FREEZE-DRIED IMMOBILIZED *Lactiplantibacillus plantarum* B13 FOR GAMMA AMINOBUTYRIC ACID BIOSYNTHESIS

By

TAN SHANG WEI

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

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Chairman : Associate Professor Murni binti Halim, PhD
Faculty : Biotechnology and Biomolecular Sciences

Gamma-aminobutyric acid (GABA) is a four carbon non-protein amino acid. GABA intended for use in food and pharmaceutical products must fulfill strict safety requirements and hence cannot be chemically synthesized. Therefore, biotechnological approach for GABA production appears to be promising due to simple procedure, high catalytic efficiency, and environmental compatibility. Although various GABA-producing strains have been isolated from different sources, much work is still needed to exploit and characterize additional GABA-producers that provide a potential basis for developing fermented health-oriented products. This study was aimed to enhance the yield of GABA by locally isolated lactic acid bacteria (LAB), *Lactiplantibacillus plantarum* B13 by using natural support for biocatalyst immobilization. *L. plantarum* B13 was classified as LAB (Gram-positive, catalase-negative and oxidase-negative strain) and potential probiotic (high survivality in acidic and bile salt (≥ 50 %); high autoaggregation (73.03 %), high viable cell counts in phenol (7 – 9 log CFU/mL), possessed hydrophilic nature due to adherence to chloroform (72.48 %), exerted antimicrobial activity (against *E. coli*, *S. aureus*, *S. typhimurium*, and *B. subtilis*) and antioxidant activity (77.27 %)). The optimum fermentation parameters were determined by using one-factor-at-a time (OFAT). The optimum conditions for yielded the highest GABA (based on thin layer chromatography and UV spectroscopy method) were 45 hours of fermentation time, 35°C, pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP and 60 g/L glucose based on the highest GABA productivity (0.424 g/L/h). To increase the cell stability and productivity, *L. plantarum* B13 was then immobilized on selected vegetables (okra, onion, sweet potato, bell pepper and taro). The best natural support was selected based on GABA production and cell retention (by using cell count method). Onion was the found as the best natural support evaluated based on GABA yield (18.854 g/L) and cell retention (83 %). It was then employed in repeated batch fermentations to compare its reusability with the free-cell fermentation. The onion-immobilized cells were able to be reused with a

greater number of fermentation batches compared to the free cell. The GABA yield was reduced to below 50 % of the initial yield after the 8th and 6th batch of fermentations for the immobilized cells and free cell, respectively. Additionally, cell viability and productivity may reduce during the storage period. Therefore, freeze-dried cell storage studies were conducted to investigate the ability to retain cell viability and synthesize GABA. The potential probiotic (free-cell and onion-immobilized *L. plantarum* B13) and its postbiotic (supernatant containing GABA) were subsequently freeze-dried with a combination of 10 % (w/v) skimmed milk and 10 % (w/v) sucrose as the cryoprotectant to obtain three different probiotic powder preparations (FCP: free cell+ postbiotic; FC: free cell+ postbiotic+ cryoprotectant; IC: immobilized cell+ postbiotic+ cryoprotectant). After 8 weeks of storage at 4ºC, the IC showed the best performances (7.29 log CFU/mL; survival rate, 81.52 %; and GABA yield, 11.31 g/L) than the FC (7.02 log CFU/mL, 72.82 % survival rate, 8.71 g/L GABA) and FCP (6.06 log CFU/mL, 62.00 % survival rate, 4.89 g/L GABA). Overall, the results proved that the immobilized cells are more effective as compared to the free cell for maintaining high cell viability, survivability, and GABA yield. The findings of this study also suggest the potential use of immobilized *L. plantarum* B13 in the development of GABA-enriched products.

Keywords: Gamma-aminobutyric acid (GABA), lactic acid bacteria, *Lactiplantibacillus plantarum,* biosynthesis, cell immobilization, freeze drying

SDG: GOAL 3: Good Health and Well-being, GOAL 9: Industry, Innovation and Infrastructure

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBEKUAN KERING *Lactiplantibacillus plantarum* B13 YANG DIIMOBILISASI UNTUK BIOSINTESIS ASID GAMA AMINOBUTIRIK

Oleh

TAN SHANG WEI

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Asid gamma-aminobutirik (GABA) ialah empat asid amino bukan protein karbon. GABA yang dimaksudkan untuk digunakan dalam produk makanan dan farmaseutikal mesti memenuhi keperluan keselamatan yang ketat dan oleh itu tidak boleh disintesis secara kimia. Oleh itu, pendekatan bioteknologi untuk pengeluaran GABA nampaknya menjanjikan kerana prosedur yang mudah, kecekapan pemangkin yang tinggi, dan keserasian alam sekitar. Walaupun pelbagai strain penghasil GABA telah diasingkan daripada sumber yang berbeza, banyak usaha masih diperlukan untuk mengeksploitasi dan mencirikan pengeluar GABA tambahan yang menyediakan asas yang berpotensi untuk membangunkan produk berorientasikan kesihatan yang ditapai. Kajian ini bertujuan untuk meningkatkan hasil GABA oleh bakteria asid laktik terpencil (LAB), *Lactiplantibacillus plantarum B13* dengan menggunakan sokongan semula jadi untuk imobilisasi biomangkin. *L. plantarum B13* dikelaskan sebagai LAB (Gram-positif, katalase-negatif dan oksidase-negatif strain) dan potensi probiotik (kemandirian tinggi dalam garam berasid dan hempedu (≥ 50 %); autoagregasi tinggi (73.03 %), bilangan sel berdaya maju tinggi dalam fenol (7 – 9 log CFU/mL), mempunyai sifat hidrofilik kerana pematuhan kepada kloroform (72.48 %), melakukan aktiviti antimikrob (terhadap *E. coli*, *S. aureus*, *S. typhimurium*, dan *B. subtilis*) dan aktiviti antioksidan (77.27 %). Parameter penapaian optimum ditentukan dengan menggunakan satu faktor pada satu masa (OFAT). Keadaan optimum untuk menghasilkan GABA tertinggi (berdasarkan kromatografi lapisan nipis dan kaedah spektroskopi UV) ialah 45 jam masa penapaian, 35°C, pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP dan 60 g/L glukosa berdasarkan produktiviti GABA tertinggi (0.424 g/L/j). Untuk meningkatkan kestabilan dan produktiviti sel, *L. plantarum B13* kemudiannya diimobilisasi pada sayur-sayuran terpilih (bendi, bawang, keledek, lada benggala dan keladi). Sokongan semula jadi terbaik dipilih berdasarkan pengeluaran GABA dan pengekalan sel (dengan menggunakan kaedah kiraan sel). Bawang didapati sebagai sokongan semula jadi terbaik yang dinilai

berdasarkan hasil GABA (18.854 g/L) dan pengekalan sel (83%). Ia kemudiannya digunakan dalam penapaian kelompok berulang untuk membandingkan kebolehgunaannya semula dengan penapaian sel bebas. Sel-sel yang tidak bergerak bawang dapat digunakan semula dengan bilangan kelompok penapaian yang lebih banyak berbanding dengan sel bebas. Hasil GABA dikurangkan kepada di bawah 50% daripada hasil awal selepas kumpulan ke-8 dan ke-6 penapaian untuk sel tidak bergerak dan sel bebas, masing-masing. Selain itu, daya maju dan produktiviti sel mungkin berkurangan semasa tempoh penyimpanan. Oleh itu, kajian penyimpanan sel beku-kering telah dijalankan untuk menyiasat keupayaan untuk mengekalkan daya maju sel dan productiviti GABA. Probiotik yang berpotensi (sel bebas dan *L. plantarum B13* yang diimobilisasi pada bawang) dan postbiotiknya (supernatan yang mengandungi GABA) kemudiannya dikeringkan beku dengan gabungan 10 % (b/v) susu skim dan 10 % (b/v) sukrosa sebagai cryoprotectant untuk mendapatkan tiga persediaan serbuk probiotik yang berbeza (FCP: sel bebas+ postbiotik; FC: sel bebas+ postbiotik+ cryoprotectant; IC: sel tak bergerak+ postbiotik+ cryoprotectant). Selepas 8 minggu penyimpanan pada suhu 4ºC, IC menunjukkan prestasi terbaik (7.29 log CFU/mL; kadar survival, 81.52 %; dan hasil GABA, 11.31 g/L) berbanding FC (7.02 log CFU/mL, 72.82 % kadar survival , 8.71 g/L GABA) dan FCP (6.06 log CFU/mL, 62.00 % kadar survival, 4.89 g/L GABA). Secara keseluruhan, keputusan membuktikan bahawa sel yang diimobilisasi adalah lebih berkesan berbanding dengan sel bebas untuk mengekalkan daya maju sel yang tinggi, kemandirian, dan hasil GABA. Dapatan kajian ini juga mencadangkan potensi penggunaan *L. plantarum B13* yang tidak bergerak dalam pembangunan produk yang diperkayakan GABA.

Kata kunci: Asid gamma-aminobutyric (GABA), bakteria asid laktik, *Lactiplantibacillus plantarum*, biosintesis, imobilisasi, pengeringan beku

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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%	Percentage
°C	degree Celcius
CFS	cell-free supernatant
CFU	colony forming units
DNS	3,5-dinitrosalicyclic acid
EPS	exopolysaccharide
g/L	gram per litre
g/mol	Gram per mole
g	gram
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamate decarboxylase
GDH	glutamate dehydrogenase
Glu/GABA	glutamate/γ-aminobutyric acid
GRAS	generally recognized as safe
Kg	kilogram
L	liter
LAB	Lactic acid bacteria
mg/L	milligram per liter
mg	milligram
MHA	Mueller Hinton Agar
min	minutes
h	hours
mL	milliter
mM	millimolar
MRS	de Man Rogosa Sharpe

MSG	monosodium glutamate
NAD	Nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
nm	nanometer
OD_{600}	optical density at 600 nm
PBS	phosphate buffer saline
P_{GABA}	GABA productivity
pKa	Acid dissociation constant
PLP	pyridoxal-5'-phosphate
rpm	Revolutions per minute
RSM	response surface methodology
subsp.	subspecies
TCA	tricarboxylic acid
TLC	thin layer chromatography
UV	ultraviolet
v/v	volume per volume
w/v	Weight per volume
x g	times gravity
Y_{GABA}	GABA yield
α	alpha
µg/µL	Microgram per microliter
µL	microliter
µm	micrometer
µM	Micromolar
FCP	Free cells with postbiotic, but without cryoprotectants
FC	Free cells with postbiotic and cryoprotectants
IC	Immobilized cells with postbiotic and cryoprotectants

CHAPTER 1

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a non-protein amino acid and having four carbon atoms (Pannerchelvan et al., 2023). The biosynthesis of GABA is carried out in the presence of glutamic acid decarboxylase (GAD) enzyme and L-glutamate as a substrate while pyridoxal-5-phosphate (PLP) acts as the cofactor (Hsueh et al., 2018; Hong & Kim, 2019; Diana et al., 2014). There are many sources for isolating GABA such as plants, animals, bacteria, yeasts and moulds (Zhong et al., 2019; Sokovic Bajic et al., 2019). GABA can also be found in the various fermented foods (i.e., fermented soybean, fermented durian, fermented cassava, fermented fish, zlatar cheese, sourdough and kimchi) and fermented beverages (ie., fermented buffalo milk, yogurt-sake, sake and mulberry beer) (Sokovic Bajic et al., 2019; Pannerchelvan et al., 2023).

GABA possesses multiple health benefits for human such as sedative, antidepressant, antihypertensive, antidiabetic, anticarcinogenic and improve the immune system (Ko et al., 2013). GABA plays different roles in microorganisms and animals. In microorganisms, GABA helps to germinate spore and provide the acid resistant properties to the bacteria, whereas, in animals, GABA acts as an inhibitory neurotransmitter in peripheral tissue and central nervous system (Dhakal et al., 2012). GABA also shows a positive effect on patients with diseases such as Parkinson's, Huntington, Alzheimer's, schizophrenia and stiff-person syndrome (Diana et al., 2014). However, the productions of GABA by animals and plants are complicated. Only a small concentration of GABA is comprising in plants while the mechanism of synthesis is obscure (Varelis et al., 2018). Therefore, the production of GABA by microorganisms is widely studied mainly because it is easier to control the factors that affect the yield of GABA (Diana et al., 2014). In addition, other advantages of using microorganisms to produce GABA include more eco-friendly and safer to consume than the chemically synthesized GABA (Diana et al., 2014; Varelis et al., 2018; Li & Cao, 2010). A wide variety of microorganisms can synthesize GABA via GAD pathway including lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Escherichia coli*, *Aspergillus oryzae* and *Listeria monocytogenes* (Diez-Gutiérrez et al., 2020). Glutamate as a substrate will be turned into GABA by the GAD enzyme. The synthesis of GABA via TCA cycle is known as GABA shunt (Watanabe et al., 2002). The a-ketoglutarate is transformed into glutamate by glutamic acid dehydrogenase, followed by the decarboxylation of glutamate into GABA. The decarboxylation of glutamate is an irreversible reaction, and it requires PLP as a cofactor (Hong & Kim, 2019; Soma et al., 2017).

LAB are among the most important GABA producers mainly because of their probiotic effects and generally recognized as safe (GRAS) status (Pannerchelvan et al., 2023). Furthermore, LAB can produce higher amount of GABA and economically viable as the starters compared to other GABA

producers such as yeasts and moulds (Diana et al., 2014). Several LAB that are known as GABA producing species include *Lactobacillus plantarum* (Siragusa et al., 2007), *Lactobacillus brevis* (Seo et al., 2013), *Lactobacillus paracasei* (Franciosi et al., 2015), *Lactobacillus fermentum* (Lin et al., 2017), *Streptococcus thermophilus* (Diana et al., 2014), *Lactobacillus futsaii* (Sanchart et al., 2017), *Lactococcus lactis* (Diana et al., 2014) and *Bifidobacterium adolescentis* (Strandwitz et al., 2019). These LAB share a common characteristic which is the presence of GAD enzyme activity allowing them to synthesize GABA. LAB as GABA producers are mostly screened and sequestered from various fermented foods and beverages such as kimchi, koumiss, kung-som and zlatar cheese, while, some are also sequestered and obtained from fresh ocean fish, and fish intestine (Han et al., 2020; Strandwitz et al., 2019).

Different LAB have different optimal conditions for producing GABA at an optimal capacity. Depending on the natural environment of LAB species, different parameters will influence the expression of the GAD gene and hence affecting the GABA production (Diez-Gutiérrez et al., 2020). These factors include temperature, pH, substrates (carbon and nitrogen sources and growth factors), L-glutamic acid and PLP concentrations and culture time (Sahab et al., 2020; Pannerchelvan et al., 2023). The optimal temperatures for GAD activity varied among LAB species ranging from 30 to 60°C (Cui et al., 2020). Most LAB's GAD enzyme exhibits optimal activity at pH in the range of 3.5 to 5.0 while some losing it activity at near-neutral pH 7.0 or above. L-glutamic acid is an indispensable compound for the biosynthesis of GABA as most LAB cannot synthesize enough L-glutamic acid for an efficient GABA production. Monosodium glutamate (MSG) is commonly supplemented in the medium as it can produce L-glutamic acid by hydrolysis. Optimizing MSG concentration can stimulate the production of GABA while excessive amount can inhibit the cell growth and reduce GABA production (Villegas et al., 2016).

There are multiple strategies can be applied to enhance the production of GABA by LAB. These include co-culturing approach, two-step fermentation, genetic engineering, and immobilization technique (Pannerchelvan et al., 2023). In particular, cell immobilization technology is widely applied in the fermentation technology due to vast advantages including ease of handling and cell separation, increase cell concentration, allows recycling of cells, higher yield and productivity of targeted product and efficient purification of secondary metabolites (Webb, 1989; Xu et al., 2020). Cell immobilization involved viable cells being enclosed or attached to support matrices without losing its biological function. Cells can be immobilized via various approaches such as physical adsorption through the formation of weak bonds, entrapment, encapsulation and self-aggregation (Homaei et al., 2013). Diverse immobilization matrices or supports either organic or inorganic materials have been shown as effective cell immobilizers. Many studies have demonstrated that immobilization techniques utilizing natural edible carriers enabled enhanced survival of probiotic cells and product yield as well as can be employed in the production of functional foods. For example, apple pieces have been shown as a good natural support for immobilization of *L. plantarum* via adsorption on the surface of fruit allowing its

reusability for several times during soy fermentation (Corbo et al., 2012). Through immobilization, microbial cells will be able to resist the harsh environmental conditions such as high temperature, very acidic or alkaline and exposure towards the toxic compounds (Junter & Jouenne, 2017). The technique is also cost-effective due to its reusability in which immobilized cells can be stored and reused for many batches of fermentation with greater performances than the free cells culture.

LAB are widely applied in various industries, particularly, the food and beverage industry as the starter culture or for probiotic formulations (Prapa et al., 2022). Therefore, maintaining high cells viability, stability, and activity during processing and storage is very crucial. Dried products are preferable by the industry as they are easy to store and transport with reduce cost as refrigeration is not required and may extend the shelf life of product. Freeze drying or lyophilization is one of the commonly used drying methods for preserving microbial cells including probiotics (Kiepś and Dembczyńsk, 2022). However, the freeze-drying process expose microorganisms to various stress factors such as excessive drying, osmotic stress and oxidative stress that may result in rupture of cell walls, cells shrinkage and protein destruction. The use of suitable protectants or cryoprotective agents can improve the survival rate of cells as it helps to fortify the cell membrane constituents and contribute a protective coating to the cells for avoiding the injury of cells as well as builds up the porous structure for easing the rehydration process (Broeckx et al., 2016).

Different culture conditions such as incubation time, temperature, pH, MSG concentration, PLP concentration and glucose concentration might affect the GABA production and various strains of one species have obvious differences in GABA productivity. Therefore, this study was focusing on the effect of fermentation parameters (incubation time, temperature, pH, MSG concentration, PLP concentration and glucose concentration) towards the biosynthesis of GABA by GABA-producing lactic acid bacteria, *Lactiplantibacillus plantarum* B13 (or formerly known as *Lactobacillus plantarum*) that was previously isolated from a traditional fermented shrimp paste (*Budu).* Free cells are less stable than immobilized cells throughout the production. Several reports also stated that the free cells had lower productivity than the immobilized cells (Zhao & Xia, 2010; Chandel et al., 2009; Nigam, 2000). Hence, this study applied the whole-cell immobilization strategy and repeated batch fermentation using natural supports (selected vegetables) to improve the GABA production and cell viability. Beside that, the use of vegetables as natural support immobilizers can avoid the safety issue and offers environmental compatibility allowing its potential applications in fermented health-oriented products. Furthermore, retaining high cell viability is an issue during storage period. During prolong storage, cell viability and stability might be lost and this will reduce the cell capability of synthesizing high yield bioproduct. This study also involved the preparation of solid forms of potential probiotic (*L. plantarum* B13 cells) and postbiotic (all metabolites produced by *L. plantarum* B13 cells including GABA) via freeze drying method. The use of an appropriate cryoprotectant can help to retain cell viability and stability during prolonged storage periods and upon rehydrated, freeze-dried cells can still have

high efficiency in producing the desired GABA product. Hence, the specific objectives of this study were:

1. To determine the probiotic potential and the effect of fermentation parameters (incubation time, temperature, pH, MSG concentration, PLP concentration and glucose concentration) towards GABA production.
2. To immobilize *L. plantarum* B13 using various vegetables for enhancing GABA production via repeated batch fermentation.
3. To investigate the cell viability on the prepared freeze-dried probiotic and postbiotic powders during storage in producing GABA.

CHAPTER 2

LITERATURE REVIEW

2.1 Gamma-aminobutyric Acid (GABA)

Gamma-aminobutyric acid, which is commonly known as GABA is a popular apo-protein, bioactive nitrogenous compound, known as amino acid (Li & Cao, 2010). The structure chemical of GABA is shown in **Figure 2.1**. It is a non-protein amino acid that is biosynthesized by glutamic acid decarboxylase (GAD) (**Figure 2.2**). GAD is a pyridoxal-5'-phosphate-dependent enzyme, which catalyzes an enzymatic reaction, known as irreversible α-decarboxylation, that transforms the L-glutamic acid to GABA (Li & Cao, 2010; Ueno, 2000).

GABA was first observed in potato tubers and later in mammalian brains (Rayavarapu et al., 2021). Ever since, GABA is noticed in many life forms including fungi, bacteria, animals, and plants (Gou et al., 2012; Bouche & Fromm, 2004). GABA can be synthesized either by biosynthesis or chemical synthesis (Dhakal et al., 2012). However, the biosynthesis method by microorganisms such as yeast, fungi and bacteria is much more favourable compared to the chemical synthesis due of its good ability in acting as a biocatalyst, simplicity in the reaction process, and environmentally friendly (Cui et al., 2020; Aoki et al., 2003; Wang et al., 2003).

GABA is the major neurotransmitter (chemical messengers) inhibitor in the brain (Petroff, 2002). GABA is known as a neurotransmitter inhibitor because it impedes, or suppresses, certain brain signals and reduces the activity of the nervous system. The equilibrium state between excitatory neuronal transmission through glutamate and inhibitory neuronal transmission via GABA is vital for proper cell membrane stability and neurologic function (Allen et al., 2018). GABA receptors are categorized into GABAa and GABAb. GABAa receptor is a ligand-gated ion channel or ionotropic receptor. They have high concentration in the limbic system and the retina (Sigel & Steinmann, 2012). GABAb receptor is a G-couple protein receptor. The location of GABAa is at the central nervous system, whereas the GABAb is located at the thalamic pathways and cerebral cortex (Padgett & Slesinger, 2010).

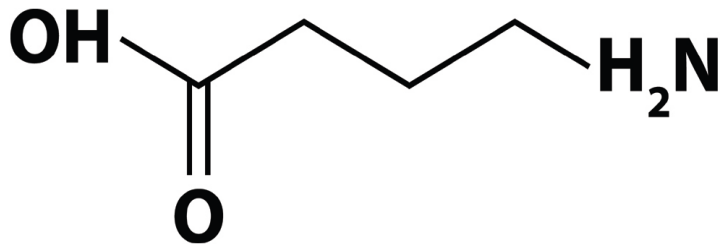


Figure 2.1 : The chemical structure of gamma-aminobutyric acid (GABA)

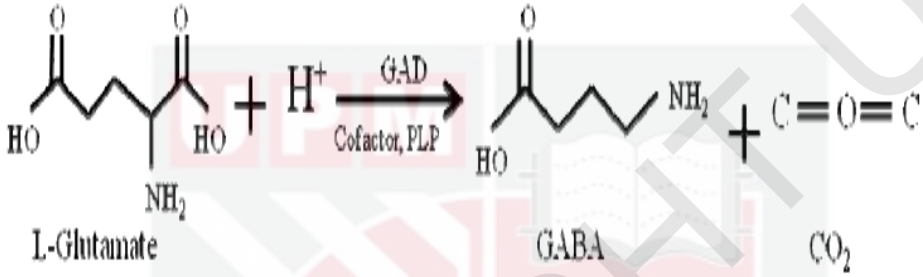


Figure 2.2 : Decarboxylation of L-glutamate to gamma-aminobutyric acid (GABA) and carbon dioxide (CO_2) by glutamate decarboxylase (GAD) in the presence of cofactor, pyridoxal-5'-phosphate (PLP) (Dhakal et al., 2012)

2.1.1 Benefits of GABA

Nowadays, the commercial demand of GABA products is surging upward. GABA plays many crucial roles. GABA has been considered as a bioactive component in pharmaceutical and functional food products due to its vast biological and beneficial functions (Kim et al., 2009). GABA is a bioactive compound with antioxidant, antidepressant, anti-insomnia, and pain-relieving effects and is used to treat autoimmune diseases, stroke, and neurological disorders. GABA is available from some food sources such as spinach, sweet potato, broccoli, kale, kimchi, and tempeh (Sahab et.al, 2020), however, the foods that naturally consist of GABA are usually not able to fulfil the people's demands as they contain a limited or little amount of GABA (Cui et al., 2020). Hence, GABA products are extensively developed as either drugs, dietary supplements or formulated as functional foods (Oketch-Rabah et al., 2021).

