diets with substances such as GABA, that can reduce mood disorders such as anxiety, depression, insomnia, irritability, and restlessness.

Finally, this study provided a comprehensive overview of past and present trends, possibilities, and challenges in producing sustainable soy sauce, emphasizing important aspects such as increased interest in sustainable practices and the significant gap in the

literature. The majority of current knowledge comes from studies on conventional production techniques and modern advances in fermentation processes, such as those used in soy sauce manufacturing. Nonetheless, there are still significant gaps about the full spectrum of sustainable practices or technologies, such as incorporating novel detection techniques and improving fermentation processes.

5.2 Recommendations

To improve the sustainability and effectiveness of soy sauce production, more research is required into these areas, especially regarding consumer preferences and industry trends, in order to formulate sustainable strategies that are in line with growing demands. In order to create a supportive environment for sustainable consumer behaviours, industry stakeholders should collaborate with policymakers and combine multiple strategies from the SHIFT framework, which includes Social influence, Habit formation, Individual self, Feelings and cognition, and Tangibility as key strategies to tailor approaches to specific audiences (White et al., 2019).

Besides, future research should also explore the effects of local and international regulations on soy sauce production and exploring policy changes to promote sustainable practices is essential (Lawrence, 2017). Effective policy implementation, cross-sector collaboration between government, educational institutions, and agricultural sectors, and global knowledge sharing should be adopted to promote sustainable practices and address global food security issues (Huang et al., 2023).

Moreover, future studies should focus more on evaluating the economic viability of sustainable production methods to ensure they are cost-effective for producers (Pusavec et al., 2010). There are several research challenges, such as developing new materials and processes that minimize environmental impact, integrating sustainability

into supply chain management, and creating metrics to measure sustainability performance that are necessary for future research (Garetti & Taisch, 2012).

Manufacturing companies play a crucial role in sustainability by influencing not just the production phase but the entire lifecycle of a product, from design and development to usage and disposal. Their decisions during the product design and development stages have far-reaching impacts, determining how a product is used, maintained, and eventually discarded (Wang et al., 2018a). Therefore, the adoption of green technologies, waste reduction, resource efficiency, and the implementation of circular economy principles into the production line is crucial for achieving truly sustainable manufacturing practices (Bjørnbet et al., 2021).

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