In the treatments of autoimmune diseases, stroke and neurological disorders, GABA strengthens the brain cell metabolism by raising blood flow and oxygen delivery (Alizadeh Behbahani et al., 2020). GABA also helps to enhance memory and the learning abilities. GABA may enhance the plasma growth hormone concentration and the synthesis rate of protein in humans' brain (Tujioka et al., 2009). GABA also impedes small airway-derived lung adenocarcinoma (Schuller et al., 2008). As an antidepressant drug, GABA helps to lessen the neurons

activity, followed by reducing the stress and anxiety. GABA possesses the anti-insomnia effect because it can calm the body and mind. A study which involved 13 people, showed that the GABA had a positive effect in alleviating the stress and anxiety (Breus, 2019). The slow brain waves were observed after the consumption of GABA. This study demonstrated that the patients who were facing an insomnia problem had a lower GABA level, which was approximately 30 % compared to the normal people who were not suffering from the insomnia. Hence, by consuming an adequate dosage of GABA, it helps to improve the quality of sleeping and increase the time of sleeping. Meanwhile, as an antihypertensive and antidiabetic drug, GABA helps to regulate growth hormone secretion, protein production, fat burning, and diminish blood pressure (Ting Wong et al., 2003). There is a study reported that the persistent consumption of GABA in the range between 0.3 to 300 mg/kg spontaneously reduced the systolic blood pressure in hypertensive rats (Kimura et al., 2002). Daily oral administration of 10 mg GABA for 12 weeks have shown a positive effect for hypertensive patients (Izquierdo et al., 2009). In the meantime, GABA also plays the role as a powerful secretagogue of insulin from the pancreas, hence, avoiding diabetes effectively (Adeghate & Ponery, 2002).

GABA-enriched foods such as are the natural way to obtain the beneficial effects of GABA at low cost (Diana et al., 2014). Many studies have been conducted to support the claims of GABA health benefits from daily consuming of GABA through natural and GABA-enriched foods (Diana et al., 2014). A study reported that 50 mg dosage of GABA that was dissolved in a beverage significantly helped to reduce the psychological and physical fatigue of 30 healthy human subjects, whom 9 of them were diagnosed with chronic fatigue (Kanehira et al., 2011). It was also discovered from the high score on Uchida-Kraepelin Psychodiagnostics Test that consuming GABA enriched beverages helped to improve task-solving ability. Another study reported candidates showed a decline in alpha waves over time while carrying out an arithmetic duty (Yoto et al., 2012). The candidates that orally received 100 mg dosage of GABA showed a smaller decline compared to the candidates that did not orally receive GABA. Meanwhile, subjects administrated with higher dosage of synthetic GABA at 800 mg showed an improved capability of prioritized planned actions and inhibitory control (Verbruggen & Logan, 2008; Steenbergen et al., 2015). A daily oral consumption of defatted rice germ containing 26.4 mg GABA was proven to be able to effectively treat neurological disorders (Okada et al., 2000). The study showed that the three time a day consumption of the GABA-enriched rice germ helped to improve the common mental symptoms such as sleeplessness, depression and somnipathy during the menopausal and a presenile period. Placebo-controlled study was carried out by Abdou et al. (2006), to evaluate the effect of orally administrated of GABA on people with acrophobia which is a type of phobia or fear of heights (Abdou et al., 2006). The study stated after 60 minutes of administration of GABA, the alpha waves were elevated in healthy participants and the levels of immunoglobulin A (IgA; an indicator of immune system functioning) declined in participants with a history of acrophobia when they were exposed to heights. Hence, they deduced that GABA can effectively function as a natural relaxant which can induce a fast effect of relaxation and diminish anxiety within 1 hour of administration. In the meantime, another study

stated that the consumption of 10 g GABA-enriched chocolate containing 28 mg GABA helped to reduce the heart rate variability and salivary chromogranin A during an arithmetic task (Nakamura et al., 2009). The result of this study showed the GABA-enriched chocolate product have the psychological stress-reducing effect in human. Besides that, GABA also can regulate the lipid levels in serum, as well as pain and anxiety (Miura et al., 2006). In addition, the ingestion of GABA-enriched foods also was found to suppress the proliferation of cancer cells (Park & Oh, 2007a).

Due to its numerous proven health benefits, todays, GABA has become as one of a famous supplement. In Europe and the United States, GABA is considered as a "dietary supplement" and "food constituent" (Oketch-Rabah et al., 2021). The manufacturers do not need to provide the evidence or proof to support the effectiveness of their products if they do not make the claims with regard to the potential benefits that relate with the specific diseases or conditions. These GABA food supplements can be purchased online via numerous websites, including web shop giants such as Amazon.com, with often very positive customer reviews. For example, "Pharma-GABA" is one of the product comprises of natural form of GABA manufactured for the Asian market from the fermentation process by a LAB strain, *Lactobacillus hilgardii* K-3 (Kanehira et al., 2011). Pharma-GABA has been ratified by the Food and Drug Administration (FDA) as a food ingredient (Food and Drug Administration, 2008). Hundreds of people reported that GABA as dietary supplements have helped them to soothe anxiety and improve sleeping quality and other beneficial effects (Boonstra et al., 2015).

2.1.2 Sources of GABA

GABA can be extracted or isolated from many different sources, including plants, animals and microorganisms (Diana et al., 2014). However, the production of GABA using plants and animals is not a simple process whilst the production mechanism is not clear and only produces a low GABA concentration than the use of microorganisms (Sahab et al., 2020).

GABA is present in almost every plant (Ramos-Ruiz et al., 2018). GABA from plant sources was first discovered in the form of alpha butyric acid in 1949 (Watanabe et al., 2002; Hajeb and Jinap, 2012). GABA is instinctively found in plants because GABA is a necessary metabolic product that interact the organism with the environment as well as playing the role as a substrate for certain metabolic processes (Lee et al., 2022). GABA also involved in various physiological processes and may play role as a signalling molecule (Shelp at al., 2017; Bown & Shelp, 2016). With the presence of glutamate decarboxylase enzyme, the plant tissues can decarboxylate the L-glutamate into GABA. Thus, the plant matrix will be rich in GABA. The accumulation of GABA in plants occurs in response to biotic and abiotic stresses (Ham et al., 2012; Ramos-Ruiz et al., 2018). Plants that are reported to be rich in GABA include tea leave, mulberry leave, tomato, cabbage, broccoli, spinach, cauliflower, and sweet potato (Zhao

et al., 2011; Yang et al., 2012). Moreover, pulses such as beans, peas and lentils are rich in bioactive compounds, dietary fibres, minerals and vitamins (Nikmaram et al., 2017). GABA is one of the bioactive compounds that composed in the pulses.

In animals, GABA is widely existing in the nervous system of mammals, insects, round worm and flat worm (Gou et al., 2012). GABA is also naturally contained in human body. There is a GABAergic system in humans' body that will involve in the process of action selection (Stagg et al., 2011). Different responses or choices will be made due to the different stimulus.

GABA is also widely found in the microorganisms such as lactic acid bacteria, yeasts and moulds (Wu et al., 2018). GABA was first extracted from acid-treated yeast extracts, followed by segregated from red yeast, *Rhodotorula glutinis* (Reed, 1950). Many microorganisms can produce GABA in their cells due to the activity of the glutamic acid decarboxylase enzyme. Moreover, most microorganisms can produce high levels of GABA due to the presence of glutamate, pyridoxyl-5-phosphate (PLP), and acidic conditions (Dhakal et al., 2012). Based on the study that disclosed by Di Cagno et al. (2010), fungi such as *Aspergillus nidulans* and *Aspergillus niger* are rich in GABA. According to Dahiya et al. (2021), the strains that can produce GABA are *L. brevis* CRL 1942, *L. plantarum* FNCC 260, *Streptococcus salivarius subsp. thermophilus* Y2, *Monascus spp.*, *Rhizopus spp.* and *Bifidobacterium* strain. In addition, many researchers have isolated and screened many different strains of *Lactococcus* and *Lactobacillus* from different fermented foods for GABA production, including *L. delbruskii*, *L. lactis*, *L. plantarum*, *L. brevis*, *L. helviticus*, *L. bulgaricus*, and *L. paracasei* (Shan et al., 2015).

2.1.3 GABA stability

According to the study that conducted by Lamberts et al. (2008), the results showed that GABA was not destroyed when it was heated in a watery solution alone, but in combination with sugars, GABA participated into the Maillard reaction, resulting in 10% GABA loss after heating for 4 hours at 110°C. Besides that, there is another study showed that the GABA content of heated germinated soymilk at pH 6.5 remained stable during storage time of 8 days at 4°C (Le et al., 2020). Moreover, based on the study by Khan et al. (2015), the GABA content reduced significantly with increasing pH (from 3.4 to 8), heating temperature (80–121°C) and heating times (15–120 minutes), respectively. The results showed that GABA was stable (ranging from 12.9 to 11.8 mg/mL) over a pH range of 5.4 – 8.0. As the pH increased to 10.8, the content of GABA decreased rapidly to 1.53 mg/mL. According to the results that stated by Khan et al. (2015), the highest GABA residue, 88.21%, was found when the Monascus-fermented rice solution was treated at 80°C with a pH of 3.4 for 15 minutes. In contrast, 1.31% GABA content was obtained when the Monascus-fermented rice solution was treated at 121°C with a pH of 5.4 for 120 minutes. GABA content was at the undetectable range (less than 0.04 µg/mL) when the

Monascus-fermented rice solution was treated at 121°C with a pH of 3.4 for 90 minutes onward. These results proved that GABA is more stable under lower temperature and shorter time as well as lower pH treatment. These findings also speculated that GABA is unstable at high temperature and higher pH.

2.2 Lactic acid bacteria (LAB)

2.2.1 LAB as probiotics

Lactic acid bacteria (LAB) have been used to produce many traditional fermented foods such as yogurt, cheese, pickles and beverages for many centuries (Cui et al., 2020). LAB are highly in demand for fermentation industry mainly due to its generally recognized as safe (GRAS) status. The concept of utilizing LAB for healthy and long life was first proposed more than a hundred years ago by Élie Metchnikoff while the probiotic term was introduced in 1965 by Lilly and Stillwell (Gibson, 2004). According to Food and Agriculture Organization, probiotics are defined as live microorganimsm which, when administered in appropriate doses, confer health benefit to the host" (Hill et al., 2014).

Generally, catalase analysis, oxidase analysis and gram staining test are always designated for the characterization of the bacteria as LAB. Catalase enzymes are playing the role of breaking down the hydrogen peroxide into water and oxygen molecules (Cappucinno and Welsh (2018). The hydrogen peroxide is the by-product of aerobic respiration. The accumulation of hydrogen peroxide will cause the bacteria to die as it is toxic to the bacteria. The catalase analysis was applied to investigate the production of catalase in the bacterial culture. For the oxidase analysis, it is often applied to examine the presence of cytochrome oxidase in the bacterial strain (Cappucino and Welsh, 2018). The cytochrome oxidase helps to catalyse the oxidation of reduced cytochrome by the oxygen molecule. This reaction will produce hydrogen peroxide and water molecules. Besides that, to determine the morphology of the bacterial strain, Gram staining method is always being applied (Moyes et al., 2009). This is a combination method of the biochemical test and microscopy method. For Gram staining tests, the bacteria are always being classified into two groups, specifically Gram-positive and gram-negative based on the composition of the bacterial cell walls. For a Gram-positive bacterium, it has a thick peptidoglycan layer and does not have the outer lipid membrane, whereas, for a gram-negative bacterium, it has a thin peptidoglycan layer that surrounded by an outer lipid-containing layer (Cappucinno and Welsh, 2018). Because of having the thick peptidoglycan layer, Gram-positive bacteria will show the purple colour after staining, whereas, for Gram-negative bacteria, it will show pink or red colour after staining (Steward, 2019).

To date, dozens of LAB have been identified as probiotics due to their ability to produce metabolite compounds with various health benefits (Petrova et al., 2022). These bioactive compounds include lactic acid, aromatic compounds

such as diacetyl and acetaldehyde, hydrogen peroxide and bacteriocins that can inhibit the growth of pathogenic organisms and cause cell lysis (Indira et al., 2019). Probiotic strains should also exhibit detoxifying properties and possess the ability to inhibit the production of toxins and inactivate or facilitate their removal from the human body (Markowiak & Śliżewska, 2017). According to FAO/WHO guidelines, even though potential probiotic strains have the GRAS status, assessments must still be done to confirm their safety before utilizing them in commercial products (Lin et al., 2006; Rolfe, 2000). The minimum assessments include identification of antibiotic resistance, evaluation of metabolic activities, antimicrobial activity, adhesion ability to epithelium of the intestine and determination of cell viability during storage. The LAB, *Lactiplantibacillus plantarum* (or formerly known as *Lactobacillus plantarum*) can be classified as probiotics based on some characteristics as shown in **Table 2.1**.

Table 2.1 : List of *Lactiplantibacillus plantarum* strains and their identified probiotic characteristics

Lactic acid bacteria (LAB)	Probiotic characteristics	Reference
L. plantarum	• Able to survive at low pH • Able to adhere on the surface of the intestinal tract	De Vries et al. (2006); Mathara et al. (2008); Georgieva et al. (2009)
L. plantarum Lp790	• Able to resist the lysozyme • Able to adapt with the mimicked gastric juice • Able to show bile tolerance • High surface hydrophobicity	Zago et al. (2011)
L. plantarum Lp813	• Able to resist the lysozyme • Able to adapt with the mimicked gastric juice • Able to show bile tolerance • High surface hydrophobicity • Presence of antibacterial activity	Zago et al. (2011)
L. plantarum Lp998	• Able to resist the lysozyme • Able to adapt with the mimicked gastric juice • Able to show bile tolerance • High surface hydrophobicity • Presence of antibacterial activity	Zago et al. (2011)
L. plantarum KCC-37	• Presence of acidic and bile tolerance • Able to autoaggregate • High cell surface hydrophobicity • Presence of antifungal activity	Muthusamy et al. (2020)
L. plantarum KCC-38	• Presence of acidic and bile tolerance • Able to autoaggregate • High cell surface hydrophobicity • Presence of antifungal activity	Muthusamy et al. (2020)

It is very important for the probiotics to tolerate acidic and alkaline conditions to be able to survive in the human's gastrointestinal tract (Prete et al., 2020). According to Bao et al. (2010), it is important to screen the LAB with the growing absorbance at pH 3.0, because the pH values in human stomach are ranged from 1.5 (during fasting) to 4.5 (after ingestion of food) and this condition could retain for around 3 hours. Generally, the ingested food will move into the stomach, followed by moving into the small intestine by passing through the duodenum (Choi & Chang, 2015). In duodenum, there is the presence of bile that secretes from the gall bladder. The bile will break down the lipid membrane of the cell and cause the leakage of the cell contents, leading to the cell death. Hence, to reach the small intestine and retain their viability for a prolonged period while exerting beneficial effects, probiotics LAB must possess bile and acidic pH tolerance (Kim et al., 2012).

Besides that, it is also very important to ensure the ability of probiotics to colonise the surface of intestine and develop the intestinal mucosal barrier, as this is one of the important characteristics for probiotics especially in health-promoting foods (Honey Chandran & Keerthi, 2018, Collado et al., 2008). The colonisation and adherence of probiotics to the surface of the epithelial cells of intestine will prevent other pathogenic bacteria from adhere to the surface of intestine, and thereby it can protect the cells from being infected by pathogenic bacteria. Therefore, the evaluation of the adherence and autoaggregation ability, as well as the cell surface hydrophobicity of the strain are necessary because these evaluations are commonly used for the characterization of probiotics (Tang et al., 2018).

Furthermore, cellular autoaggregation is a crucial factor for probiotics to develop intestinal colonisation with the ability of self-aggregation (Farid et al., 2021). According to Kumari et al. (2016), bacterial cell surface consisted of proteins and exopolysaccharides, thereby providing the aggregation ability to the strain.

Moreover, EPS is the natural sugar polymer that has many important functions such as enhancing the food texture and boosting the food flavour (Farid et al., 2021). Besides that, EPS also has antioxidant, anticancer and anti-inflammatory properties which are beneficial for health. For LAB, EPS is one of the crucial components of the extracellular biofilm matrix (Nguyen et al., 2020). The biofilm plays a role as a protective barrier, which protects the lactic acid bacteria from some unfavourable and stressful factors such as temperature, pH, antibiotics and so on. *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus, Streptococcus* and *Weissella* sp. are among the most prominent EPS producing lactic acid bacteria (Angelin and Kavitha, 2020).

2.2.2 LAB as GABA producers

Nowadays, LAB have also gained interests as GABA-producing microorganisms due to their probiotic effects and various production characteristics (Cui et al., 2020). Among genera of LAB, Lactobacillus has a high number of GRAS species

which are also identified as GABA-producing species. These include *Lactobacillus plantarum* (Franciosi et al., 2015; Zhuang et al., 2018), *Lactobacillus brevis* (Li et al., 2010a; Park & Oh, 2007b; Diana et al., 2014), *Lactobacillus buchneri* (Park & Oh, 2006; Cho et al., 2007), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Siragusa et al., 2007; Gangaraju et al., 2014), *Lactobacillus fermentum* (Woraharn et al., 2016; Lin et al., 2017), *Lactobacillus helveticus* (Sun et al., 2009), and *Lactobacillus paracasei* (Siragusa et al., 2007; Franciosi et al., 2015). Besides Lactobacillus, probiotics such as *Streptococcus thermophilus* (Han et al., 2020) and *Lactococcus lactis* (Laroute et al., 2023) that are widely used as starter cultures of fermented dairy products such as milk, yogurt and cheese also showed the ability of producing GABA. Furthermore, some other species from the genera *Enterococcus*, *Leuconostoc*, *Pediococcus*, *Propionibacterium* and *Weissella* also have been found to possess the capacity to produce GABA through fermentations (Cui et al., 2020).

Generally, the GABA yield of various species is greatly different. Many studies showed that *Lactobacillus brevis* was able to synthesize high amounts of GABA compared to the other LAB species. GABA produced by various strains of *Lactobacillus brevis* was reported in the range from 0.015 to 205 g/L (Siragusa et al., 2007; Wang et al., 2018). Meanwhile, several *Lactobacillus plantarum* strains that were isolated from different cheeses also have been shown with noticeably different GABA yields (Siragusa et al., 2007; Franciosi et al., 2015; Zhuang et al., 2018). Additionally, as summarized in Table 2.2, different strains of one species exhibited different concentration of GABA.

Table 2.2 : List of the gamma-aminobutyric acid produced by different strains of lactic acid bacteria from various isolation sources

Lactic acid bacteria	Isolation sources	GABA concentration (g/L)	Reference
L. paraplantarum N-15	Sourdough	0.67	Demirbaş et al. (2017)
L. plantarum BC114	Sichuan pickle	3.82	Zhang et al. (2017)
L. plantarum C48	Cheese	0.02	Siragusa et al. (2007)
L. plantarum CGMCC 1.2437^T	Fermented cabbage	74.37	Zhuang et al. (2018)
L. plantarum DW12	Fermented food	4.00	Ratanaburee et al. (2011)
L. plantarum ED-10	Sourdough	1.59	Demirbaş et al. (2017)
L. plantarum $FC_2$10	Fermented dairy product	0.04	Nejati et al. (2013)
L. plantarum IFK-10	Fermented soy bean	2.68	Yogeswara et al. (2018)
L. plantarum K154	Korean kimchi	0.2	Park et al. (2014)
L. plantarum L2A21R1	Azorean cheese	0.94	Ribeiro et al. (2018)
L. plantarum L2C21E8	Azorean cheese	0.46	Ribeiro et al. (2018)
L. plantarum L3C1E8	Azorean cheese	0.65	Ribeiro et al. (2018)
L. plantarum LMG 6907	Pickled cabbage	0.39	Parmentier (2018)
L. plantarum NDC75017	Fermented milk	3.15*	Shan et al. (2015)
L. plantarum NTU 102	Cabbage pickles	0.63	Tung et al. (2011)
L. plantarum PU11	Fermented dairy product	0.08 *	Nejati et al. (2013)
L. plantarum SC-9	Sourdough	0.51	Demirbaş et al. (2017)
L. plantarum Taj-Apis362	Honeybee	0.74	Tajabadi et al. (2015)
L. brevis 340G	Kimchi	7.09	Seo et al. (2013a)
L. brevis BH2	Kimchi	20.00	Kim et al. (2007)
L. brevis BJ20	Salt-fermented Jot-gal (cod gut)	2.47	Lee et al. (2010)
L. fermentum HP3	Thai fermented foods	2.11	Woraharn et al. (2016)
L. fermentum YS2	Chinese traditional pickled vegetable	5.15	Lin et al. (2017)
L. paracasei NFRI 7415	Fermented fish	31.14	Komatsuzaki et al. (2005)
L. paracasei PF6	Cheese	0.10 *	Siragusa et al. (2007)
L. lactis subsp. lactis B	Kimchi	6.41	Lu et al. (2008)

*= g/Kg

Essentially, the key sources for isolating the GABA-producing LAB are fermented foods that are rich in L-glutamate. For example, cheese consists of high concentration of caseins that allow for the synthesize of an ample amount of L-glutamate. Many studies reported that LAB strains such as *Lactobacillus buchneri*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp*. bulgaricus* and *Lactococcus lactis* that were isolated from either traditional or commercial cheeses showed the capability of producing GABA (Siragusa et al., 2007; Tres et al., 2014; Park & Oh, 2006; Franciosi et al., 2015). In addition, high GABA concentrations were also reported for *Lactobacillus paracasei* PF6, *Lactobacillus delbrueckii* subsp. *bulgaricus* PR1, *Lactococcus lactis* PU1 and *Lactobacillus brevis* PM17 that were isolated from Pecorino di Filiano, Pecorino del Reatino, Pecorino Umbro and Pecorino Marchigiano cheeses, respectively (Siragusa et al., 2007). Meanwhile, a Japanese distilled alcoholic beverage or known as shochu distillery-lees that composed of 10.50 mM free glutamate was able to transform into 10.18 mM of GABA by *Lactobacillus brevis* IFO-12005 (Yokoyama et al., 2002). Besides that, by using *Lactobacillus plantarum* DSM19463 in the fermentation process, it can produce grape beverage that rich in GABA and has a good anti-hypertensive effect and dermatological protection (Di Cagno et al., 2010; Li & Cao, 2010).

Commonly, GABA-producing LAB are favourable towards an acidic environment. The acidic environment can promote the growth of GABA-producing LAB such as in Korean kimchi and Chinese paocai (Chinese fermented cabbage) (Cui et al., 2020). For instant, *Lactococcus lactis* subsp. *lactis* that was screened and isolated from kimchi was found to exhibit 3.68 g/L of GABA in de Man, Rogosa and Sharpe (MRS) broth (Lu et al., 2008). Moreover, various strains of LAB which can synthesize GABA have been screened and isolated from many other fermented foods and beverages including sourdough, yoghurt, fermented cassava, fermented fish, fermented soybeans, sake, fermented buffalo milk and mulberry beer (Cui et al., 2020; Sahab et al., 2020).

2.3 Biosynthesis of GABA by LAB

2.3.1 GABA biosynthetic pathway in LAB

Glutamic acid decarboxylase (GAD) system of the microorganisms plays an important role in carrying out the biosynthesis of GABA (**Figure 2.3**). This system consists of GAD enzyme which is encoded by GadA and GadB as well as the glutamate or GABA antiporter GadC (Lyu et al., 2018; Wu et al., 2017; Wu & Shah, 2019; Yunes et al., 2016). *L-glutamate* is transferred into a cell by passing through GadC. GAD catalyzes the decarboxylation of *L-glutamate* in the presence of cofactor pyridoxal-5'-phosphate (PLP) resulting in the formation of GABA and produces carbon dioxide as the by-product. The decarboxylated product which is GABA will be then transported out from the intracellular to extracellular matrix by once again passing through the GadC (Cui et al., 2020).

There are some species of LAB such as *Lactobacillus fermentum*, *Lactobacillus frumenti, Lactobacillus gastricus, Lactobacillus gorilla, Lactobacillus mucosae, Lactobacillus oris, Lactobacillus plantarum* (Kleerebezem et al., 2003), *Lactobacillus pontis, Lactobacillus reuteri* (Morita et al., 2008), Lactobacillus similis, *Lactobacillus vaginalis*, *Leuconostoc citreum* (Kim et al., 2008), *Leuconostoc gasicomitatum* (Johansson et. al., 2011), Leuconostoc kimchii (Oh et al., 2010), *Leuconostoc mesenteroides subsp. mesenteroides* and *Oenococcus oeni* (Makarova et al., 2006), are being recognized to have the GABA aminotransferase (GABA-AT) encoding gene gadT (Zhuang et al., 2018). It was found that the gene gadT expression was down-regulated by a factor of 0.26 times, showing that the GABA-AT activity was impeded (Cui et al., 2020). The results indicate that the activity of GABA-AT has a direct relationship with the GABA production. Furthermore, the molecular mechanisms of GABA degradation in some bacteria such as Listeria monocytogenes and Escherichia coli also has been elucidated. Firstly, the major GABA-degradative enzyme, which is GABA-AT degrades GABA to become succinic semialdehyde (SSA). Next, SSA will be transformed to become succinic acid due to the catalysis activity of the enzyme, succinate semialdehyde dehydrogenase (SSADH) to move into the TCA cycle.

Generally, glucose is a readily fermentable sugar. It is the most important energy source and the main substrate for carrying out the glycolysis process (Cherkas et al., 2020). However, when there is insufficiency of glucose, adenosine triphosphate molecules would be produced by replacing glucose with amino acids and ketone bodies. For the other metabolic pathway, which is the pentose phosphate pathway (PPP), glucose is a non-replaceable with other metabolic molecules. The PPP pathway is very important for cells to survive under the utmost oxidative and toxic conditions. This is because PPP pathway helped to control the cellular redox reaction (reduction-oxidation) to achieve homeostasis and battle with the oxidative stress. According to Cui et al. (2020), glucose can be used as substrate, and it will undergo glycolysis process to become pyruvate, then, converting into Acetyl-CoA and enter the TCA cycle to become α-ketoglutarate. After that, the α-ketoglutarate will be catalyzed by glutamate dehydrogenase (GDH) to form glutamate. Then, the glutamate will be decarboxylated to become GABA.

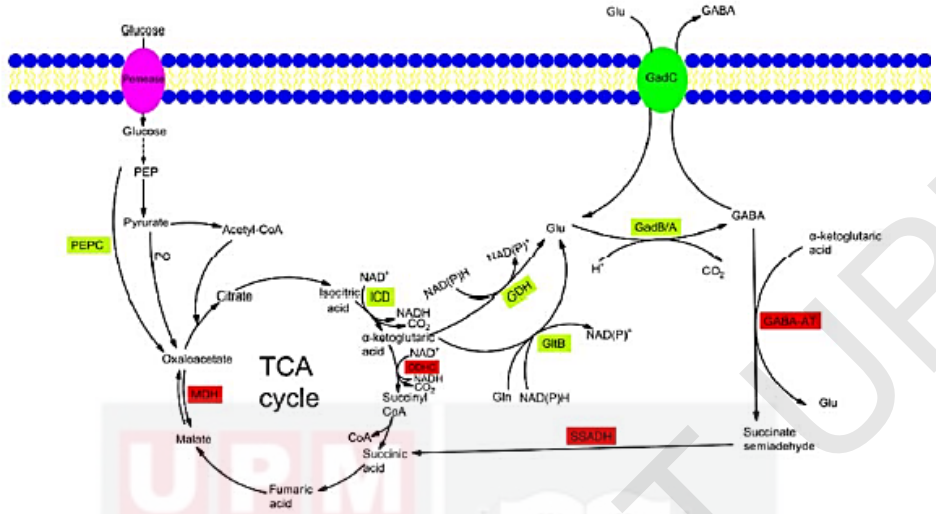


Figure 2.3 : The biosynthetic pathway of GABA by microorganisms. GABA-AT, GABA aminotransferase; GadB/GadA, glutamate decarboxylase; GadC, glutamate: γ-aminobutyrate antiporter; GDH, L-glutamate dehydrogenase; GltB, glutamate synthase; Icd, isocitrate dehydrogenase; MDH, malate dehydrogenase; ODHC, 2-oxoglutarate dehydrogenase complex; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; SSADH, succinate semialdehyde dehydrogenase; TCA cycle, tricarboxylic acid cycle. The expression of enzymes in green font will increase GABA production, and the expression of enzymes in red font will decrease GABA (Cui et al., 2020)

2.3.2 Glutamic acid decarboxylase (GAD) system of LAB

Several species of LAB including the genera *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Streptococcus* have been found to comprise the GAD system (Cui et al., 2020). Although various LAB species, including *Lactobacillus casei*, *Lactobacillus paracasei*, *Leuconostoc mesenteroide*s and *Weissella hellenica* can synthesize GABA, but there is still limited information about the GABA production genes in the genomes (Nejati et al., 2013; Barla et al., 2016; Siragusa et al., 2007). Most of the GAD systems of LAB species are located in the chromosome. However, unlike the other LAB species, the GAD system of *Lactobacillus sakei* WiKim0074 was discovered to be located in the plasmid (Cui et al., 2020). The glutamate-tRNA ligase gene gltX is located at the upstream of the gadB-gadC operon of some species. Besides that, the glutamate synthases encoding gene gltB and gltC are placed closely to the GadB-GadC gene in *Lactococcus lactis* (Wegmann et al., 2007; Gao et al., 2011). Researchers conjectured that these glutamate metabolism related genes could ease the GABA production.

GAD is the main enzyme to carry out the catalytic activity for producing GABA. It is concentrated in the cytoplasm. Most commonly, LAB which can produce GABA are having the GAD-encoding gene. For example, *Enterococcus avium* 352 has three GADs, and *Lactobacillus brevis* strains consist of two recognizable GAD encoding genes (Wu & Shah, 2018; Lyu et al., 2018; Yu et al., 2019; Li et al., 2013). However, there is a study reported that a high GABA-producing *Lactobacillus brevis* CD0817 consisted only of a GadB-GadC operon while the GadA gene was absent (Gao et al., 2019). There was also a significant difference between the GadB gene of *L. brevis* CD0817 and GadB gene of other GABA-producing *L. brevis* strains which consist of 91 % of amino acid sequence identity compared to *L. brevis* CD0817. The results also showed that the GadB gene was mainly affecting the GAD activity.

2.4 Factors affecting GABA biosynthesis by LAB

The rate of GABA production is affected by various factors such as temperature, pH, cultivation time and composition of culture media (Dhakal et al., 2012). The optimization of GABA production can be done based on the biochemical characteristics of GAD of the fermenting microorganisms. According to Figure 2.3, decarboxylation of glutamate in LAB consumes a proton (H^+) and releases the end product GABA. This results in increasing of the pH value of the cytosol and environment. Different GABA-producing LAB will have different optimum fermentation environments due to their GABA system. Therefore, the biochemical traits of the GAD are needed to be characterized to obtain the maximum yield of GABA.

2.4.1 Effect of pH value

The GABA production by LAB is predominantly controlled by pH, which has the most noticeable effect for the fermentation process (Komatsuzaki et al., 2005; Tsai et al., 2006; Yang et al., 2008). The effective pH value for various LAB is varied due to the differences of biochemical properties of the GAD. Hence, the optimal pH value for GABA production is species-dependent (Li et al., 2010b; Yang et al., 2008).

During the GABA fermentation process, pH varies from time to time due to the glutamic acid decarboxylase system in the LAB. Hence, pH value of the medium during the fermentation process should be timely modified or controlled in order to make sure the pH value of the medium is always optimum for the LAB (Lu et al., 2009; Li et al., 2010b; Kim et al., 2009). A study stated that the *Lactobacillus plantarum* DSM19463 produced the maximum GABA, which was 59 μM/h at the pH 6.0 (Di Cagno et al., 2010), whereas *L. plantarum* Y7 was able to produce the maximum GABA concentration (375.29 μg/mL) when the pH of the culture medium was at 6.5 (Kim et al., 2022b). Another work reported *L. plantarum* FBT215 produced the maximum GABA concentration (121.76 μg/mL) when the pH was 7.5 (Kim et al., 2022a). However, increasing the pH of the fermentation

medium to 8.5 resulted in a decrease in GABA concentration (114.75 μg/mL) by *L. plantarum* FBT215. In the meantime, some *L. plantarum* strains preferred lower pH to optimally producing GABA. For instant *L. plantarum* KB 1253 was shown to synthesize the maximum GABA concentration (245.8 mM) with the optimum pH of 4.0 (Nakatani et al., 2022). Meanwhile, when *L. plantarum* MNZ was grown at the optimal pH 4.5, the production of GABA was observed to be increased from 3 to 3.35 fold (Zareian et al., 2013).

Another LAB species, *Lactobacillus paracasei* NFRI 7415 was demonstrated to synthesize the maximum GABA of 210 mM at pH 5.0 (Kumar et al., 2000). Meanwhile, the optimization of the culture medium of *L. paracasei* NFRI 7415 at pH 5.0 by adding pyridoxal-5'-phosphate managed to convert the 500 mM glutamate content into 302 mM GABA (Komatsuzaki et al., 2005). Besides that, the *Lactobacillus paracasei* PF6, *Lactobacillus paracasei* PF8, *Lactobacillus paracasei* PF13, *Lactobacillus plantarum* PF14, *Lactobacillus* sp. strain PF7 and *Enterococcus durans* PF15 that were isolated from cheese did obtain high GABA yield between the pH range of 4.68 and 5.70 (Siragusa et al., 2007). There is another study stating that the *Streptococcus salivarus* subsp. *thermophilus* Y2 synthesized the maximum GABA, which was 7084 mg/L at pH 4.5 (Yang et al., 2008).

Furthermore, to obtain high GABA yield, the activities of glutamic acid decarboxylase should be activated, meanwhile, the activities of GABA-decomposing enzymes such as GABA transaminase and succinic semi-aldehyde dehydrogenase should be impeded (Dhakal et al., 2012). GABA-transaminase will convert GABA to succinic semi-aldehyde by using either pyruvate or α-ketoglutarate as the amino acceptor and succinic semi-aldehyde dehydrogenase will convert the succinic semi-aldehyde to succinate (Shelp et al., 1999; Kumar et al., 2000). Thus, in order to obtain a maximum GABA yield, the pH value also plays an important role in inhibiting the catalytic activities of these GABA-decomposing enzymes.

2.4.2 Effect of temperature

The optimal incubation temperature also has a significant effect on the production of GABA by LAB (Dhakal et al., 2012). This is because the fermentation temperature not only affecting the activity and stability of the biocatalyst but it also affects the thermodynamic equilibrium of a reaction. High cell density and the optimum culture temperature will result in the productive conversion of glutamic acid to GABA (Kim et al., 2009). Commonly, the optimum temperatures for LAB to synthesize the maximum amount of GABA are between 25°C to 40°C (Dhakal et al., 2012).

A study that conducted by Zareian et al. (2013) disclosed that the optimum temperature to increase the GABA yield up to 3.35 folds by *L. plantarum* MNZ was determined at 37°C. Likewise, *L. plantarum* K154 was able to obtain the

maximum GABA yield (154.86 μg/mL) when the cultivation temperature was at 37°C. In addition, *L. plantarum* Y7 was shown to produce the highest amount of GABA (375.29 μg/mL) at 37°C while the concentration of GABA was significantly reduced (79.86 μg/mL) when the temperature was below 30°C (Kim et al., 2022b). The *L. plantarum* GB01-21 strain was reported to attained it optimum GABA production (80.5 g/L) and dry cell weight (2.16 g/L) at a culturing temperature of 35°C. *L. plantarum* BC114 was able to produce the maximum GABA concentration (3.45 g/L) at lower culturing temperature of 30°C (Zhang et al., 2017). Meanwhile, *L. plantarum* DSM19463 produced the most amount of GABA, which was 59 μM/h at temperatures ranging from 30°C to 35°C (Di Cagno et al., 2010).

In the meantime, another LAB species, *Lactobacillus brevis* NCL912 had the optimum growth at the temperature of 35°C and when the temperature was higher than 35°C, the cell growth and GABA production were found to be significantly declined (Li et al., 2010a). Additionally, the optimum temperatures for *L. brevis* GAD and *Lactobacillus* CGMCC 1306 were at 30°C and 37°C, respectively (Ueno, 2000). Besides that, a study reported that the immobilized *L. brevis* cells that was grown within 8 hours of fermentation process managed to synthesize the optimum amount of GABA with corresponding yield of 92 % at the temperature of 40°C (Huang et al., 2007b). Further increase of the temperature decreased the GABA yield by the *L. brevis*. Furthermore, for two different strains of *Lactobacillus lactis*, the highest amounts of GABA corresponding to 310 mg/mL and 439 mg/mL were reached at slightly different optimum temperatures of 33°C (Cao et al., 2010) and 34°C (Lu et al., 2008), respectively.

2.4.3 Effect of the concentration of monosodium glutamate (MSG)

The optimal GABA production by LAB is correlated by the amount of MSG that can be converted to GABA via irreversible decarboxylation reaction (Pannerchelvan et al., 2023). By increasing the concentration of MSG, it enhances the GABA yield by the glutamic acid decarboxylase of LAB (Cui et al., 2020). However, an excessive concentration of MSG impeded the LAB cell growth and consequently lowered the production of GABA (Zhuang et al., 2018; Yang et al., 2008; Villegas et al., 2016). Different LAB are having different optimum concentration of MSG to achieve their maximum GABA yield (Cui et al., 2020).

The MSG concentration was found to significantly affecting the GABA production by *Lactobacillus plantarum* CGMCC 1.234^{T} (Zhuang et al., 2018). By adding 100 mM L-MSG, 721.35 mM GABA was produced by *L. plantarum* CGMCC 1.234^{T} that corrsponding to 7.7 folds higher than the fermentation process without adding MSG. Through the transcriptomic analysis, it showed that MSG enhanced the crucial enzymes of carbohydrate metabolism, amino acid metabolism and fatty acid synthesis. According to Park et al. (2014), the *L. plantarum* K154 strain was able to produce the GABA concentrations of 154.86

μg/mL, 170.42 μg/mL and 201.78 μg/mL at the MSG concentrations of 1 %, 2 % and 3 %, respectively. Another study showed that *L. plantarum* BC114, which was isolated from Sichuan paocai (a Chinese traditional fermented vegetable), produced the maximum GABA concentration of 3.45 g/L, when the MSG concentration of the culture medium was 20 g/L. Besides that, *L. plantarum* EJ2014 was able to synthesize the maximum GABA concentration of 19.8 g/L when the culture medium was supplemented with 2.25 % MSG (Park et al., 2021).

GABA productions by other LAB species such as *L. paracasei* and *L. brevis* were also reported to be increased by the supplementation of glutamate in the medium (Hayakawa et al., 1997; Komatsuzaki et al., 2005; Huang et al., 2007b). In contrast, the GABA yield of *Streptococcus salivarus* subsp. *thermophilus* Y2 did not have a great difference when MSG was added in the range from 10 g/L to 20 g/L (Yang et al., 2008). The maximum GABA yield by *P. pentosaceus* MN12 was measured to be 17.6 mM when the medium was supplemented with 60 mM MSG (Thuy et al., 2021). Nonetheless, the GABA yields were decreased at higher concentrations of MSG in between 90 to 120 mM. Meanwhile, the optimum concentration of MSG for synthesizing the GABA by using *Lactobacillus brevis* CRL 1942 was determined to be 270 mM (Villegas et al., 2016).

2.4.4 Effect of the concentration of pyridoxal 5'-phosphate (PLP)

PLP is the cofactor for the glutamic acid decarboxylase enzyme (Cui et al., 2020). PLP enhances the activity of the glutamic acid decarboxylase resulting in the increase of GABA production. The effect of PLP is varied depending on the concentrations of PLP. A study reported that PLP that was added at 10 or 100 μM concentrations in the initial culture medium was significantly enhanced the GABA yield (Komaztsuzaki et al., 2005).

Lactobacillus plantarum FNCC 260 was reported to produce the highest yield of GABA of 909.2 mg/L after 60 hours of fermentation (Yogeswara et al., 2020). However, the GABA yield was further increased to 945.3 mg/L by adding 0.6 mM PLP, whereas, the addition of 0.1 mM pyridoxine increased the GABA concentration to 969.5 mg/L . Meanwhile, *L. plantarum* NDC75017 that was used to produce GABA-enriched yoghurt produced the maximum GABA yield of 314.56 mg/100g when supplemented with 18 μM PLP and 80 mM of glutamate (Shan et al., 2015). In contrast, the production of GABA by *L. brevis* NCL912 did not increase with the addition of PLP in the fermentative medium (Li & Cao, 2010). This study showed that some LAB can self-synthesize enough PLP to produce GABA.

2.4.5 Effect of fermentation time

Fermentation time is another crucial factors that will affect the GABA yield (Dhakal et al., 2012). For LAB fermentations, GAD is actively involved in the coversion of GABA starting from the late exponential growth phase and reaching the maximum GABA yield at the stationary phase (Hayakawa et al., 2004).

A study reported that *Lactobacillus plantarum* K16 required 96 hours of fermentation period to produce the maximum amount of GABA corresponding to 1000 mg/L (Diez-Gutiérrez et al., 2022). Meanwhile, the LAB strain *L. plantarum* DSM19463 attained the maximum GABA yield of 4.83 mM at the fermentation time of 72 hours (Di Cagno et al., 2010; Higuchi et al., 1997). Yogeswara et al. (2020), reported the strain *L. plantarum* FNCC 260 obtained the highest GABA yield (909.2 mg/L) after 60 hours of incubation. Likewise, *L. plantarum* Taj-Apis362 was also able to produce the maximum GABA concentration (497.97 mM) with the incubation time of 60 hours (Tajabadi et al., 2015). Another study revealed that the strain *L. plantarum* subsp. *plantarum* IBRC10817 produced the highest yield of GABA (295. 04 mg/L) within a shorter incubation time of 30.9 hours (Rezaei et al., 2023).

Furthermore, the fermentations of black raspberry juice by *Lactobacillus brevis* GABA 100 were reported to reach the maximum GABA yields on the fifteenth day of the fermentation process resulted in 25.4 mg/mL GABA under parameters of 25°C, pH 4.0 and 26.5 mg/mL GABA under parameters of 37℃ and pH 5.5 (Kim et al., 2009). Whereas, when the temperature was set at 30℃ and pH value of 3.5, it achieved the optimum GABA yield in a shorter period of twelfth days. Meanwhile, the fermentation of *Lactobacillus brevis* NCL 912 under controlled pH of 5.0, temperature of 32°C and 400 mM initial glutamate followed by a subsequent addition of glutamate at 12 hours (280.70 g glutamate) and 24 hours (224.56 g glutamate) of the fermentation process, required 36 hours to obtain the maximum GABA production (Li et al., 2010a). After the 36 hours of fermentation period, GABA was producing at a very small amount. In the meantime, another LAB species, *Lactobacillus paracasei* NFRI 7415 was showed to produce the maximum GABA yield of 60 mM at the fermentation time of 144 hours (Di Cagno et al., 2010; Higuchi et al., 1997).

2.5 Whole-cell immobilization method

The improvement of productivity and shelf life of probiotics is a major demand for it to be commercially applied in the biotechnology industry. The survivability of probiotic bacteria can be enhanced by various methods such as cell immobilization, choosing the suitable acid and bile resistant strains, application of oxygen impermeable containers, and stress adaptation (Hassan et al., 2019). In particular, immobilization technology has found many practical uses including for pharmaceutical manufacturing, food and beverages industry, bioremediation and biosensor technology (Nemati & Webb, 2011). Generally, immobilization

process can be divided into two types which are enzyme immobilization and whole-cell immobilization (Datta et al., 2013). Whole-cell immobilization can be the alternative to solve several disadvantages of enzyme immobilization method (Chibata & Tosa, 1983). These include the difficulty in obtaining the pure intracellular enzyme while the downstream processing such as cell lysing and purification steps can be very costly. Furthermore, enzyme also can be highly unstable during and after the immobilization process.

Whole-cell immobilization is a method that involved the localising of intact cells onto specified locations or matrices without compromising their biological functions (Kulkarni et al., 2020). To date, various types of whole-cell immobilization methods have been established. These include cell entrapment, microencapsulation, physical adsorption, cross-linking and covalent binding (**Figure 2.4**). Cells are immobilized with the aims for it to be reusable for multiple times while enhancing the productivity (Hassan et al., 2019). Moreover, the immobilization of biomolecules is more advantageous compared to the free biomolecules as the immobilized biomolecules is able to be reused continuously, convenient and simple recovery as well as may offer a lower production cost (Magdy et al., 2014). For example, product recovery from the immobilized cell culture is simpler than the free-suspended culture as there will be no free cells in the product stream hence the cell separation steps such as filtration and centrifugation can be eliminated and reducing the production cost (Nedovic et al., 2011).

The method of physical adsorption is the easiest and most convenient for cell immobilization (Hassan et al., 2019). The biomolecules or cells are attached on the matrix surface (Woodward, 1985; Sugahara & Varéa, 2014). The matrix can be either organic or inorganic. This method is depending on the weak bonds that are formed between the matrix and biomolecules or cells. Examples of the weak bonds are van der Waal's force, hydrogen bond, ionic bond, electrostatic and hydrophobic interaction (Flickinger & Drew, 1999; Jegannathan et al., 2008). The physical adsorption is performed through immersing the solid matrix in the solution that containing biomolecules or cells at the particular conditions of temperature, pH and ionic strength for a certain period of time (Hassan et al., 2019). The immobilization method via physical adsorption has some common advantages and disadvantages as shown in **Table 2.3**.

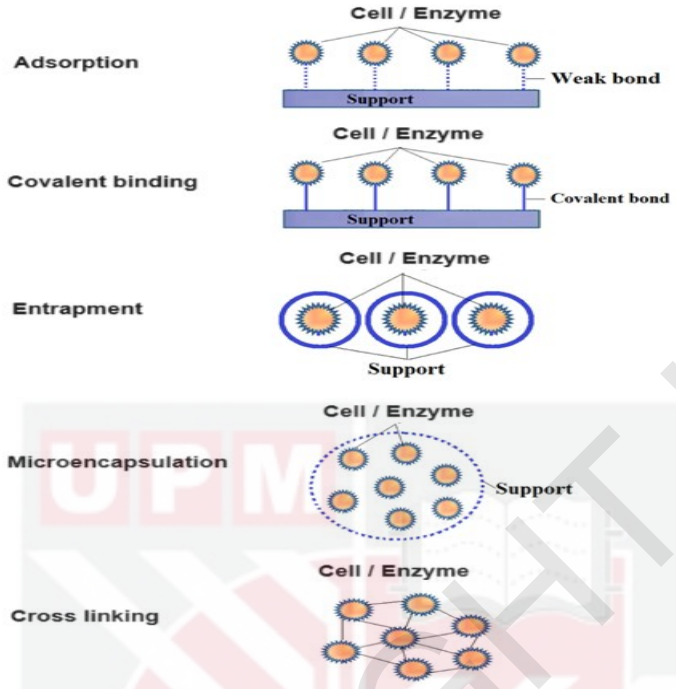


Figure 2.4 : Methods of cell and enzyme immobilization
(Hassan et al., 2019)

Table 2.3 : Benefits and drawbacks of whole cell immobilization by adsorption method

Benefits	Drawbacks
Negligible damage towards the cells Easy Cheap Rapid process Time saving Reversible process	Loss of cells from the support Binding of cells towards support is non-specific Product separation is complicated

(Hassan et al., 2019)

Cell immobilization via physical adsorption can be accomplished by the use of various supports or matrices (Kourkoutas et al., 2006). The desired properties of natural supports include high loading efficiency, possess chemical and mechanical stability, high biocompatibility, wide applicability and low cost (Lou et al., 2021). Immobilization supports or carriers varied from natural origin matrixes to artificial synthetic materials. For examples, supports such as calcium pectate gel and chemically modified chitosan beads were effectively used to immobilize *L. casei* (Kourkoutas et al., 2005). Some other supports used for the immobilization of cells include gluten pellets (Chronopoulos et al., 2002), porous foam glass particles, ceramic beads or porous glass, and poraver beads

(Kourkoutas et al., 2005). In the food-related aspect, even though there are established and well-known natural carriers or supports matrices such as alginate, carrageenan, chitosan, xanthan and gluten pellets (Nedovic et al., 2011), however the search for novel cell support materials are encouraging to obtain more efficient materials that can be best employed for probiotic strains. The use of natural-support materials from food sources such as fruits and vegetables are becoming more common as it created a "food-like system" that is compatible for the immobilization of probiotics or cell starter culture and allows their direct applications in foods, medications and cosmetic products (Mitropoulou et al., 2013; Altieri et al., 2019). Furthermore, natural-support materials are often readily available, low cost and easy to be utilized for cell immobilization (Kourkoutas et al., 2004).

Apple (Kourkoutas et al, 2001; Kourkoutas et al., 2002) and quince pieces (Kourkoutas et al., 2003; Kourkoutas et al., 2005) have been suggested to be used as immobilization supports of yeast strains for the wine production at room temperature and low temperature. Apple pieces was also used for the immobilization of LAB, *L. casei* cells for the production of lactic acid in the probiotic drinks (Kourkoutas et al., 2006). The apple pieces-immobilized LAB helped to increase the production of lactic acid as the immobilized cells were highly stable that allowed it to be stored and reused for multiple times even after 129 days. The protective effect exerted by the fruits or plant immobilizers towards cells is also due to the cellulose content which are made from non-digestible carbohydrates or cellulosic materials (Campaniello et al., 2020). In addition, when consuming, the presence of small and intricate ridges on the surface of fruits or plants can help to protect microbial cells from the rough acidic condition in the stomach. Beside improving the product yield, these food-grade immobilizer materials can result in foods and beverages products with better sensory qualities by providing extra flavours and distinct fruity aromas. For example, the use of grape peels-immobilized yeast for wine making produced better fermentation productivity than the free-cell culture and at the same time improved the wine aroma (Mallouchos et al., 2002). These kind of food grade supports are safe to consume, cheap, ample in nature and acid resistant, meanwhile, the immobilization method is uncomplicated and showing good operational stability as well as having a significant enhancement in product yield (Kourkoutas et al., 2005).

2.6 Freeze drying technology for preparation of freeze-dried probiotics

Freeze-drying technology is a highly efficient dehydration technology that is primarily use by the western European countries before spreading worldwide especially across developed countries including United States, France, England and Japan (Liu et al., 2022). Freeze-drying technology has become the most crucial drying method in food industry and it is extensively applied for preparation of foods that required special conditions for certain activities such as aerospace, navigation, military, mountaineering and exploration. Through the application of the freeze-drying technology, the water in foods is frozen into solid ice crystals,

followed by undergoing the sublimation process, which transforms the solid ice crystals into the gas particles directly in a vacuum (Hua et al., 2010).

As depicted in **Figure 2.5**, the freeze-drying process includes three main stages change of water: quick freezing, sublimation drying and desorption drying (Liu et al, 2022; Han-Shan et al., 2008). In accordance with the theory of the thermodynamic phase equilibrium, the triple point temperature of water is 0.0098°C with the pressure of 4.579 mmHg. During quick freezing, the products will be rapidly frozen. The liquid water will turn into a solid ice. This can avoid the denaturation that caused by heat and effervescence during the vacuum drying (Zhang, 2005). When the pressure is lower than the triple point, the solid ice or frozen product will undergo the sublimation drying process, which cause the direct transformation of solid ice into gaseous water vapour (Guo-Yan et al., 2010; Zhang, 2005). During the sublimation process, the solid ice will not be transformed into liquid water. The dehydration and drying of the product is taken place during the process of sublimation drying, in which the water (approximately 90 to 95 %) will be removed (Liu et al., 2022). After that, the product will undergo the desorption drying as there is a little of water residual left on the surface of the product. Thus, it is necessary to remove the water residual through the desorption drying at a higher temperature. However, the temperature is a critical factor during the freeze-drying process and it cannot be higher than the denaturation temperature.

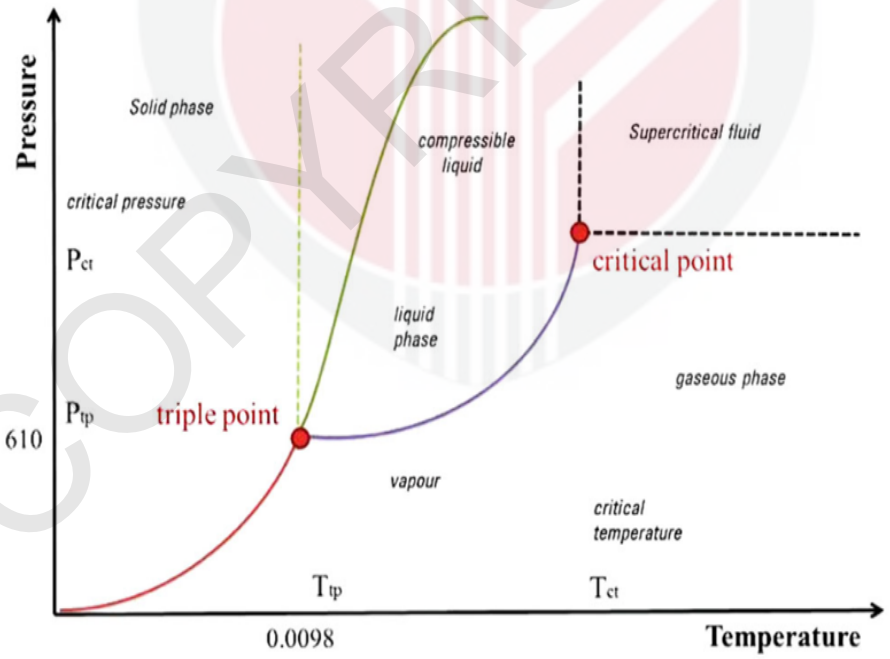


Figure 2.5 : Three-phase diagram of water that involved in the freeze-drying process (Liu et al., 2022)

The freeze-drying technology offers a lot of benefits to the food industry (**Table 2.4**). This drying technology helps to retain the indigenous characteristics of the heat labile food such as colour, flavour, fragrance, taste and appearance as well as avoid the depletion of nutrients (Zeki, 2009; Cheng-Hai et al., 2008). Furthermore, it is very easy to rehydrate the freeze-dried food and easy to be stored for a long period of time due to its low moisture content (Cheng-Hai et al., 2008). Other than the advantages, the freeze-drying technology also has some disadvantages such as high cost and it consumes a very long period of time to complete the process due to its complexity (Hua et al., 2010).

Table 2.4 : Benefits and drawbacks of freeze-drying process

Benefits	Drawbacks
Operate with a low temperature Operate in a closing environment Avoid the contamination Moisture content of the product is well-controlled Quick recovery	High production cost Operating time is long Complexity High maintenance cost Accessible to the interface of ice-water

(Degobert & Aydin, 2021)

Product formulations containing probiotic strains must have been clinically proven to provide the expected benefits and the strains must present in adequate numbers upon administration (Oluwatosin et al., 2022). According to the World Health Organization (WHO) and Food and Agricultural Organization of the United Nations (FAO), probiotic preparations should contain a minimum number of live bacteria of at least 10^6 CFU/g upon consumption (Yuste etal., 2021; Kiepś & Dembczyński, 2022). Nevertheless, the effectiveness and performance of probiotics can be affected due to deprivation of viability over the storage (Broeckx et al., 2017). Cell dehydration is required and it is frequently applied as the final step of probiotic production in order to maintain the probiotic cells in an inactivated form during storage, thereby making sure that there are sufficient viable probiotic cells upon ingested. Among the available drying methods, freeze drying technique is frequently use for drying of various microorganisms including probiotic strains (Kiepś & Dembczyński, 2022). The method is suggested to be applied for drying probiotic cells as it is operated under low temperature and pressure which assist to retain the native biochemical properties and structure as well as the cellular activities (Fatemeh et al., 2011; Perdana et al., 2013). Furthermore, the conditions used for the freeze-drying technique is generally milder than those of spraying drying technique and often resulting in higher cells survival rate (Barbosa et al., 2015).

Despite various advantages, the process of freeze drying will expose microorganisms to stress factors such as deformation, cellular structure damage due the formation of ice crystals, osmotic shock, change of membrane lipids structure, loss of semipermeable properties of cell membranes and denaturation of protein components (Broeckx et al., 2016). Hence, to lessen the cell damage during freeze drying and to obtain high cell viability, the protective agents, which

are known as cryoprotectants, can be applied to probiotic cells before undergoing the freeze drying process. An effective cryoprotectant can coat and protect the embedded probiotic cells easily throughout the freeze drying process and during storage. Selecting the ideal protective agents is very crucial to improve cell survivability during storage (Savini et al., 2010). Commonly, to select the right cryoprotectant, trial-and-error will be performed based on several factors such as effectiveness of protection, availability, cost and physical properties of the final product (Flores-Ramírez et al., 2019). **Table 2.5** summarizes several commonly used cryoprotectants for freeze drying of lactic acid bacteria under certain conditions that resulted in different cell survival rate. Among various cryoprotectant substances, skimmed milk and sucrose are commonly used as cryoprotectants for microorganisms especially probiotics (Hubalek, 2003). Skimmed milk which is milk proteins can build layers around the cells walls which offers protection against ice crystal and allow for pH stabilization (Yeo et al., 2018). Skimmed milk can also avoid the cell damage by stabilizing the cell membrane while sucrose has the ability to prevent harmful eutectic fluid cell freezing making them a suitable synergistic combination as a cryoprotectant for high viable cell counts and survival rate (Carvalho et al., 2004; Gisela et al., 2014).

Table 2.5 : Survival rate of freeze-dried LAB prepared under different freeze-drying conditions and various cryoprotectants

Lactic acid bacteria	Type of cryoprotectants	Conditions of freeze-drying	Survival rate (%)	References
L. plantarum	10 % skim milk	Freeze-dried at −35°C for 24 h under the pressure of 300 mTorr	91	Oluwatosin et al. (2022)
L. plantarum	11 % skim milk	Freeze-dried under pressure of 50 mTorr for 48 h at room temperature	93	Carvalho et al. (2003)
L. plantarum TISTR 2083	10 % Skim milk	NA	92.17	AL et al. (2021)
L. plantarum TISTR 2075	15 % plant protein and 5 % trehalose	Freeze-dried at −50°C under the pressure of 0.110 mbar	98.13	Savedboworn et al. (2017)
L. curvatus N19	20 % skim milk, 3.57 % lactose and 10 % sucrose	Freeze-dried for 18 h under the pressure of 0.061 mbar	98.59	Gul et al. (2020)
L. fermentum FXJCJ6-1	Sucrose (mass ratio 1:1 towards the cells)	Freeze-dried at −30°C for 30 h, then secondary-dried at 25°C for 20 h under the pressure of 0.2 mbar	38.21	Cui et al. (2022)
L. lactis Sr. 3.54	10% skimmed milk and 10 % sucrose	Freeze-dried for 24 hours at a pressure of 0.1Pa	62%	Berner & Viernstein, (2006)
L. salivarius W13	10 % skim milk, 10 % sucrose	NA	59.5	Yeo et al. (2018)
Streptoccous termophilus 937	8 % skim milk, 6 % sucrose	NA	90.59 %	Di et. al., 2022

*NA indicates not available

CHAPTER 3

MATERIALS AND METHOD/ METHODOLOGY

3.1 Materials, chemicals, and reagents

de Man Rogosa Sharpe (MRS) broth and agar, Mueller-Hinton agar and nutrient broth and agar were purchased from Oxoid (Massachusetts, United States). L-glutamic acid monosodium glutamate salt monohydrate, γ-aminobutyric acid, pyridoxal-5-phosphate, *p*-aminodimethylaniline oxalate, boric acid, sodium tetrabonate, oxgall, 2,2-diphenyl-1-picrylhydrazyl (DPPH), phenol, iron (III) chloride, skim milk and sucrose were purchased from Sigma-Aldrich™ (St. Louis, MO, USA). Glucose was purchased from MERCK (Darmstadt, Germany). Ninhydrin was purchased from Macklin® (Shanghai, China). Sodium potassium tartrate, sodium chloride, hydrochloric acid, chloroform, glycerol, methanol and hydrogen peroxide were purchased from Chemiz (Selangor, Malaysia). 3,5-dinitrosalicyclic acid was purchased from Alfa Aesar (Massachusetts, United States). Sodium hydroxide, 99 % ethanol, crystal violet, Gram's iodine, safranin, phosphate-buffered saline (PBS) (pH 7.4) and toluene were purchased from R&M Chemicals (Selangor, Malaysia). Acetone and acetic acid were obtained from EMSURE® (Darmstadt, Germany). Ethyl acetate and n-butanol were obtained from Systerm® (Selangor, Malaysia). Onion (*Allium cepa*), taro (*Colocasia esculenta*), bell pepper (*Capsicum annuum*), sweet potato (*Ipomoea batatas*) and okra (*Abelmoschus esculentus*) were purchased from Whole Foods Express (Selangor, Malaysia). TLC $60F_{254}$ aluminium sheet was purchased from Merck (Darmstadt, Germany).

3.2 Methods

3.2.1 Glycerol stocks of *L. plantarum* B13 strain

L. plantarum B13 strain that was used in this research project was acquired from Bioprocessing and Biomanufacturing Research Centre (BBRC), Universiti Putra Malaysia. The strain was previously isolated from budu (fermented fish sauce), a Malaysian traditional fermented food. The whole-genome sequence of this bacterium has been deposited in NCBI under GenBank accession number of ON482178.1 and Microbial Culture Collection unit (UNiCC), UPM: UPMC 1492 which belongs to *Lactiplantibacillus plantarum* B13. The stock culture of *L. plantarum* B13 that was stored at -20°C was taken out from the freezer. Then, the stock culture was being thawed and 100 μL of the stock culture was transferred into 10 mL de Man Rogosa-Sharpe (MRS) broth media and incubated at 37°C for 48 hours in an incubator (SI-50D, Protech, Malaysia) under an aerobic condition. After that, the culture was transferred onto MRS agar plate and incubated inside an incubator (SI-50D, Protech, Malaysia) for 48 hours at 37°C without oxygen limitation. Then, the single colony was picked and transferred into new MRS broth and incubated again for another 48 hours at

37°C without oxygen limitation. Lastly, the glycerol stock was completely prepared by mixing 80 % (v/v) glycerol with the cell culture in the ratio of 1:1 under sterile condition followed by storing at -20°C for further usage.

3.2.2 Inoculum preparation

The 1% inoculum was prepared by transferring 100 μL of stock culture into 10 mL of MRS broth. The MRS broth contained 100 μL of stock culture was then incubated at 37°C for 24 hours. After overnight incubation, the inoculum was ready to be used in further experiments.

3.2.3 Substrate preparation

a) DNS solution

3,5-DNS and sodium potassium tartrate solution were prepared separately. For DNS solution, 2 M sodium hydroxide was initially prepared by dissolving 8 g of sodium hydroxide in 100 mL of distilled water, followed by dissolving 15 g of DNS reagent in 30 mL of 2 M sodium hydroxide, whereas the sodium potassium tartrate solution was prepared by dissolving 45 g of sodium potassium tartrate in 75 mL of distilled water. Lastly, the DNS solution was mixed with sodium potassium tartrate solution, followed by adding distilled water into the mixture to make the final volume of the mixture to become 150 mL.

b) DPPH solution

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was freshly prepared by dissolving 6 mg DPPH in 100 mL methanol. The mixture solution was then stirred to ensure the DPPH was fully dissolved in the methanol.

c) Mixture of cryoprotectant solution

A mixture of 10 % (w/v) skim milk and 10 % (w/v) sucrose was prepared and being used as the cryoprotectant. 10 g skim milk powder and 10 g sucrose were mixed with 80 mL of distilled water, followed by topping up with distilled water until the final volume of the mixture achieved 100 mL. Then, the mixture was stirred to ensure both skim milk and sucrose were completely dissolved. Lastly, the mixture was autoclaved under 121°C for 5 minutes.

3.3 Analyses

3.3.1 Bacterial growth

3.3.1.1 Optical density (OD)

A volume of 1 mL of the sample was transferred from the fermentation medium to a cuvette, followed by using the UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) to measure the absorbance of the sample under the wavelength of 600 nm for the determination of cell growth. The absorbance readings were taken in triplicate.

3.3.1.2 Colony forming unit (CFU)

The samples were serial diluted, followed by inoculation on the agar by using spread plate method (Buck & Cleverdon, 1960). Then, the agar plates were incubated at 37°C for 48 hours. After incubation, the cell enumeration was performed. Valid cell counts ranged between 25 and 250 colonies. The agar plates with more than 250 colonies are considered as "too numerous to count" (TNTC), whereas the agar plates with less than 25 colonies are considered as "too few to count" (TFTC) (Murray & Baron, 2007). The number of cells per mL of the original cell culture (CFU/mL) was enumerated by using the formula as below:

$$\text{Number of cells per mL}\left(\frac{\text{CFUs}}{\text{mL}}\right)=\frac{\text{Number of colonies} \times \text{dilution factor}}{0.1\ \text{mL}}$$

3.3.2 Characterization of *L. plantarum* B13 strain

The procedures for the characterization of LAB strains were carried out according to Wu et al. (2018). 100 μL of the stock culture was transferred into MRS broth and incubated for 48 hours at 37°C. Then, a single loop of the culture was streaked on the MRS agar plate followed by 48 hours incubation at 37°C. Single colonies were used for running the gram staining, catalase and oxidase tests in order to characterize the bacteria as LAB.

3.3.2.1 Catalase test

This test used the slide method according to Cappucinno and Welsh (2018). A single colony from the MRS agar plate was transferred and smeared on a drop of water on the glass slide. Next, the glass slide was put into a Petri dish, followed by a drop of 3 % (w/v) hydrogen peroxide was put on the sample. Then, the Petri dish was immediately covered. After that, the observation was carried

out for the immediate formation of bubbles. The emergence of bubbles indicates catalase-positive strains and vice versa.

3.3.2.2 Oxidase test

This test applied the plate method according to Cappucinno and Welsh (2018). Either 2 or 3 drops of the p-aminodimethylaniline oxalate was placed on the surface of a single colony and the colour change was observed. The colour would change from pink to purple in 10 to 30 seconds if the strain is oxidase-positive, whereas, if the strain is oxidase-negative, there is no colour change for the colony.

3.3.2.3 Gram staining test

By following the procedure that stated by Cappucinno and Welsh (2018), a single colony from MRS agar plate was transferred and smeared on a drop of water on the glass slide. Then, the smear was air dried and heat-fixed. After that, the smear was stained with crystal violet and left for 1 minute. Then, Gram's iodine was washed off with tap water. Next, 95 % alcohol was applied drop by drop as a decolorizing agent for washing off the colour stain on the smear until it was clear. Then, the smear was washed again by tap water, followed by adding safranin as the counterstain and left it for 45 seconds. After that, the smear was washed again with tap water. Then, the smear was air dried, and it was ready to be observed under a light microscope (CX21, Olympus, Japan) with an oil immersion for the gram staining test. Gram positive cells are purple in colour, whereas gram negative cells are pink in colour.

3.3.2.4 *In vitro* probiotic characterization

a) Acidic pH, bile salt and phenol tolerance test

According to the procedure stated by Mohd Yusof et al. (2020), the inoculum was initially prepared by transferring 100 mL of stock culture into 10 mL of MRS broth. The MRS broth contained 100 mL of stock culture and was then incubated at 37°C for 24 hours. For the acidic pH tolerance test, 1 mL of overnight culture was transferred into 9 mL of MRS broths with pH adjusted to 2.5 and 3.5 by using hydrochloric acid, followed by incubation at 37°C for 4 hours.

For bile salt tolerance test, the culture which was incubated overnight was being transferred into MRS broths that contained 0.1 %, 0.3 % and 0.5 % (w/v) oxygall, followed by incubation at 37°C for 4 hours. The serial dilution was then being carried out and the diluted samples were inoculated onto MRS agar by using spread plate method to count the number of viable cells (colony forming units (CFU)) at 0 hour and 4 hours. After that, the number of viable cells was used for calculating the survival rate of the cells by using the formula as shown below:

$$\text{Survival rate (\%)} = \frac{\text{Number of viable cells survived (CFU/mL)}}{\text{Number of initial viable cells inoculated (CFU/mL)}} \times 100$$

Moreover, for the phenol tolerance test, the overnight culture was inoculated into 9 mL MRS broths with 0.2 % and 0.5 % (w/v) phenol. The MRS broth without phenol was used as a control in this experiment. Then, the cultures were incubated at 37°C for 24 hours. After that, the readings of OD_{600} were recorded by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany). Next, these three different cultures were inoculated onto MRS agar by using spread plate method and incubated for 24 hours. Then, the viable cells were counted. These three experiments were conducted in triplicate, along with the calculation of each sample's standard deviations (**Appendix A3** and **A4,** respectively) and mean values.

b) Cell surface hydrophobicity and cellular autoaggregation

These experiments were conducted by using the procedure of microbial adhesion to solvents (MATS) that as reported by Mohd Yusof et al. (2020) with some minor modifications. The culture was initially prepared and incubated at 37°C for 24 hours. Then, the overnight culture was centrifuged (Microfuge® 16, Beckman Coulter, USA) at 2097 xg for 9 minutes, followed by washing twice with phosphate-buffered saline (pH 7.4). The pellets were collected and mixed with phosphate-buffered saline to achieve the absorbance reading of 0.8 at 600 nm (A_0) that was at an early logarithmic growth phase. This was because the cells at early logarithmic growth phase would able to show a more distinguishing adhering ability towards various liquid hydrocarbons compared to the cells at stationary phase (Rosenberg et al., 1980). Cells grown to early exponential phase will show a low affinity towards hexadecane and xylene and non towards octane, whereas cells that was grown to stationary phase will show the same adhering ability towards all three hydrocarbons. This is due to the cell biomass, certain cell mixtures and hydrophobic cell components. Then, the solvent and culture suspension were mixed with the ratio of 1:1 and vortexed for 2 minutes. Next, the mixture was phase split up at 37°C for 30 minutes. After that, the aqueous phase was collected, and the absorbance was taken under 600 nm (A_1). In this assessment, three different solvents were used: toluene (an apolar solvent), chloroform (monopolar and acidic solvent) and ethyl acetate (monopolar and basic solvent). The following formula was used for calculating the percentage of hydrophobicity:

$$\text{Cell surface hydrophobicity (\%)} = \frac{[\text{Initial optical density (A0)} - \text{Final optical density (A1)}]}{\text{Initial optical density (A0)}} \times 100$$

According to Mohd Yusof et al. (2020), for the experiment of cellular autoaggregation, the overnight grown culture was centrifuged (Microfuge® 16, Beckman Coulter, USA) with 2097 xg for 9 minutes, followed by washing twice with phosphate-buffered saline. Then, the pellets were resuspended with

phosphate-buffered saline to get the absorbance reading that was approximately 1.0 at 600nm (A_0) for standardizing the number of cells (approximately 10^8 to 10^9 cfu/mL). Next, the culture suspension was vortexed for 30 seconds and incubated at 37°C. After that, the OD_{600} readings were taken and recorded by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) at certain time points (1, 4 and 24 hours). The calculation formula for percentage of cellular autoaggregation was as shown below:

$$\text{Autoaggregation (\%)} = \frac{[\text{Initial optical density (A0)} - \text{Final optical density (A1)}]}{\text{Initial optical density (A0)}} \times 100$$

Both experiments were conducted in triplicate, along with the calculation of each sample's standard deviation (**Appendix A5**) and mean values.

c) Exopolysaccharide (EPS) production

According to Mohd Yusof et al. (2020), the MRS agar was pre-added with 5 % (w/v) glucose, followed by transferring *L. plantarum* B13 onto it. Then, the MRS agar with *L. plantarum* B13 was incubated at 37°C for 24 to 48 hours. By applying the method of loop touch, the sticky or viscous characteristics of the strain was observed by naked eyes to ascertain the EPS-producing strain.

d) Antibacterial activity

The experiment of antibacterial activity was performed by applying agar well diffusion method in accordance with Mohd Yusof et al. (2020) with some minor modifications. The pathogens that were used in this experiment were the Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*). These pathogens were acquired from the Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia.

First, all the pathogens (*S. aureus*, *B. subtilis*, *E. coli* and *S. typhimurium*) were prepared by culturing them on the nutrient agar, followed by incubation at 37°C for 24 hours. Then, a single colony of each pathogen was looped and inoculated into 10 mL nutrient broth, and subsequently incubated at 37°C for 24 hours. After that, each pathogen was inoculated onto the whole surface of Mueller Hinton Agar (MHA) by using the lawn culture method. Then, the sterile pipette tip was used to make the well with approximately 8 mm of diameter on the MHA agar. Next, 10 mL of *L. plantarum* B13 culture suspension was centrifuged (Microfuge® 16, Beckman Coulter, USA) at 9000 x g with 10 minutes and filtered. 20 μL cell free supernatant was pipetted into the well. Meanwhile, 20 μL of ampicillin was used as the positive control, whereas 20 μL of sterile distilled water was used as the negative in this test. The appearance of the clear zone, also known as the inhibition zone was observed and the diameter was measured and recorded in

millimetres (mm). The diameter of inhibition zone defines the bacteria as resistant (≤9 mm), moderately sensitive (10-11 mm), or sensitive (≥12 mm) to the sample (Sarker et al, 2014). This experiment was conducted in triplicate.

3.3.3 Determination of antioxidant properties by DPPH free radical scavenging

The antioxidant properties of the sample were determined by using the method that was described by Mohd Yusof et al. (2020). Firstly, *L. plantarum* B13 was incubated at 37°C for 24 hours. Then, the overnight cell culture was centrifuged (Microfuge® 16, Beckman Coulter, USA) at 5000 rpm for 10 minutes. The cell-free supernatant was transferred into a new Eppendorf tube for further analysis. Next, the DPPH solution was freshly prepared (as described in **Section 3.2.3 (b)**). After that, 10 mL cell-free supernatant (CFS) of *L. plantarum* B13 and 0.5 % (w/v) ascorbic acid (as a positive control) were added into 190 mL methanol, respectively and mixed well. Subsequently, 200 mL DPPH solution was added into the mixture of samples (denoted as A_{test} for either for CFS or ascorbic acid) and methanol, respectively, followed by mixing thoroughly with the vortex mixer (RX3, VELP Scientifica, Italy). The mixtures were left in a dark place at room temperature for 30 minutes. The control (denoted as $A_{control}$) was prepared by mixing DPPH with methanol at a volume ratio of 1:1, whereas the methanol was used as the blank for this experiment. After 30 minutes of incubation, the absorbance was taken by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) with the wavelength of 517 nm. This experiment was conducted in triplicate. The percentage of DPPH free radical scavenging activity (RSA) was determined by using the formula as shown below:

$$\text{DPPH free RSA}(\%) = \frac{(\text{Acontrol} - \text{Atest})}{\text{Acontrol}} \times 100$$

3.3.4 Screening the effects of fermentation conditions on GABA production

A volume of 80 mL MRS broth was prepared in a 250 mL shake flask and pre-added with 1 % (w/v) monosodium glutamate (MSG) and 0.5 mM pyridoxal-5-phosphate (PLP). The pH was adjusted to pH 6.0 before sterilization. Then, 10 % (v/v) stock culture of *L. plantarum* B13 was transferred into MRS broth. Another fermentation medium with the same conditions was prepared as a control without transferring 10 % (v/v) stock culture of *L. plantarum* B13 into it. After that, both fermentation mediums (with and without having *L. plantarum* B13 inside) were incubated in an incubator shaker (IS-971R, Lab Companion, USA) with the agitation speed of 150 rpm and temperature of 37°C for 96 hours. Sampling was carried out for every 3 hours' time intervals at the first 24 hours, followed by every 24 hours' time intervals until 96 hours. After sampling, the data were used for the determination of cell growth, GABA concentration and residual glucose concentrations. Then, the graphs (growth profile of *Lb. plantarum* B13, GABA yield against time and residual glucose concentration against time) were

plotted. Besides that, the correlation graph between log (CFU/mL) and OD_{600} for the cell profile of *L. plantarum* B13 was also plotted for further evaluation and measurement by referring to the graph equation.

Next, the experiments for determining the effects of several fermentation parameters such as incubation time, temperature, pH, MSG concentration, PLP concentration and glucose concentration were conducted respectively by using the method of One-Factor-At-Time. The data were then being collected and recorded for further investigation and analysis to determine the optimum parameters for GABA production. The standard graph of glucose and GABA concentration were initially prepared (as shown in **Appendix A1** and **Appendix A2,** respectively).

a) Effect of the incubation time on GABA production

To perform the fermentation by using *L. plantarum* B13, the fermentation conditions were fixed with the initial pH 6.0, temperature of 37°C, 1 % (w/v) MSG concentration, 0.5 mM PLP concentration and agitation speed of 150 rpm. The fermentation was carried out for the total time of 72 hours. Then, the data were collected from the experiment within the optimum range of fermentation time, from 48 hours to 72 hours (based on the results that obtained earlier from cell profiling of**Section 3.3.4**). The cell growth and GABA concentration that produced from the fermentation process were recorded with the sampling time intervals of every 6 hours (the sampling points were 48 hours, 54 hours, 60 hours, 66 hours and 72 hours of fermentation time).

b) Effect of the temperature on GABA production

To perform the fermentation by using *L. plantarum* B13, the fermentation conditions were fixed with the incubation time of 66 hours, initial pH 6.0, 1 % (w/v) MSG concentration, 0.5 mM PLP concentration and agitation speed of 150 rpm. However, different temperatures (33°C, 35°C, 37°C, 39°C and 41°C) were used to conduct the experiments, respectively. The cell growth and GABA production were evaluated for each sample after fermentation time of 66 hours to determine the optimal temperature.

c) Effect of pH on GABA production

To perform the fermentation by using *L. plantarum* B13, the fermentation conditions were fixed with the incubation time of 66 hours, temperature of 37°C, 1 % (w/v) MSG concentration, 0.5 mM PLP concentration and agitation speed of 150 rpm. However, different pH values (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) were used to conduct the experiments, respectively. The cell growth and GABA production were evaluated for each sample after fermentation time of 66 hours in order to determine the optimal pH value.

d) Effect of MSG concentration on GABA production

To perform the fermentation by using *L. plantarum* B13, the fermentation conditions were fixed with the incubation time of 66 hours, initial pH 6.0, temperature of 37°C, 0.5 mM PLP concentration and agitation speed of 150 rpm. However, different MSG concentrations (1 %, 3 %, 5 %, 7 % and 9 % (w/v)) were used to conduct the experiments, respectively. The cell growth and GABA production were evaluated for each sample after fermentation time of 66 hours in order to determine the optimal MSG concentration.

e) Effect of PLP concentration on GABA production

To perform the fermentation by using *L. plantarum* B13, the fermentation conditions were fixed with the incubation time of 66 hours, initial pH 6.0, temperature of 37°C, 1 % (w/v) MSG concentration and agitation speed of 150 rpm. However, different PLP concentrations (0 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM and 0.9 mM) were initially added into the different media, respectively. Then, the fermentation was being conducted. The cell growth and GABA production were evaluated for each sample after fermentation time of 66 hours to determine the optimal PLP concentration.

f) Effect of glucose concentration on GABA production

To perform the fermentation by using *L. plantarum* B13, the optimized fermentation conditions were used (incubation time of 66 hours, initial pH 5.5, temperature of 35°C, 5 % (w/v) MSG concentration and 0.7 mM PLP concentration) with the agitation speed of 150 rpm. However, different glucose concentrations (20 g/L, 40 g/L, 60 g/L, 80 g/L and 100 g/L) were used to conduct the experiments, respectively. The cell growth, GABA production and reducing sugar concentration were evaluated for each sample at the time intervals.

3.3.4.1 Determination of glucose concentration using 3,5-Dinitrosalicylic acid (DNS) method

According to the method that was stated by HiMedia Laboratories (n.d.), the consumption of glucose by *L. plantarum* B13 was identified and determined by the 3,5-dinitrosalicylic acid (DNS) method. The DNS solution was initially prepared (as described in **Section 3.2.3 a**). The standard curve was prepared by mixing the glucose with distilled water at different volumes for making the total volume of 200 mL. Next, 1 mL of DNS solution was added into the diluted glucose solutions and mixed well. Then, the mixture was placed in the boiling water bath for 5 minutes. After that, the mixture was left to cool for 5 minutes. Lastly, the absorbance of the solutions with different glucose concentrations was measured by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) with the wavelength of 540 nm. For the DNS analysis of samples, the fermentation samples were initially centrifuged (Microfuge® 16, Beckman Coulter, USA) at 9000 x g for 10 minutes. Then, the supernatant was collected.

Next, the procedure that applied for the preparation of the standard curve was repeated by replacing the glucose solutions with the supernatant samples in order to determine the glucose concentration of the samples. The glucose concentration of the samples was then calculated based on the equation of the standard curve (as shown in **Appendix A1**).

3.3.4.2 GABA analysis

GABA that was produced by *L. plantarum* B13 through the fermentation process was evaluated and analysed by using thin layer chromatography (TLC) and colorimetric estimation methods. The samples were collected at certain time points of the fermentation process. Then, the samples were centrifuged at 9000 x g for 10 minutes by using a benchtop centrifuge (Microfuge® 16, Beckman Coulter, USA). After that, the cell pellets were discarded, whereas the supernatant was collected for further analysis.

a) Thin layer chromatography (TLC)

TLC was used to qualitatively determine the GABA yield following the method described by Rayavarapu et al. (2021). Firstly, 2 μL of standard GABA solution and 2 μL of the supernatant were spotted on TLC $60F_{254}$ aluminium sheet. The 2 μL of standard GABA solution was used as the positive control. Then, the TLC sheet was soaked in the running solvent (mobile phase) composed of n-butanol, acetic acid and water with the ratio of 5:3:2. After immersing in the running solvent, the TLC sheet was dried at room temperature and sprayed with 1 % (w/v) ninhydrin reagent (1 % (w/v) ninhydrin dissolved in 99 % ethanol), followed by incubation at 60°C for 30 minutes in the dryer (FDD-720, Protech, Malaysia). The emergence of red colour spots indicated the presence of GABA. The red spot that appeared on the column of supernatant was compared with the standard GABA solution (positive control) (as shown in **Appendix A7**).

b) Colorimetric estimation of GABA

The GABA spots (red spots) that appeared after running TLC were further to be used in colorimetric estimation methods (Rayavarapu et al., 2021). First, the GABA spots were determined and scrapped into powder form. The powder was then put into a glass tube that contained 3 mL borate buffer (pH 7) and 0.5 mL ninhydrin reagent (0.8 % (w/v) ninhydrin dissolved in acetone), followed by vortexing using a vortex mixer (RX3, VELP Scientifica, Italy). Next, the glass tube contained 3 mL borate buffer (pH 7), 0.5 mL ninhydrin reagent and the powder were incubated in the water bath (WB-22, WiseBath®, South Korea) at 70°C for 20 minutes. Then, the absorbance of the sample was measured by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) with the wavelength of 570 nm. The standard curve of GABA was prepared by using the standard GABA compound with different concentrations (0, 2, 4, 6, 8 and 10 mg/mL). The standard curve was used to estimate and determine the GABA concentration that was contained in the samples (as shown in **Appendix A2**).

Besides that, the mixture solution composed of 3 mL borate buffer and 0.5 mL ninhydrin reagent was used as the blank in this experiment.

3.3.5 Cell immobilization on natural supports and repeated batch fermentation

3.3.5.1 Cell immobilization

There were five different vegetables (okra, onion, potato, taro and bell pepper) being selected to act as the natural supports for cell immobilization. According to Kourkoutas et al. (2005), each sample was cut into small pieces with the size of 1 cm^3 (approximately 1 g) (as shown in **Appendix A9**). Then, each small piece of the sample was sterilized under the temperature of 121°C for 15 minutes (as shown in **Appendix A8**). Next, the sterilized small pieces of different vegetable samples (okra, onion, garlic, potato, taro and bell pepper) were transferred into the liquid culture containing 1 % of active culture (~9 log CFU/mL) of *L. plantarum* B13, respectively with the ratio of 1:2, followed by incubation at 37°C for 15 hours without agitation. After incubation, the fermented liquid was decanted, whereas the immobilization supports were washed twice with MRS broth. The number of cells that was successfully immobilized on each immobilization support was determined by using the cell count method based on the colony forming units (refer to **Section 3.3.1.2**). The formula of cell retention was as shown below:-

$$\text{Cell Retention (\%)} = \frac{\text{Initial colony forming units} - \text{Final colony forming units}}{\text{Initial colony forming units}} \times 100$$

3.3.5.2 GABA fermentation by immobilized *L. plantarum* B13 cells

The immobilization supports were transferred into the optimum fermentation medium (initial pH 5.5, 5 % (w/v) MSG concentration, 0.7 mM PLP concentration and 60 g/L glucose concentration) respectively and incubated at 35°C for 45 hours without agitation. After fermentation, the samples were collected and centrifuged (Microfuge® 16, Beckman Coulter, USA), followed by the cell supernatants were stored at -20°C for further analysis. The best immobilization support was selected based on the cell retention and GABA yield, for further used in the repeated batch fermentation.

3.3.5.3 GABA fermentation by repeated batch fermentation

For the repeated batch fermentation, the immobilized cells were collected from the fermentation medium of the previous batch, followed by washing twice with 0.5 mL MRS broth. After washing, the immobilized cells were transferred into a

new MRS medium to run the subsequent batch of fermentation. The viable cell count, GABA and lactic acid productions, pH changes were evaluated for each sample at the time intervals. In addition, the immobilized biocatalysts used in the repeated batch fermentation (before fermentation and the final batch of fermentation) were viewed by scanning electron microscope (SEM).

3.3.5.4 Lactic acid analysis

By using the method described by Borshchevskaya et al. (2016), the lactic acid concentration was measured by spectrophotometric method. For plotting the standard curve, lactic acid solutions with different concentrations were prepared. Then, a volume of 25 μL lactic acid solution with different concentrations was mixed with 1 mL of 0.2 % (w/v) iron (III) chloride solution respectively at room temperature. Then, the mixtures were mixed thoroughly by using the vortex mixer (RX3, VELP Scientifica, Italy). After that, the readings were taken by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) with the wavelength of 390 nm. For determining the lactic acid concentration of the centrifuged sample, the same procedures as the preparation of standard curve were applied by replacing different concentrations of lactic acid solution with the centrifuged samples. The centrifuged samples were diluted 5-fold by using the distilled water. The concentrations of lactic acid were determined by using the standard curve (as shown in **Appendix A22**).

3.3.5.5 pH changes analysis

According to JoVE Science Education Database (2023), the pH electrode of the Eutech pH 700 Meter (Thermo Fisher Scientific Inc, Massachusetts, US) was initially calibrated by using the standard buffer solutions with known pH which covered the range of the pH values that was desired for the measurement. Then, the electrode was rinsed with distilled water and dried with a piece of Scott paper. After that, the electrode was immersed in the fermented samples until a steady reading was obtained. The reading was then recorded.

3.3.5.6 Scanning electron microscopy (SEM) analysis

Firstly, the immobilized biocatalysts were washed with the MRS broth containing 1 % (w/v) of MSG. The immobilized biocatalysts were dried at 30°C for 24 hours. The dried immobilized biocatalysts were then coated with gold in the BAL-TEC SCD 005 Sputter coater for 2 minutes. After coating, the dried immobilized biocatalysts were observed and investigated by using the JEOL JSM IT-100 scanning electron microscope.

3.3.6 Freeze drying of free-cells and immobilized *L. plantarum* B13

The free-cells and onion-immobilized *L. plantarum* B13 were initially prepared in the optimum fermentation medium (initial pH 5.5, 5 % (w/v) MSG concentration, 0.7 mM PLP concentration and 60 g/L glucose concentration) and incubated at 35°C for 45 hours without agitation. After fermentation, the samples (free-cells and onion immobilized *L. plantarum* B13) were collected and mixed with the mixture of cryoprotectants. The mixture of cryoprotectants that was used in this experiment was composed of 10% (w/v) skim milk and 10% (w/v) sucrose based on the study that proposed by Yeo et al. (2018).

Prior to freeze drying, according to the method proposed by Dimitrellou et al. (2016), the onion-immobilized *L. plantarum* B13 were transferred into the sterile bottles and immersed in the mixture of cryoprotectants (ratio 1:1). Then, the immobilized cells were left to immerse in the mixture of cryoprotectants for 1 hour at 20°C. Meanwhile, for the free-cells, it was separated into 2 different samples, with and without mixing with the mixture of cryoprotectants. According to Cui et al. (2022), the free-cells were mixed thoroughly with the mixture of cryoprotectants with the ratio of 1:1.

After that, all the samples (FCP: free-cells with postbiotic only; FC: free-cells with postbiotic and cryoprotectant; and IC: cells with postbiotic and cryoprotectant) were collected and put into the freeze-drying machine (FD-30F-RE, LabFreez, China). In this experiment, the free cells without mixing with cryoprotectant (FCP) was prepared in order to show that the cryoprotectant mixture was playing its role to protect the cells from freeze-drying process. Thus, the immobilized cells without mixing with cryoprotectant was not being prepared. The samples were frozen at -50°C for 4 hours, followed by primary drying at -30°C for 30 hours with the pressure below 5 Pa (Cui et al., 2022). After that, the samples were dried at 25°C for 20 hours with the pressure below 5 Pa. After the freeze-drying process, the samples were collected and stored at 4°C to be used for the storage study.

3.3.6.1 Rehydration of the freeze-dried free and immobilized *L. plantarum* B13

According to Prapa et al. (2023), for the rehydration of onion-immobilized *L. plantarum* B13, the immobilized cells were mixed with the sterilized water at a ratio of 1:9 and left for 1 hour at 25°C to 30°C. For the rehydration of free-cells *L. plantarum* B13, the free cells were mixed with the sterilized water which was the same volume as the initial volume (prior freeze-drying) and left for 15 minutes at room temperature.

3.3.6.2 Moisture content analysis

As described by Shamekhi et al. (2012), the moisture content analysis was conducted by drying 1 g of the samples in the hot-air oven (INE 400, Memmert, Germany) at 100°C for 4 hours. The percentage of moisture content was calculated by using the formula as shown below:

$$\text{Moisture content}(\%) = \frac{\text{loss of sample weight during drying}}{\text{initial sample weight}} \times 100$$

3.4 Statistical analysis

All the experimental data were analysed by using GraphPad Prism 9.0 (San Diego, CA, USA). To identify the statistical differences between the means of the results, the evaluation was performed by using one-way analysis of variance (one-way ANOVA) with the significance level set at $p < 0.05$. For the multiple comparisons of the means, it was conducted by using Tukey's multiple range tests. All the experiments were carried out in triplicate and the mean values and standard error of each sample were shown and demonstrated as means ± standard error of means (means ± SEM).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of *L. plantarum* B13 as lactic acid bacteria

From **Figure 4.1 (A)**, there were no bubbles formed when the hydrogen peroxide was dropped onto the *L. plantarum* B13 smear. It showed that the *L. plantarum* B13 strain did not have the catalase enzyme. There was no enzyme reacting with hydrogen peroxide to form the oxygen gas, which caused the absence of bubbles formation. This represented that this bacterium strain was a catalase negative. The catalase negative bacteria have the peroxidase enzyme to prevent the formation of oxygen and gas production when they are exposed to the hydrogen peroxide (Sulmiyati et al., 2018).

Based on the result as shown in **Figure 4.1 (B)**, the *L. plantarum* B13 strain was a Gram-positive as it showed the purple colour. Besides that, the result as shown in **Figure 4.1 (B)** also showed that the shape of *L. plantarum B13* was rod-shaped. As stated by Ismail et al. (2018), LAB are Gram-positive bacteria, and the cells are rod or coccus shaped.

Based on the result as shown in **Figure 4.1 (C)**, the colour of the *L. plantarum* B13 bacterial colonies that were grown on the agar plate were yellowish white. The colour of the bacterial colonies was remained unchanged even after the addition of p-aminodimethylaniline oxalate. For the oxidase positive bacterial strains, the colonies will turn into purple colour after the addition of p-aminodimethylaniline oxalate. This showed that the *L. plantarum* B13 was an oxidase negative bacterial strain.

Lactic acid bacteria (LAB) are commonly identified as catalase negative, cytochrome-free, Gram-positive, and anaerobic or aerotolerant bacteria (Teneva-Angelova et al., 2018). Based on the test results, hence it confirmed that *L. plantarum* B13 was sharing the identical characteristics.

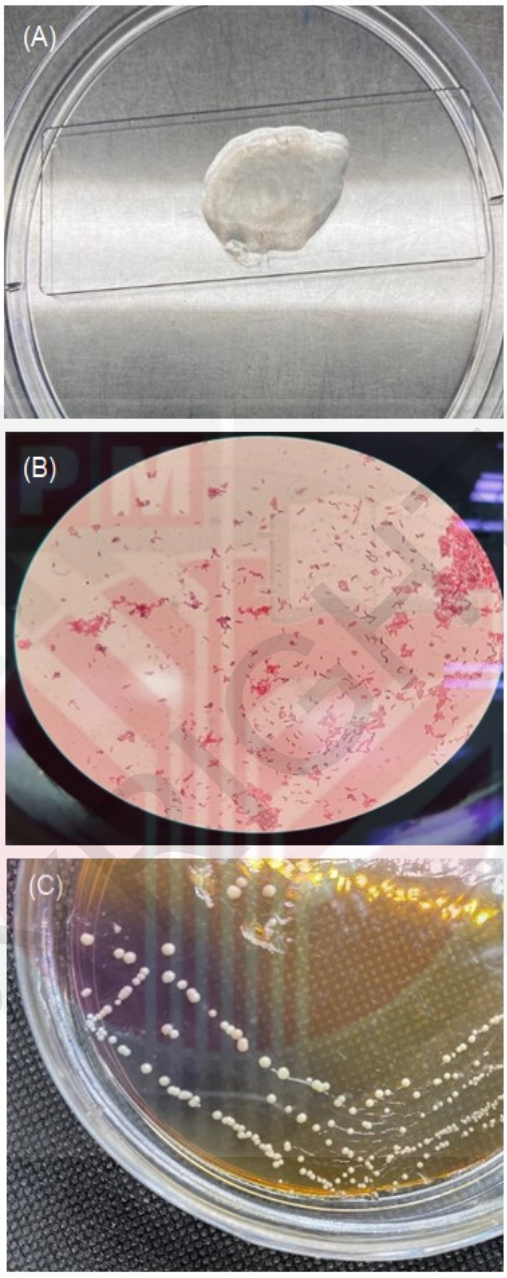


Figure 4.1 : The characterization of *Lactiplantibacillus plantarum* B13 as lactic acid bacteria through (A) catalase test which showing the strain as a catalase negative, (B) Gram staining test under magnification of 1000X under oil immersion, showing the strain is a Gram-positive, and (C) oxidase test, that showing it is an oxidase negative strain

4.2 *In vitro* probiotic characterization of *L. plantarum* B13

4.2.1 Assessments of acidic pH, bile salts and phenol tolerance

It is very important for the probiotics to tolerate acidic and alkaline conditions to be able to survive in the human's gastrointestinal tract (Prete et al., 2020). According to Bao et al. (2010), it is important to screen the LAB with the growing absorbance at pH 3.0, because the pH values in human stomach are ranged from 1.5 (during fasting) to 4.5 (after ingestion of food) and this condition could retain for around 3 hours. Generally, the ingested food will move into the stomach, followed by moving into the small intestine by passing through the duodenum (Choi & Chang, 2015). In duodenum, there is the presence of bile that secretes from the gall bladder. The bile will break down the lipid membrane of the cell and cause the leakage of the cell contents, leading to the cell death. Hence, to reach the small intestine and retain their viability for a prolonged period while exerting beneficial effects, probiotics LAB must possess bile and acidic pH tolerance (Kim et al., 2012).

Based on the results shown in **Table 4.1**, *L. plantarum* B13 was able to remain viable at pH 2.5 and 3.5 after 4 hours of incubation time. At both pH 2.5 and 3.5, the log CFU/mL were slightly decreased. For pH 2.5, the reduction of log CFU/mL was 0.191, whilst, for pH 3.5, the reduction of log CFU/mL was 0.039. These results showed that the cells were non-significantly different between the incubation time of 0 hour and 4 hours for both pH 2.5 and 3.5. Besides that, by comparing the log CFU/mL between pH 2.5 and 3.5 after the incubation time of 4 hours, the *L. plantarum* B13 that was grown in pH 3.5 had a higher number of viable cells, which was 8.255 log CFU/mL than pH 2.5, which was 8.050 log CFU/mL. The results also showed *L. plantarum* B13 was able to retain higher survival rate in pH 3.5 than pH 2.5 that equivalent to 91.62 % and 65.62 %, respectively.

Table 4.1 : Tolerance of *L. plantarum* B13 under different pH for the incubation time of 0 hour and 4 hours

pH	log CFU/mL		Survival rate (%)
	0 h	4 h	
2.5	8.241 ± 0.0299[a]	8.050 ± 0.1223[a]	65.62
3.5	8.294 ± 0.0375[a]	8.255 ± 0.0563[a]	91.62

Note: The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Furthermore, based on the results shown in **Table 4.2**, *L. plantarum* B13 also managed to survive in the presence of bile salts. The cell viabilities were slightly reduced after the incubation time of 4 hours for 0.1 % (w/v), 0.3 % (w/v) and 0.5 % (w/v) bile salt concentrations. All these three different bile salt concentrations

showed the viability (log CFU/mL) of *L. plantarum* B13 were non-significantly different between the incubation time of 0 hour and 4 hours. The *L. plantarum* B13 was able to remain viable with the survival rate of 87.93 % and 60.44 % when it was grown in the medium with the bile salt concentrations of 0.1 % (w/v) and 0.3 % (w/v), respectively. However, when the bile salt concentration was adjusted to 0.5 % (w/v), the survival rate of *L. plantarum* B13 was reduced to 50.00 %. This showed that the higher the concentration of the bile salts, the lower the viability (log CFU/mL) of cells. According to Nath et al. (2020), high concentration of bile salt would damage the cell membrane by breaking down the lipids and fatty acids that are composed in the cell membrane, followed by causing the permeabilization of the cells and leading to cell death.

The result of this study was in accordance with a study previously reported study by Mohd Yusof et al. (2020) for *L. plantarum* TA4 that was isolated from Malay traditional fermented food (Tapai pulut). They observed, that *L. plantarum* TA4 was able to survive at pH 2.5 and 3.5 with the survival rates of 79 % and 90.5 %, respectively. Meanwhile, in bile concentration of 0.3 % (w/v) the strain retained its survival rate up to 83.2 %. Meanwhile, another study reported that a potential probiotic, *L. plantarum* L7, was able to remain viable at pH 2.0 and 3.0, as well as in the bile salt concentration of 0.3 % (Giri et al., 2018). Likewise, Patil and Vishwanath (2012), also stated that the strains of *Lactobacillus* possessed the tolerance towards acidic conditions (pH 2.0 and 3.0). Therefore, based on the results shown in **Table 4.1** and **Table 4.2**, it is likely that *L. plantarum* B13 could survive and remain viable in acidic conditions and the presence of bile salts while in the gastrointestinal tract, highlighting one of the important characteristics possess by probiotic strains.

Table 4.2 : Tolerance of *L. plantarum* B13 under different bile salt concentrations for the incubation time of 0 hour and 4 hours

Oxgall concentration (%)	log CFU/mL		Survival rate (%)
	0 h	4 h	
0.1	6.461 ± 0.0424^{a}	6.406 ± 0.0120^{a}	87.93
0.3	5.259 ± 0.0507^{b}	5.034 ± 0.1129^{b}	60.44
0.5	4.040 ± 0.0560^{c}	3.690 ± 0.3012^{c}	50.00

Note: The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

In addition, *L. plantarum* B13 also showed the ability to survive in medium containing phenol. This proved that *L. plantarum* B13 was tolerant to the phenol concentrations of 0.2 % (w/v) and 0.5 % (w/v) (**Table 4.3**). In comparison, after the incubation time of 24 hours, the control (without phenol) was having the highest cell viability (8.947 log CFU/mL), followed by 0.2 % (w/v) phenol with the cell viability of 8.644 log CFU/mL and 0.5 % (w/v) phenol with the cell viability of 7.893 log CFU/mL. Sharma et al. (2022) stated that the phenol is a toxic metabolite, and it will suppress the growth of bacteria in the gut. Therefore, it is

crucial for probiotics to tolerate a limited amounts of phenol for surviving in the gastrointestinal tract because phenol will be produced when gut microorganisms deaminated various aromatic amino acids that are derived from dietary-based and endogenous proteins. Generally, the researchers always use the phenol concentration (range between 0.1 % and 0.6 %) for *in vitro* probiotic characterization. Phenol concentration of 0.4 % or above might show strong inhibitory effect towards the cell viability of probiotics (Yadav et al., 2016; Kılıç et al., 2013; Nandha & Shukla, 2023). Likewise, *L. plantarum* TA4 was previously demonstrated to be able to tolerate high phenol concentrations by maintaining considerably high numbers of viable cells of approximately 8 and 5 log CFU/mL for 0.2 % and 0.5 % (w/v) phenol, respectively (Mohd Yusof et al. 2020). Besides that, another study reported that all three lactobacilli strains, *L. acidophilus*, *L. casei* and *L. plantarum* were able to have high viable cell counts of approximately 10 and 9 log CFU/mL in the presence of 0.4 % phenol after 24 hours of incubation period (Soliman et al., 2015).

Table 4.3 : Tolerance of *L. plantarum* B13 under different phenolic concentration with the incubation time of 24 hours

Phenol concentration (%)	log CFU/mL
Control	8.947 ± 0.0058^{a}
0.2	8.644 ± 0.0081^{b}
0.5	7.893 ± 0.0037^{a}

Note: The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

4.2.2 Assessments of cell surface hydrophobicity, cellular autoaggregation and EPS production

Autoaggregation ability and cell surface hydrophobicity of bacteria are two independent traits, and they are commonly used as an indirect method for evaluating the adhesion ability of bacteria. Several researchers have reported good relation between autoaggregation and adhesion ability (Del Re et al., 2000; Perez et al., 1998) and surface hydrophobicity and adhesion ability (Marin et al. 1997; Pan et al. 2006). Autoaggregation ability of probiotic strains are necessary for bacterial adherence ability, meanwhile, the bacterial adherence ability is related to the cell surface characteristics (Canzi et al., 2005). In addition, the autoaggregation plays an important role in biofilm formation. Generally, EPS is one of the crucial components of the extracellular biofilm matrix (Nguyen et al., 2020). The autoaggregation can cause the formation of biofilm in two ways: the probiotic strain can attach to the intestinal surface as single cells and then recruit more cells via aggregation to form a single microcolony or the probiotic cells aggregate in suspension and then settle on the intestinal surface, followed by forming the biofilm (Trunk et al., 2018).

According to Schär-Zammaretti and Ubbink (2003), the cell wall constituents, such as phosphate and carboxylate groups and proteins, will cause the bacteria to have variable surface charges and hydrophobicity. This complicated behaviour has been shown to be the result of electrostatic and van der Waals interactions and surface hydrophobic interaction (Bustos et al., 2012). Moreover, the extracellular substances excreted by bacteria, such as bacterial polysaccharides, which either attach to the outer surfaces of cells or exist in the surrounding medium, have been shown to influence the adhesion as well (Lebeer et al., 2008).

Toluene, chloroform and ethyl acetate represent non polar solvent, mono-polar electron accepting solvent and mono-polar electron donating solvent, respectively (Klopper et al., 2018). As shown in **Table 4.4**, *L. plantarum* B13 had the strongest adherence towards chloroform (72.48 %), followed by toluene (25.31 %) and ethyl acetate (15.77 %). The results showed that *L. plantarum* B13 was having a low adherence towards the non-polar solvent (toluene), which pointed out that this strain was having a low hydrophobicity. According to Colloca et al. (2000), the cell surface hydrophobicity was attributed to the composition on surface polymers that involved in the hydrophobic interaction. Whereas, by making a comparison between polar solvents (chloroform and ethyl acetate), the results have shown that *L. plantarum* B13 tends to adhere to the acidic solvent (electron accepting), chloroform (72.48 %) than to the basic solvent (electron donating), ethyl acetate (15.77 %). This involved the electrostatic interaction between the cell surface and the solvent. Electrostatic interaction of ions involves the interaction between the opposite electic charges, such as cations (positively charged) and anions (negatively charged). Additionally, high affinity and low affinity of cells towards chloroform and ethyl acetate suggested that this strain may able to colonize mucus on the intestinal region due to the mucus has a net negative charge (Klopper et al., 2018). According to Polak-Berecka et al. (2014), *L. rhamnosus* PEN and *L. rhamnosus* E/N were also having a higher tendency to adhere to chloroform, which were 81.62 % and 76.49 %, respectively compared to ethyl acetate and hexadecane, that proved their hydrophilic characteristic.

As reported by Rahman et al. (2008), for the autoaggregation percentage which is more than 70 %, is ranked as high, followed by the autoaggregation percentage which is between 20 % and 70 %, is ranked as medium. Whereas for autoaggregation percentage which is below 20 %, is ranked as low. Based on **Table 4.4**, it showed that the longer the incubation time, the higher the autoaggregation activity of *L. plantarum* B13. The highest autoaggregation activity of *L. plantarum* B13 was achieved at the incubation time of 24 hours, which was 73.03 % compared to the autoaggregation activity at the incubation times of 1 hour (19.98 %) and 4 hours (33.80 %). Mohd Yusof et al. (2020) stated that *L. plantarum* TA4 obtained the highest autoaggregation activity (99.33 %) after incubating for 24 hours. Meanwhile, during the assessment of the aggregation of several *Lactobacillus* isolates (from native (desi) chicken gut), it was discovered that 3 out of 20 *L. reuteri* showed significantly high autoaggregation potential in the range of 57 to 98 % after 5 hours of incubation (Aziz et al., 2019).

Based on **Table 4.4**, it showed that *L. plantarum* B13 was not producing EPS. According to Wang et al. (2010), the medium composition and the conditions of cultivation (ie., temperature and pH) would affect the EPS production from the microorganisms. For example, in the optimization of medium formulation for *L. plantarum* MTCC 9510, the profound influence in the EPS production were lactose, yeast extract and ammonium sulphate that reached maximum at 72 hours of incubation (Ismail and Nampoothiri, 2010). In the meantime, changes in EPS production caused by growth temperatures (20, 30 and 37°C) were observed for several *Lactobacillus paracasei* strains (isolated from kefir grains) with an increment of EPS was recorded at the lowest temperature (Bengoa et al., 2018). Besides that, several researchers stated that *Lactobacillus* and *Streptococcus* species were able to produce high amounts of EPS at 35°C and pH ranging from 6.5 to 7.0 (Ismail & Nampoothiri, 2010; Kanmani et al., 2011). In this study, the initial pH of the culture medium that was adjusted to pH 5.5 seemed do not favour the EPS production by *L. plantarum* B13.

Table 4.4 : Cell surface hydrophobicity activity with different organic solvents, cellular autoaggregation assessment with different incubation time and EPS production ability of the *L. plantarum* B13 strain

Cell surface hydrophobicity (%)			Cellular autoaggregation (%)			EPS Production
Chloroform	Toluene	Ethyl acetate	1h	4h	24h	
72.48 ± 0.1245^{a}	25.31 ± 0.2138^{b}	15.77 ± 0.4304^{c}	19.98 ± 0.1004^{c}	33.80 ± 0.2090^{b}	73.03 ± 0.0580^{a}	-

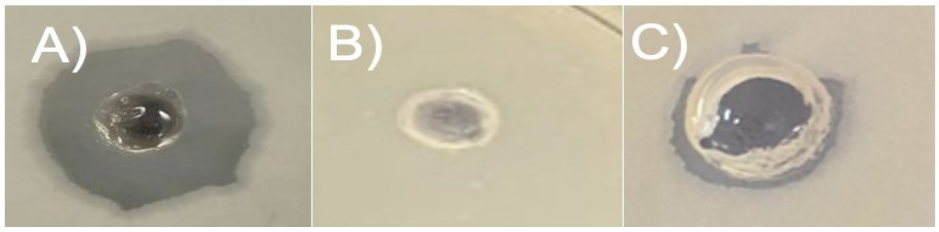
Note: The results are expressed as mean (n = 3) ± SD. Different letters in the rows of each group are significantly different based on Tukey's test at $p < 0.05$.

4.2.3 Determination of the antibacterial activity

Nowadays, the trend of research and development of new antimicrobial agents from different sources is growing to address the increasing problem of antimicrobial resistance (Balouiri et al., 2016). Meanwhile, in developing countries, diarrhoea is one of the major public health problems, especially among the children (Karimi et al., 2018). *Escherichia coli*, *Salmonella* sp., *Listeria* sp. and *Staphylococcus* sp., are among the pathogenic bacteria that cause the food-borne diseases (Bintsis, 2017, Nataro et al., 2006). Accordingly, probiotics are gaining increasing public attention and are more commonly used to combat those pathogenic bacteria while bringing health benefits to humans (Plaza-Diaz et al., 2019). Probiotics are frequently used to perform the antagonistic effect towards pathogenic bacteria and prolong the shelf life of foods (Karimi et al., 2018). Besides that, according to Soccol et al. (2010) and Ng et al. (2009), probiotics also took part in the battle with pathogens that might cause some diseases including infectious diarrhoea, recurrent *Clostridium difficile*-induced infection and inflammatory bowel diseases. Generally, the type of food being consumed would affect the intestinal microflora (Karimi et al., 2018). Consuming healthy foods such as probiotic foods can help to replace the harmful with useful microorganisms (Torkan et al., 2011).

In this study, the antimicrobial effect of cell-free supernatant (CFS) of *L. plantarum* B13 towards both Gram-positive and Gram-negative pathogenic bacteria were examined. The formation of zones of inhibition by the CFS of *L. plantarum* B13 on the agar plates for all four tested pathogens can be viewed in **Figure 4.2**. As reported by Sarker et al. (2014), the diameter of inhibition zone defines the bacteria as resistant (≤9 mm), moderately sensitive (10-11 mm), or sensitive (≥12 mm) to the sample. Based on the results shown in **Table 4.5**, the CFS of *L. plantarum* B13 showed the highest antimicrobial activity towards *S. aureus*, corresponding to the largest diameter of inhibition zone of 11.50 mm, whereas the other three different pathogenic bacteria (*E. coli*, *S. typhimurium* and *B. subtilis*) recorded a similar diameter of inhibition zone of 10 mm. Besides that, the results also showed that the diameters of inhibition zone that formed by using the positive control (ampicillin) on each pathogen (*E. coli*: 11 mm, *S. typhimurium*: 14 mm and *B. subtilis*: 18mm) were significantly different to the diameters of inhibition zone that formed by using the CFS of *L. plantarum B13* on each pathogen (*E. coli*: 10 mm, *S. typhimurium*: 10 mm and *B. subtilis*: 10 mm) except for the *S. aureus*, there was non-significant difference between the diameter of inhibition zone that formed by using positive control (11 mm) and CFS of *L. plantarum* B13 (11.50 mm).

Previously, the antimicrobial properties of the CFS (or fermented extract) of *Lactobacillus brevis* A3 which had also been evaluated by an agar well diffusion method, showed inhibition zone diameters for *S. typhimurium* and *E. coli,* which were 11 mm and 13 mm, respectively (Alizadeh Behbahani et al., 2020). Likewise, Benítez-Serrano et al. (2018) also showed the antimicrobial activity of the CFS of *L. plantarum* and *L. brevis* towards *S. typhimurium* and *E. coli*. Meanwhile, *L. plantarum strain* GCC_19M1 was shown to perform the antimicrobial effect towards a broad spectrum of pathogens including *Bacillus cereus, Acinetobacter johnsonii, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Cedecea davisae* and *Achromobacter spanius* (Nath et al., 2020). Besides that, there are several other studies that disclosed the *Lactobacillus* sp. had the antimicrobial characteristic and was antagonistic to many pathogenic bacteria such as *E. coli, Staphylococcus aureus, B. cereus, Listeria monocytogenes, Salmonella typhimurium, Salmonella enteritidis, Enterococcus faecalis, and Enterobacter cloacae* (Kaboosi, 2011; Palachum et al., 2018).



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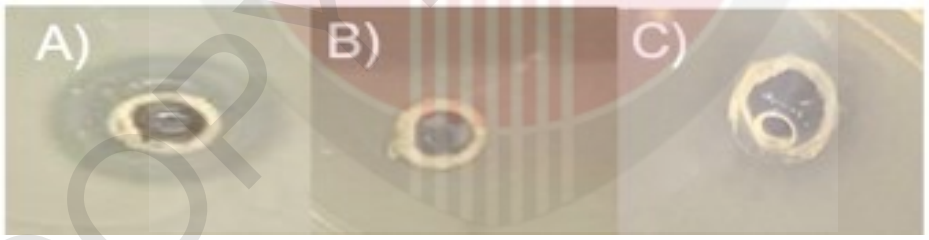

(4)

Figure 4.2 : Antibacterial activity of A) positive control (ampicillin); B) negative control (distilled water); C) *L. plantarum* B13 against different bacteria strains; (1) *B. subtilis*; (2) *S. typhimurium*; (3) *S. aureus*; (4) *E. coli*

Table 4.5 : Antibacterial activity of cell-free supernatant (CFS) of *L. plantarum* B13 strain, positive control (ampicillin) and negative control (distilled water) against different bacteria strains

	Tested bacteria strain	Zone of inhibition (mm)
***L. plantarum* B13**	**Gram-negative**	
	E. coli	10.00 ± 0.1414^{d}
	S. typhimurium	10.00^{d}
	Gram-positive	
	S. aureus	11.50 ± 0.0707^{c}
	B. subtilis	10.00^{d}
Positive control (ampicillin)	**Gram-negative**	
	E. coli	11.0 ± 0.0707^{b}
	S. typhimurium	14.0 ± 0.1061^{a}
	Gram-positive	
	S. aureus	11.0 ± 0.1414^{c}
	B. subtilis	18.0 ± 0.1414^{b}
Negative control (distilled water)	**Gram-negative**	
	E. coli	0.00^{e}
	S. typhimurium	0.00^{e}
	Gram-positive	
	S. aureus	0.00^{e}
	B. subtilis	0.00^{e}

Note: The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

In addition, there are studies that used viable probiotic cells instead of CFS to conduct antimicrobial tests. According to a study disclosed by Adeniyi et al. (2015), both forms of viable cells and CFS of *Enterococcus hirae* CO6A were able to exert an antimicrobial effect that suppressed the growth of pathogenic bacteria. However, the viable cells exhibited higher antimicrobial activity compared to CFS. They observed that the viable cells of *E. hirae* CO6A showed a positive antimicrobial effect against *E. coli* CB6 with the formation of a large diameter of inhibition zone of 26 mm while the CFS only10 mm. Therefore, it is expected that higher antimicrobial activity may be attained by *L. plantarum* B13 if viable cells are used instead of CFS.

In general, the antimicrobial activity is often related to bacteriocins produced by the strain and its stability reaction towards acidic conditions (Alizadeh Behbahani et al., 2020). Beside bacteriocins, probiotics can synthesise many other antimicrobial compounds such as organic acids (ie., lactic acid, acetic acid,

propionic acid, and succinic acids), hydrogen peroxide, ethanol, diacetyl, acetoine, acetaldehyde, carbon dioxide, reuterin and reutericyclin during their metabolism pathways (Soccol et al., 2010, Adeniyi et al., 2015). Organic acids produced by probiotics are acidic and the accumulation may cause the environment to become acidic, which is not suitable for certain pathogenic bacteria to proliferate (Pinto et al., 2006). Besides that, organic acids would permeate the cell membrane of pathogenic bacteria, causing the structural changes in the phospholipid content that occurred inside the cells (Arena et al., 2016). There are studies stating that lipopeptides were being synthesised during the fermentation by *L. plantarum*, and consequently, exerted the antimicrobial effect towards *Streptococcus* mutans (Park et al., 2012; Benítez-Serrano et al., 2018). In addition, the hydrolysis of protein by the probiotic bacteria produced two products of high-hydrolysis proteins and low molecular weight peptides that could also provide the antimicrobial effect (Adak et al., 2011). Cationic antimicrobial peptides of Gram-negative bacteria tend to attach on the lipopolysaccharides with a negative charge, while cationic antimicrobial peptides of Gram-positive bacteria tend to attach to teichoic and lipoteichoic acids thus exerting an antimicrobial effect (Ramos et al., 2013; Yazdi et al., 2015).

4.3 Determination of the antioxidant activity

Nowadays, ageing and aging-related diseases are becoming worldwide issue that leads to expanding research and development for finding methods in preventing the aging-related diseases (Tu et al., 2022). Ageing is a process where the vitality is slowly decrease and cells are getting older and stop to proliferate in an accelerated way (Ferrando-Martínez et al., 2011). The ageing phenomenon may cause an oxidative stress to increase, followed by the aging-damage due to the oxygen-derived free radicals, that can severely damage the cells and tissues (Fusco et al., 2007). Most living organisms possess enzymatic defences and repair systems. However, these systems are generally not enough to provide protection against an oxidative stress (Wang et al., 2017). Furthermore, the ageing phenomenon also breaks the equilibrium balance of the oxidants, antioxidants, and biomolecules. Both synthetic and natural antioxidant additives have demonstrated the capacity to protect the human body against an oxidative damage. Nevertheless, natural antioxidants are gaining more interests due to the increasing issues in relation to the safety and carcinogenicity of the synthetic antioxidants (Luo and Fang, 2008). Therefore, based on recent *in-vivo* and *in-vitro* studies that have shown the presence of significant antioxidant abilities in probiotics, it has highlighted another interesting feature of probiotics as natural antioxidant agents (Shen et at., 2011; Wang et al., 2016; Persichetti et al., 2014).

In this study, the antioxidant activity of cell free supernatant of *L. plantarum B13* was compared with the antioxidant activity of vitamin C. The vitamin C is also known as ascorbic acid and it is a naturally occurring organic compound with known antioxidant properties (Njus et al., 2020). The results demonstrated that the cell free supernatant of *L. plantarum* B13 possessed a considerably high antioxidant activity (77.27 %) by comparing with the antioxidant activity of

ascorbic acid or vitamin C (**Figure 4.3**). According to a study reported by Alizadeh Behbahani et al. (2020), the fermented extract of *L. brevis* A3 had been shown to have a positive antioxidant activity corresponding to 59.67 %. Meanwhile, the cell-free supernatants of several lactic acid bacteria isolates have been shown to possess different antioxidant activity of 84.7 %, 80.5 %, 70.5 % and 66.3 % for strains SC8, TA4, MPJ10 and FF2, respectively (Mohd Yusof et al., 2020).

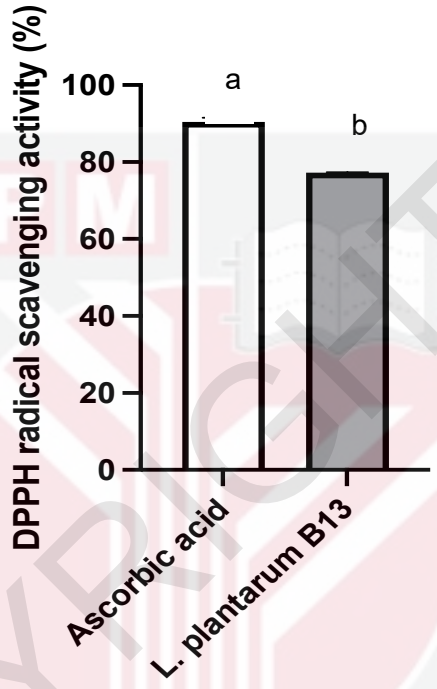

Figure 4.3 : Antioxidant activity (% DPPH) of cell-free supernatant (CFS) of *L. plantarum* B13 strain and ascorbic acid (control). The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$

4.4 Cell profile for the culture of *L. plantarum* B13

4.4.1 Time course of cell growth, residual glucose concentration and GABA yield

Prior to screening of optimum fermentation parameters, the lactic acid bacteria, *L. plantarum* B13 was cultured in MRS broth containing 1 % (w/v) MSG and 0.5 mM PLP with adjusted pH of 6.0 for 96 hours of fermentation period to obtain the basis cell growth profile. The cell growth, yielding amount of GABA and residual glucose concentration against time were shown in **Figure 4.4**. From the

cell growth profile, it can be observed that *L. plantarum* B13 was undergoing a lag phase for a short period of time and after the first 3 hours it started to grow exponentially, while the residual glucose concentration started to drop drastically. *L. plantarum* B13 was consuming the glucose to carry out metabolic activity of cells. The metabolic activities are important and essential for lactic acid bacteria to survive, proliferate and grow (Saeed and Salam, 2013). The most important metabolic activity for lactic acid bacteria is the breakdown of carbohydrates and corresponding compounds to acquire energy and carbon molecules (Sanchez & Demain, 2008; Hoefnagel et al., 2002). After 21 hours of exponential growth, *L. plantarum* B13 reached a stationary phase. The maximum cell growth (OD_{600} of 2.710) was obtained at 21 hours of incubation while glucose was almost completely consumed by the cells. Afterward, the cell growth of *L. plantarum* B13 did not show an increasing trend and it remained constant until the fermentation was stopped at 96 hours. During this long stationary phase of 75 hours fermentation period, the remaining glucose was slowly reduced in a non-significant amount.

Based on the **Figure 4.4**, for the first 12 hours of fermentation period, GABA was observed to be produced in a very small amount and the bacterial cells were in the lag growth phase and exponential growth phase, whereas, starting from the 15 hours onwards (stationary growth phase), the yield of GABA was gradually increased. The maximum GABA yield, which was 7.740 g/L, was obtained at the 72 hours of fermentation time during the stationary growth phase of *L. plantarum* B13. This result is in accordance with a study reported by Di Cagno et al. (2010) that discovered the maximum GABA yield by *L. plantarum* DSM19463 was also obtained during the late-stationary phase of 72 hours fermentation time. In addition, Castanie-Cornet and Foster (2001), also showed that the maximum GABA yield was achieved at the stationary phase of the cell growth (7 Log CFU/mL) rather than the logarithmic phase (with a maximum cell growth of 10 log CFU/mL). The production of GABA is influenced by the stress conditions such as the acidity and starvation which initiate the expression of GAD gene (Cotter & Hill, 2003; Shao et al., 2008; Castanie-Cornet & Foster, 2001). According to a study by Castanie-Cornet and Foster (2001), the GAD protein of the *Escherichia coli*, was being produced in the exponential phase, but the GAD protein would only turn activated when it encountered and processed with some forms of the stationary phase.

Secondary metabolites are usually produced during stationary growth phase of microorganisms (Sanchez and Demain, 2011). They are not essential for the bacterial growth and reproduction, but they may serve various survival functions in nature. Based on **Figure 4.4**, the GABA was mainly produced during the stationary growth phase of *L. plantarum* B13, so, it showed that GABA was the secondary metabolites that produced by *L. plantarum* B13 during the fermentation.

Besides that, Zhang et al., 2020 also reported on the fermentation of thermophilic bacteria, *Saccharomyces cerevisiae* SC125 and *L. plantarum* BC114 at 300°C in the mulberry beer that pre-mixed with 5 g/L glutamic acid

also required 72 hours to obtain the maximum yield of GABA (2.42 g/L). Several studies showed that the optimum GABA productions by the strains *L. plantarum* ED-10 (Demirbas et al., 2017), *L. plantarum* IFK-10 (Yogeswara et al., 2018), *L. plantarum* K154 (Park et al., 2014) and *L. plantarum* BC 114 (Zhang et al., 201) were 1.59 g/L, 2.68 g/L, 0.2 g/L and 3.82 g/L, respectively. By comparing the strain (*L. plantarum* B13) that was used in this study with the other aforementioned strains that used in the other studies, the results as shown in **Figure 4.4** proved that the strain *L. plantarum B13* possessed a better GABA production ability because the maximum GABA production by *L. plantarum* B13 was higher corresponding to 7.740 g/L.

After that, the fermentation period was extended up to 96 hours in order to observe whether the GABA production would be increased or reduced after 72 hours of fermentation. Through this, the optimum range of fermentation time could be identified and used in further experiment for determining the optimum point of fermentation time that yield the maximum amount of GABA. Based on **Figure 4.4**, the production of GABA showed a decline trend after reaching the maximum GABA production at 72 hours. However, the cell viability remained stable at stationary growth phase which indicated that was no cell growth or proliferation. This showed that the extension of fermentation time did not affect the cell viability. Meanwhile the glucose depletion was remained stable as well. This might because there was no cell proliferation or growth. Thus, no nutrient was being consumed by the strain *L. plantarum* B13. This may be due to the enzymatic activity of GABA transaminase (GABA-T), which degrades the GABA and transforms it into another form of compounds (Cho et al., 2011; Ko et al., 2013; Han et al., 2017). The GABA-T enzyme utilized either pyruvate or a-ketoglutarate as the acceptor of amino for the degradation of GABA into succinic semialdehyde. The succinic semialdehyde is then converted into succinate irreversibly by succinic semialdehyde dehydrogenase enzyme. Therefore, based on the GABA production profile, the range between 48 hours and 72 hours of the fermentation time was being applied in the subsequent experiments to specifically obtain the optimum time point for the maximum GABA production.

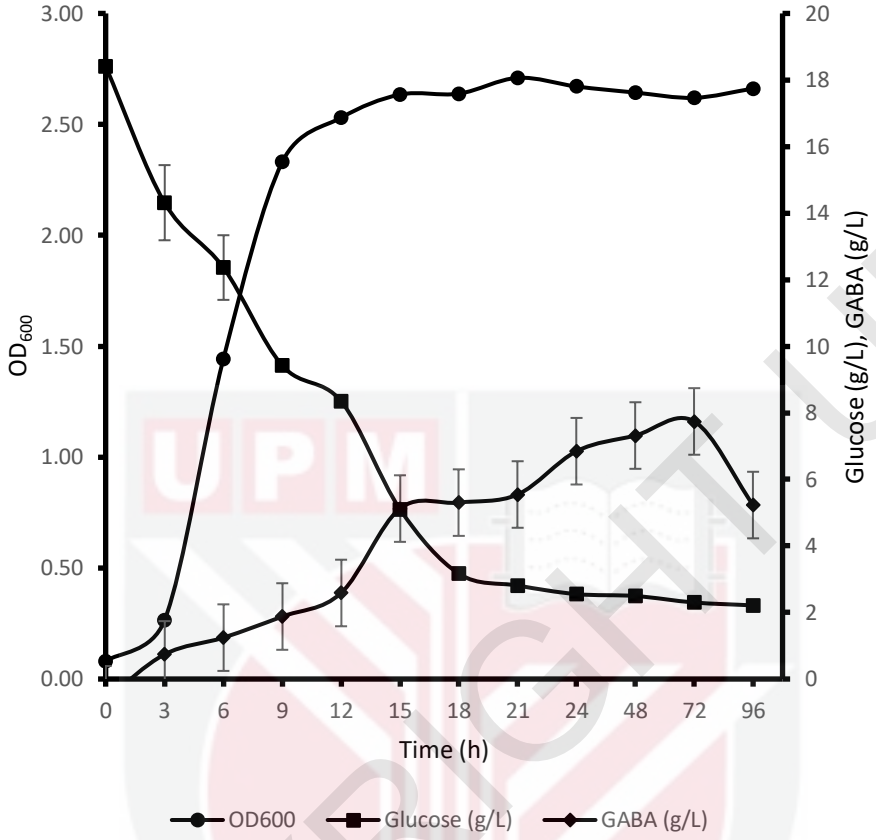

Figure 4.4 : The cell profile with the absorbance reading (OD_{600}), residual glucose concentration (g/L) and GABA concentration (g/L) for the shake flask culture of *L. plantarum* B13 in MRS medium containing 1 % (w/v) MSG, 0.5 mM PLP, pH 6.0, 37 °C, 96 hours of incubation. The error bars represent the standard deviations about the mean (n=3)

4.4.2 Correlation between the absorbance reading and cell viability

To quantify and assess the cell concentration for the bacteria strain, usually, the measurement is carried out by applying the UV-spectrophotometer method. Through the measurement of optical density at 600 nm (OD_{600}) by using the UV-spectrophotometer and enumeration of the number of viable cells in the units of colony-forming units per unit volume of culture (CFU/mL), the cell concentration for the bacteria strain will be able to be determined. In comparison between these two methods, the calculation of the viable cells is more reliable (Peñuelas-Urquides et al., 2013). However, the method of calculating the number of viable cells is more tedious and it is also time-consuming to run the experiments. Therefore, a correlation graph between OD_{600} and log CFU/mL is often established by researchers for the determination of the number of viable cells that is equivalent to the OD_{600} measurement. Based on **Figure 4.5**, it showed

that there is a linear relationship between log CFU/mL and OD_{600}, which expressed in the graph equation of y = 0.7769x +7.1751. The OD_{600} measurement could be considered as highly correlated with the number of viable cells, as the R_2 value was high (0.994) and approximately equal to 1.0. This correlation graph was used to estimate the number of viable cells for further experiments based on the results of OD_{600} measurement.

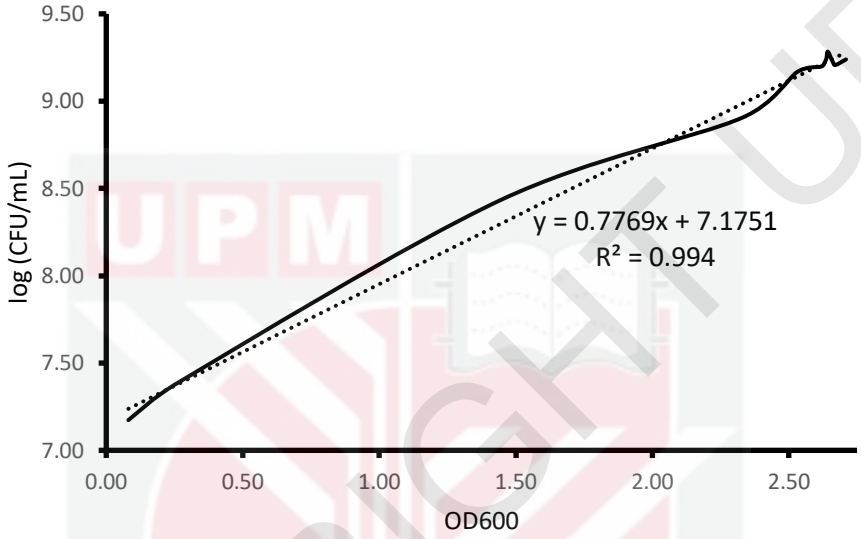

Figure 4.5 : Correlation graph between log CFU/mL and OD_{600} for the shake flask culture of *L. plantarum* B13. The results were expressed as mean (n = 3) ± SD

4.5 Determination of the effects of fermentation parameters on GABA production

4.5.1 Incubation time

Based on **Figure 4.4**, the optimum range of incubation time to produce GABA by *L. plantarum* B13 was between 48 hours (7.317 g/L GABA) and 72 hours (7.740 g/L GABA) of incubation time. Optimization method by One-Factor-At-Time (OFAT) was hence applied to determine the accurate optimum incubation time for the GABA production. In this study, the incubation time was varied (48, 54, 60, 66, and 72 hours), whereas the other fermentation parameters including initial pH, temperature, concentration of MSG and concentration of PLP were fixed at initial pH 6.0, 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively.

According to **Figure 4.6**, the results showed that the GABA yield was gradually increased from 7.317 g/L (at the incubation time of 48 hours) to reach 7.951 g/L

at the incubation time of 66 hours. Although it was in the stationary growth phase, a slight increment of cell growth could still be observed within this period of fermentation. The cells were achieving its maximum growth at the incubation time of 60 hours, with OD_{600} reading of 2.703 and 9.275 log CFU/mL. Generally, the stationary phase is a phase where the cells are still active to carry out the metabolic activities, but the growth is stopped and halted (Jaishankar and Srivastava, 2017). This can be due to the accumulation of the inhibitory products such as organic acid and exhaustion of the vital nutrients. Therefore, the environment conditions became harsh for the cells to survive, thereby, it caused the cells to activate the stringent response mechanism for the sake of survival. This mechanism would allow the cells to reconfigure and reset the pattern of gene expression to let the cells adapt to the stresses, and consequently, the cells utilised the resources to produce the amino acids for enhancing their survivability rather than for the growth. As observed earlier, the GABA yield (7.740 g/L) was started to decline when the incubation time was extended to 72 hours. The trend of GABA production observed in this study was in accordance with a study by Yogeswara et al. (2020) for the GABA synthesis by *L. plantarum* FNCC 260. They reported that GABA (450 mg/L) began to be rapidly synthesised during the stationary phase after 48 hours of incubation, which was higher than the GABA yield obtained at the 12 hours of incubation (at an exponential growth phase). The maximum GABA production (809.2 mg/L) was achieved after 60 hours of incubation. Likewise, the highest GABA yield (497.97 mM) attained by *L. plantarum* Taj-Apis362 was also at the incubation time of 60 hours (Tajabadi et al., 2015). Meanwhile, *L. plantarum* BC114 was able to produce the maximum concentration of GABA (3.45 g/L) at the incubation time of 72 hours, while further prolonged the incubation time reduced the GABA yield (Zhang et al., 2017). In addition, several other studies stated that the range of incubation time for *L. brevis* L-32 to obtain the maximum GABA production was between 36 to 72 hours (Cho et al., 2011; Kono & Himeno, 2000; Shelp et al., 1999).

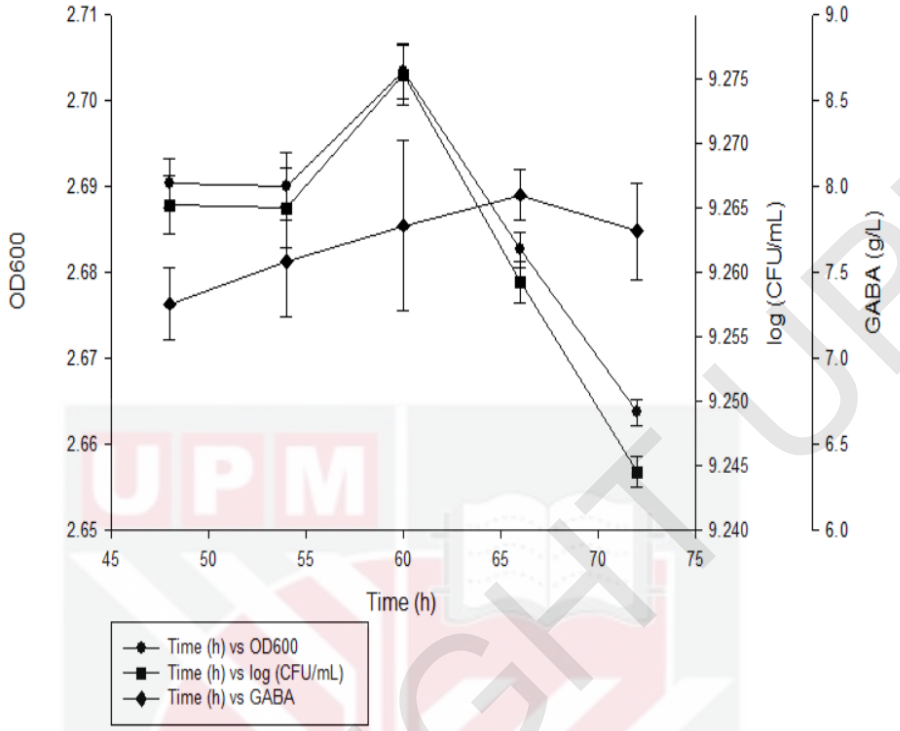

Figure 4.6 : Cell growth (OD_{600} and CFU/mL) and GABA production of *L. plantarum* B13 at different incubation times (48, 54, 60, 66, and 72 hours). The other fermentation parameters: 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively. The error bars represent the standard deviations about the mean (n=3)

4.5.2 pH value

GABA synthesis is highly correlated with the pH of the fermentation medium (Rayavarapu et al., 2021). This is because the mechanism for synthesising GABA is important and playing the role of perpetuating the pH inside the cell as a proton (H^+) will be taken up during the process of decarboxylation, which results in the increment of pH inside the cell (Kook & Cho, 2013). To determine the effect of pH value on GABA production and cell growth of *L. plantarum* B13 by OFAT, the initial pH value of the culture medium was varied (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5), whereas the other fermentation parameters including incubation time, temperature, concentration of MSG and concentration of PLP were fixed at incubation time of 66 hours, 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively.

Based on **Figure 4.7,** the results showed that the initial pH value of the culture medium was significantly affecting the GABA production. The OD_{600} reading and

the log CFU/mL for the maximum cell growth at initial pH 5.5 were 2.660 and 9.242, respectively. When the initial pH values were adjusted to 4.0, 4.5 and 5.0, the growth of *L. plantarum* B13 was inhibited and recorded lower maximum log CFU/mL corresponding to 8.723, 8.833 and 8.987, respectively. Likewise, the highest production of GABA was observed for the fermentation with an initial pH 5.5 that yielded 9.268 g/L, while for fermentations with initial pH 4.0, 5.4 and 5.0 attained lower GABA productions of 3.732 g/L, 3.837 g/L and 5.626 g/L, respectively. Adjusting the fermentation medium higher than pH 5.5 also did not favour the GABA production despite supporting the cell growth of *L. plantarum* B13. When the initial pH values were adjusted to 6.0, 6.5, 7.0 and 7.5, the maximum cell growth were 9.237, 9.186, 9.151 and 9.142 log CFU/mL, respectively. However, the GABA yields were significantly lower than that observed for *L. plantarum* B13 cultured in the medium with an initial pH 5.5 that attained approximately a similar maximum cell growth. The GABA yields were 7.268 g/L for initial pH of 6.0; 4.715 g/L for initial pH of 6.5; 3.967 g/L for initial pH of 7.0; and 3.805 g/L for initial pH of 7.5.

Based on **Figure 4.7**, the optimum GABA production (9.268 g/L) was obtained when the culture medium was slightly acidic (pH 5.5). This study which used the strain *L. plantarum* B13 for GABA production showed a different result from the study that reported by Lu et al. (2009). Lu et al. (2009) reported that the production of GABA by *Lactococcus lactis* subsp. *lactis* was gradually increasing with the pH values that ranged from pH 5.5 to 7.0, and retained the maximum GABA yield at the pH that ranged between 7.0 to 8.0. After optimizing the fermentation parameters using the Response Surface Methodology, they discovered that the optimum initial pH was at pH 7.1, resulting the maximum amount of GABA of 7.2 g/L. By comparing the results that obtained in this study with the results that obtained in another study as demonstrated by Lu et al. (2009), the optimum pH for GABA production was different. This indicated that the optimum pH for GABA production was strain-dependent.

pH regulation is very crucial for lactic acid bacteria to produce GABA as the initial pH value would affect the activity of glutamic acid decarboxylase (Li et al., 2010a; Siragusa et al., 2017). Glutamic acid decarboxylase needs H^+ ions to carry out the catalysis. Thus, the production of GABA can be enhanced by adjusting the initial pH of the culture medium to be slightly acidic. Nonetheless, the activity of the glutamic acid decarboxylase will be partially lost if the pH is too high (highly alkaline) or too low (highly acidic). Besides that, adjusting the initial pH of fermentation medium is also important since different bacterial species will have different optimum pH for glutamic acid decarboxylase to function at its best (Diana et al., 2014). For instant, the pH for the glutamic acid decarboxylase of *E. coli* to perform at its optimum rate was found at pH 3.8, while the most suitable pH to obtain a high activity of glutamic acid decarboxylase for *N. crassa* and *L. brevis* were pH 5.0 and pH 4.2, respectively (Yang et al., 2006). According to a study conducted by Tajabadi et al. (2015), the highest GABA concentration (7.15 mM) produced by *L. plantarum* Taj-Apis362 was obtained at the optimum initial pH of 5.31. They also stated that the initial pH would affect the final biomass beside the GABA yield. Furthermore, several other studies also reported that the highest amount of GABA could be attained at an initial pH 5.0

for bacterial species such as *L. fermentum* (Rayavarapu et al., 2021) and *L. brevis* NCL912 (Li et al., 2010a). In a nutshell, the initial pH value for the optimal biosynthesis of GABA is strain-dependent, however, it can be deduced that the maximum production of GABA by LAB is commonly achieved at the range of initial pH of 5.0 to 7.0.

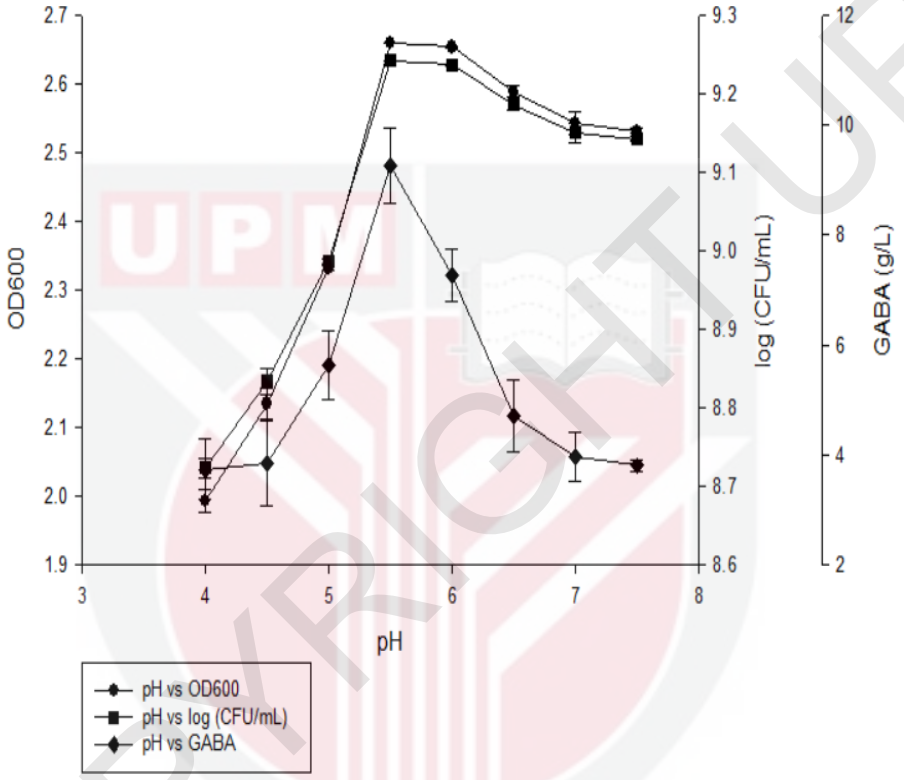

Figure 4.7 : Cell growth and GABA production of *L. plantarum* B13 with different pH values (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) within 66 hours cultivation. The other fermentation parameters: 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively. The error bars represent the standard deviations about the mean (n=3)

4.5.3 Temperature

It is necessary to regulate temperature for the biosynthesis activity as the optimal temperature could help to improve the efficiency of biosynthesis resulting in an optimal GABA yield (Zhang et al. 2012, Shan et al., 2015). Generally, most lactic acid bacteria possess high rate of GABA biosynthesis at the optimum temperature that ranged between 30°C to 50°C (Li and Cao, 2010). Nevertheless, when the cultivation temperature is too high and exceeds the

temperature that the bacteria can withstand, it will lead to the inactivation or degradation of glutamate decarboxylase and cell senescence (Shan et al., 2015). Hence, to determines the effect of temperature on GABA biosynthesis and cell growth of *L. plantarum* B13 by OFAT, temperature of the culture medium was varied (33°C, 35°C, 37°C, 39°C and 41°C), whereas the other fermentation parameters including incubation time, initial pH, concentration of MSG and concentration of PLP were fixed at incubation time of 66 hours, initial pH of 6.0, 1 % (w/v) MSG and 0.5 mM PLP, respectively.

Based on **Figure 4.8,** the results showed that the cultivation temperature was significantly affecting the GABA production by *L. plantarum* B13. The cell growth and GABA production of *L. plantarum* B13 were increased when the cultivation temperatures were increased from 33°C to 35°C. The maximum cell growth (9.272 log CFU/mL; OD_{600} reading of 2.700) and the highest GABA production (8.902 g/L) were achieved at 35°C. High cell concentration indicated high number of viable cells that may result in the improvement of the activity of glutamate decarboxylase leading to the enhancement of GABA production (Li and Cao, 2010). The result of this study was in line with the study reported for *L. plantarum* NDC75017 that produced the maximum concentration of GABA (314. 56 mg/100 g) at the incubation temperature of 36°C (Shan et al., 2015). However, *L. plantarum* NDC75017 attained the maximum number of viable cells at a lower temperature of 30°C. Meanwhile, *L. plantarum* BC114 (Zhang et al., 2017) and *L. plantarum* DSM19463 (Di Cagno et al., 2010) were able to attain the highest amount of GABA corresponding to 3.45 g/L and 0.497 g/L, respectively at the cultivation temperature of 30°C. In this study, both the cell growth and GABA production of *L. plantarum* B13 were observed to decline when the cultivation temperature was increased within 37°C to 41°C. At 41°C, *L. plantarum* B13 showed the lowest cell growth (8.191 log CFU/mL; OD_{600} reading of 1.307) and GABA yield (3.797 g/L) compared to the other tested cultivation temperatures (from 33°C to 39°C).

Based on extensive reported works on GABA production by lactic acid bacteria, the optimal temperature for yielding the maximum amount of GABA is species dependent. For example, *L. brevis* IFO 12005 had a high rate of GABA biotransformation at the incubation temperature of 30°C (Huang et al., 2007b), while at the same temperature, *L. brevis* TCCC13007 had a high number of viable cells (Zhang et al., 2012). Lu et al. (2008) demonstrated that the most suitable temperature for *Lactococcus lactis* for attaining the highest amount of GABA (0.27 mg/mL) was at 34°C. Both *L. paracasei* NFRI 7415 (Komatsuzaki et al., 2005) and *L. brevis* CGMCC 1306 (Ueno et al., 1997) were able to produce the highest amount of GABA at the cultivation temperature of 37°C. In the meantime, the optimum temperature to produce the maximum amount of GABA (7984.75 ± 293.33 mg/L) by *Streptococcus salivarius* ssp. *thermophilus* Y2 was at 40°C (Yang et al., 2008). In the nutshell, controlling the fermentative parameter, such as cultivation temperature is very crucial to achieve the maximum yield of GABA as it will affect the activity of glutamate decarboxylase (Dhakal et al., 2013). From the results obtained in this experiment, the optimum temperature for yielding the highest amount of GABA by *L. plantarum* B13 was

at 35°C, which is in the range of the optimum temperature (30°C to 50°C) for lactic acid bacteria that were reported by other researchers.

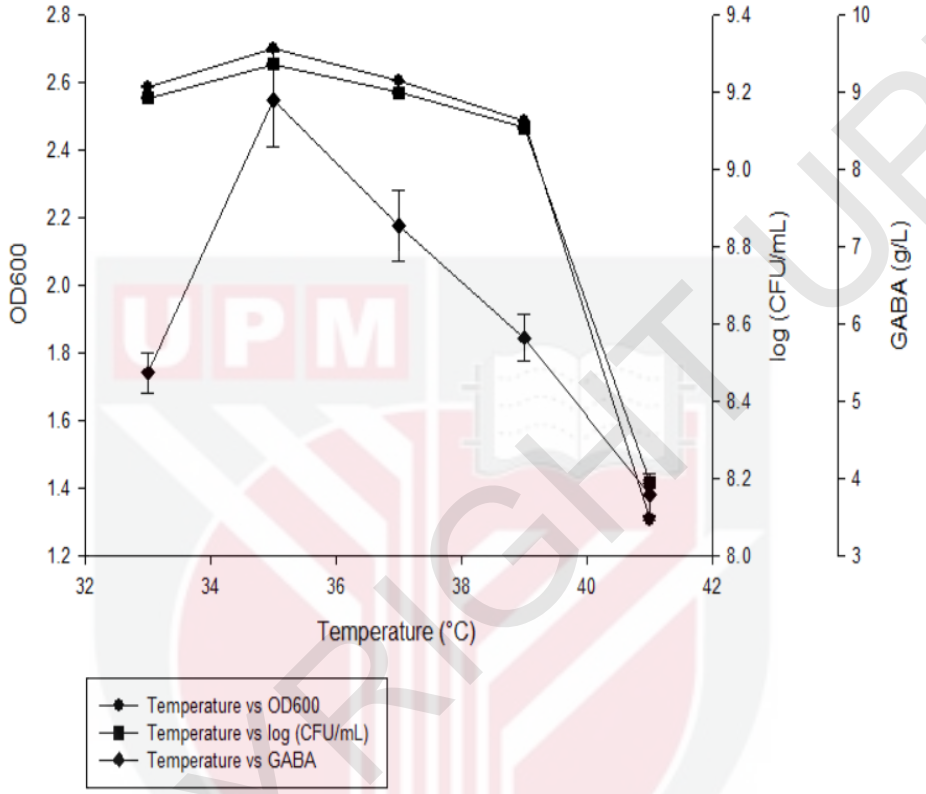

Figure 4.8 : Cell growth and GABA production of *L. plantarum* B13 with different temperatures (33, 35, 37, 39 and 41°C) within 66 hours cultivation. The other fermentation parameters: initial pH of 6.0, 1 % (w/v) MSG and 0.5 mM PLP. The error bars represent the standard deviations about the mean (n=3)

4.5.4 Monosodium glutamate (MSG) concentration

Various studies have reported that the supplementation of MSG onto the culture medium would affect the activity of glutamic acid decarboxylase, hence influencing the GABA production by *L. plantarum* strains (Di Cagno et al., 2010; Park et al., 2013; Park et al., 2014, Park et al., 2021; Shan et al., 2015; Tajabadi et al, 2015). Hence, to determines the effect of MSG concentration on GABA biosynthesis and cell growth of *L. plantarum* B13 by OFAT, the MSG concentration added into the MRS medium was varied (0 % (control), 1 %, 3 %, 5 %, 7 %, and 9 % (w/v)), whereas the other fermentation parameters including the incubation time, initial pH, temperature, and concentration of PLP were fixed

at incubation time of 66 hours, initial pH of 6.0, 37°C and 0.5 mM PLP, respectively.

As depicted in **Figure 4.9,** the results showed that the concentration of MSG was significantly affecting the GABA production and cell growth of *L. plantarum* B13. By comparing the MRS medium with and without (control) the addition of MSG, the results showed that the addition of MSG in the range of 1 % to 7 % (w/v) helped the *L. plantarum* B13 to obtain higher cell growth and GABA yield. The results of this study demonstrated that the cell growth was stimulated by adding glutamate to the culture medium. The addition of MSG in the concentration ranges from 0.02 % to 6 % in the cultivation medium also increased the viable cell counts (in the range of 1 x 10^9 to 4 x 10^9 CFU/mL) of *L. plantarum* FL-664 and improved the ability of cells to produce the desired product (IL-12(p40)), however, it reduced the long diameter of cells (Kawamoto-Miyamoto et al., 2022). This study also suggested that the addition of MSG up to 6%, may protect *L. plantarum* FL-664 from the osmotic stress by increasing the expression of genes involved in the cell wall synthesis and cell division. Meanwhile, according to a study by Villegas et al. (2016), cell growth of *Lactobacillus brevis* CRL 1942 was also improved by supplementing the medium with glutamate as it facilitated the expression of genes related to cell division and build-up of cell wall, as well as acting as a substrate for GABA biosynthesis through the decarboxylation activity. They also stated that the addition of MSG into the culture medium plays the role of alkalinization of the medium through the decarboxylation activity. The alkalinization process may offer a more suitable medium environment for the cells to proliferate and divide, as at certain time point of incubation, the medium might be accumulated with high concentration of organic acids as the result of by-products produced by the lactic acid bacteria. This will cause the culture medium to be more acidic that may inhibit the cell growth.

In this study, it was discovered that the supplementation of 9 % (w/v) of MSG did not favour the GABA production and cell growth of *L. plantarum* B13. The cells growth of lactic acid bacteria tends to be inhibited by high concentrations of glutamate (Tajabadi et al., 2015). Previously, the fed-batch fermentation of *L. brevis* NCL912 also showed that the higher the concentration of glutamate (0.25 M, 0.5 M, 0.75 M, and 1.0 M), the lower the cell growth obtained (Li et al., 2010a). Gandhi and Shah (2016) stated that the addition of salt instigated the osmotic pressure to the lactic acid bacteria, resulting in a defective formation of cell wall and cell extension. The cells are extended due to the pressure that came from the growing environment (Molina-Höppner et al., 2003). In this study, within the range of tested concentration of MSG of 0 % to 9 % (w/v), the highest GABA yield (8.423 g/L) was obtained at 5 % (w/v) MSG. However, the highest *L. plantarum* B13 cell density (9.281 log CFU/mL) was achieved at the lowest MSG concentration of 1 % (w/v). In the meantime, the results showed a declination trend for the cell growth for the medium supplemented with 3 %, 5 %, 7 %, and 9 % (w/v) MSG. In particular, the lowest cell growth (9.099 log CFU/mL) was observed for medium with 9 % (w/v) MSG that was even lower than that obtained for the medium without the supplementation of MSG (control), but the GABA production (7.187 g/L) was higher than that obtained for the medium without the

supplementation of MSG (3.691 g/L). Although the cell growth showed a decreasing trend, however the GABA yield was considerably higher when the medium was supplemented with MSG concentrations of 3 %, 5 % and 7 % (w/v) compared to the GABA yield obtained from the culture medium with 1 % (w/v) MSG (that attained the highest cell growth). This is similar to the statement made by Kawamoto-Miyamoto et al. (2022), in which the ability of lactic acid bacteria to produce the desired product would be enhanced with the supplementation of a high concentration (ie., up to 6 %) of MSG. Likewise, *L. bulgaricus* CFR 2028 showed a higher cell growth with a lower GABA yield with the supplementation of 1 % (w/v) MSG compared to the culture mediums that composed of 2 %, 3 %, 4 % and 5 % (w/v) MSG, whereas the highest GABA yield (46.64 ± 1.35 mM) was observed in the medium with the addition of 2 % (w/v) MSG (Gangaraju et al., 2014). Meanwhile, a study disclosed by Seo et al. (2013), showed that the addition of 3 % MSG into the culture medium boosted the GABA yield by *L. brevis* 340G, however, when the concentration of MSG was higher than 3 %, it caused a reduction of GABA yield. These results pointed out that the manipulated factor, which was MSG concentrations, can improve the cell growth but at certain concentrations, it may reduce the efficiency of glutamate decarboxylase (Castanie-Cornet & Foster, 2001) and would increase the osmotic pressure of the cells thus inhibiting the metabolic activities and cell growth (Yang et al., 2008).

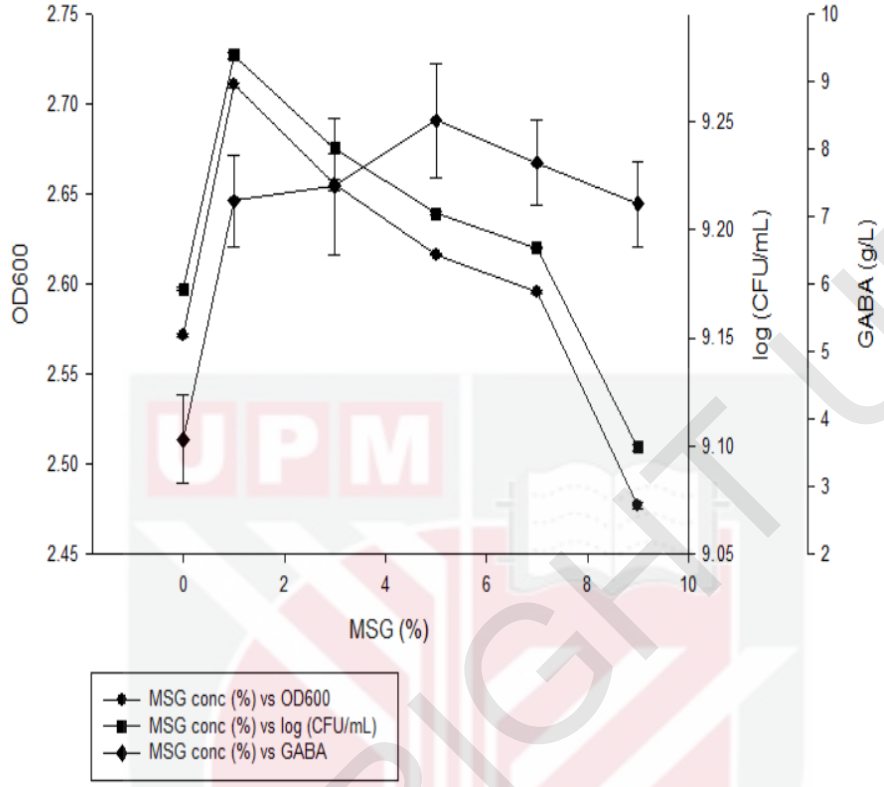

Figure 4.9 : Cell growth and GABA production of *L. plantarum* B13 with different MSG concentrations (0 % (control), 1 %, 3 %, 5 %, 7 %, and 9 % (w/v)) within 66 hours cultivation. The other fermentation parameters: initial pH of 6.0, 37°C and 0.5 mM PLP. The error bars represent the standard deviations about the mean (n=3)

4.5.5 Pyridoxal-5-phosphate (PLP) concentration

PLP is an essential co-factor for enhancing the GABA yield by lactic acid bacteria (Coda et al., 2010; Komatsuzaki et al., 2005; Yang et al., 2008). Generally, glutamate decarboxylase which is the enzyme that plays an important role for transforming glutamate into GABA, is initiated, and could be enhanced by the presence of coenzyme PLP (Kook et al., 2010). PLP may recover the activity of glutamate decarboxylase especially during the later stages of bacterial growth (Altaib et al., 2022). Hence, to determine the effect of the concentration of PLP for the biosynthesis of GABA and cell growth of *L. plantarum* B13 by OFAT, the PLP concentration added into the culture medium was varied (0 mM (control), 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, and 0.9 mM), whereas the other fermentation parameters including incubation time, initial pH, temperature, and concentration of MSG were fixed at incubation time of 66 hours, initial pH of 6.0, 37°C and 1 % (w/v) MSG, respectively.

Based on **Figure 4.10,** the results showed that the concentration of PLP significantly affecting both GABA production and cell growth of *L. plantarum* B13. The fermentation without the supplementation of PLP (control) had resulted with viable cell count of 9.170 log CFU/mL and GABA yield of 4.854 g/L. Increasing trend of both cell growth and GABA yield was observed when PLP was added in the range of 0.3 to 0.7 mM. Likewise, *L. plantarum* FNCC 260 also obtained higher yield of GABA when it was fermented in a medium supplemented with PLP compared to the medium without the addition of PLP (Yogeswara et al., 2020). The addition of 0.1 mM PLP resulted in GABA yield of 969.5 mg/L by *L. plantarum* FNCC, however, when PLP concentration was increased to 0.6 mM, the GABA yield was slightly reduced to 945.3 mg/L. In this study, the highest cell growth of *L. plantarum* B13 (9.267 log CFU/mL) and its GABA yield (8.439 g/L) were attained in the fermentation with the supplementation of 0.7 mM PLP. At 0.8 mM PLP, the cell growth of *L. plantarum* B13 retained at high viable cell count of 9.257 log CFU/mL, however, the GABA production was drastically dropped to 5.033 g/L. This is equivalent to 40 % reduction from the highest GABA attained in the fermentation with 0.7 mM PLP. Further increasing the PLP concentration to 0.9 mM, both the cell growth of *L. plantarum* B13 (9.149 log CFU/mL) and its GABA production (4.553 g/L) were at the lowest. The result of this study is in contrast with the work reported by Shan et al. (2015), that observed the influence of PLP on the GABA yield and glutamate decarboxylase activity, but it did not possess a significant effect on the cell growth of *L. plantarum* NDC75017. In the meantime, Li et al. (2010a) stated that the supplementation of PLP did not affect both GABA yield and cell growth of *L. brevis* NCL912.

The trend obtained by *L. plantarum* B13 is similar to the trend observed for *L. plantarum* NDC75017 as reported earlier by Shan et al. (2015). In the fermentation of *L. plantarum* NDC75017, a maximum glutamate decarboxylase activity was attained when the fermentation medium was supplemented with 20 mM PLP, resulting the maximum GABA production of 165.6 ± 0.3 mg/100g. Nevertheless, when the PLP concentration supplied exceeded 20 mM, the cell growth, activity of glutamate decarboxylase and GABA yield were all significantly reduced. These proved that PLP plays its role as a cofactor of glutamate decarboxylase, which helped to improve the enzyme catalytic activity (Cho et al., 2007; Komatsuzaki et al., 2005; Li et al., 2010b, Lu et al., 2008; Yang et al., 2008; Yogeswara et al., 2020). In the nutshell, PLP is beneficial for enhancing the GABA production by lactic acid bacteria, however an excessive concentration of PLP will cause a negative effect to both cell growth and GABA yield.

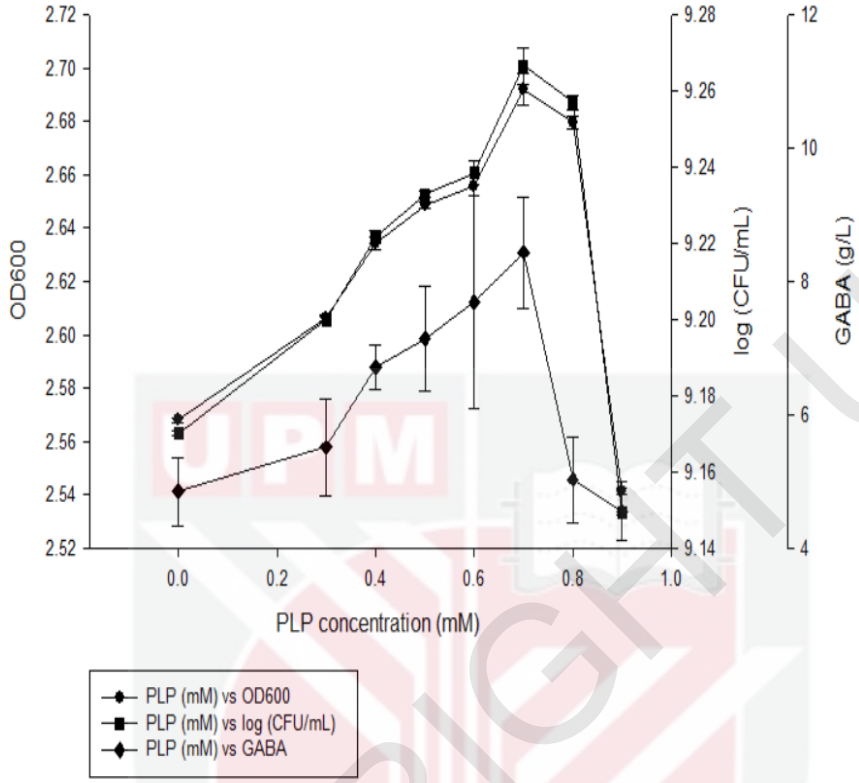

Figure 4.10 : Cell growth and GABA production of *L. plantarum* B13 with different PLP concentrations (0 (control), 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mM) within 66 hours cultivation. The other fermentation parameters: initial pH of 6.0, 37°C and 1 % (w/v) MSG. The error bars represent the standard deviations about the mean (n=3)

4.5.6 Glucose concentration

Generally, for high yield and efficient productivity of targeted products such as GABA, supplementing the batch fermentation with an appropriate amount of initial substrate concentration is important to avoid the substrate inhibition effect and may reduce the production cost. In this study, for determination of the effect of glucose concentration on the cell growth and GABA yield of *L. plantarum* B13, glucose concentrations were varied (20 g/L, 40 g/L, 60 g/L, 80 g/L, and 100 g/L) with the incubation time of 114 hours while the other fermentative parameters were fixed at the optimum conditions (initial pH: 5.5; temperature: 35°C; concentration of MSG: 5 % (w/v); and concentration of PLP: 0.7 mM) based on the results that obtained earlier.

Based on **Figure 4.11**, *L. plantarum* B13 was undergoing a lag phase for the first 3 hours of the incubation time for all tested glucose concentrations ranging from 20 g/L to 100 g/L corresponding to cell growth with OD_{600} reading and log CFU/mL that ranged from OD_{600} 0.075 to OD_{600} 0.223, and 7.233 log CFU/mL to 7.349 log CFU/mL, respectively. This showed that the initial concentration of glucose did not influence the duration of bacterial lag phase. However, in certain conditions such as when cells are inoculated into poor or unsuitable media, a longer duration of lag phase with no production of biomass will be observed ((Schultz and Kishony, 2013). This is because cells will be concentrating on the synthesis of carbon source utilization genes instead of cell growth and proliferation. For example, during the lactic acid fermentation in shrimp waste ensilation by *Lactobacillus* sp. (B2), lag phase was observed to be extended when an initial glucose concentration was higher than 10 % (Shirai et al., 2001).

Afterward, *L. plantarum.* B13 started to enter the exponential growth phase until 45 hours of incubation period for all tested glucose concentrations except for 20 g/L that reached the stationary phase after 21 hours. During the exponential phase, the readings of OD_{600} for all mediums with different glucose concentrations were rapidly increased. The range of the maximum OD_{600} reading for all the tested glucose concentrations was between OD_{600} 2.7 to 3.0 (**Figure 4.11 (A)**) while the range of viable cells count is between 9.3 to 9.5 log CFU/mL (**Figure 4.11 (B)**). These observations showed that the initial concentrations of glucose affecting the biomass density beside influencing the duration of exponential phase. The exponential growth of phase was prolonged when *L. plantarum* B13 was cultivated in medium with initial glucose concentrations of above 20 g/L. Among the tested glucose concentrations, the highest maximum cell growth (9.526 log CFU/mL and OD_{600} reading of 3.026 at 45 hours) for *L. plantarum* B13 was attained in the fermentation with 40 g/L glucose. Glucose supplies exceeded 40 g/L were not favourable for the enhancement of the cell growth of *L. plantarum* B13. This might be due to the inhibition effect resulted from the excessive glucose concentration in the culture medium. According to Abdel-Rahman et al. (2013), high concentration of glucose will increase the osmotic pressure towards cells leading in higher rate of cell death.

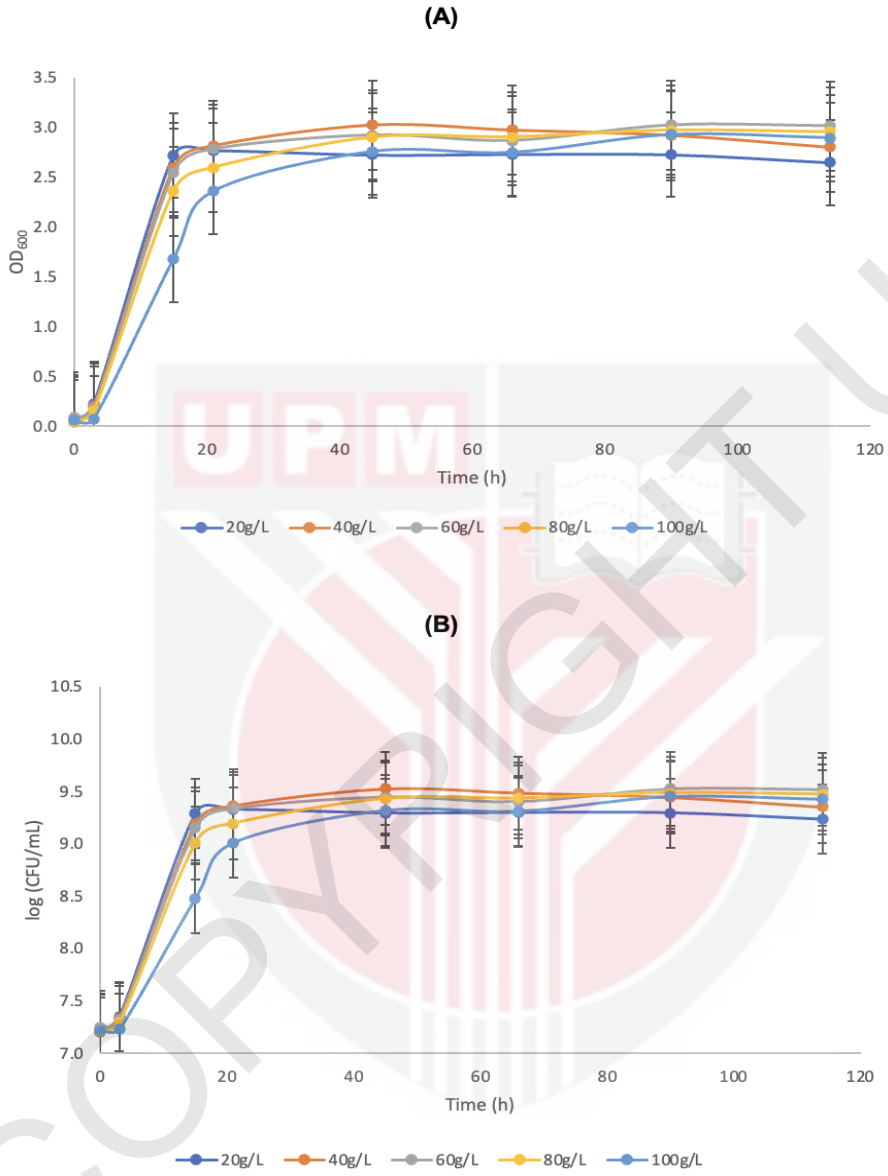

Figure 4.11 : OD_{600} readings (A) and log CFU/mL (B) for the cell growth of *L. plantarum* B13 in medium composed of different glucose concentrations (20, 40, 60, 80 and 100 g/L) within 114 hours incubation period. The fermentations conditions: initial pH: 5.5; temperature: 35°C; concentration of MSG: 5 % (w/v); and concentration of PLP: 0.7 mM. The error bars represent the standard deviations about the mean (n=3)

In general, glucose consumption was decreasing as the fermentation period was increasing for all tested glucose concentrations (**Figure 4.12)**. For the concentration of 20 g/L, glucose was drastically depleted to 6.801 g/L during the exponential phase (from 3 to 21 hours of the incubation time), then it remained almost constant during the stationary phase. The same trends were observed for the other glucose concentrations (from 40 to 100 g/L), in which the glucose concentrations were rapidly depleted between 3 to 45 hours (exponential phase) and remained almost constant until the end of the fermentation process (within a stationary phase). Approximately 82 % and 68 % glucose were depleted for the fermentations with glucose concentrations of 40 g/L and 60 g/L, respectively. Meanwhile, for 80g/L and 100 g/L glucose concentrations, only around 50 % of the initial glucose was consumed within the same fermentation period.

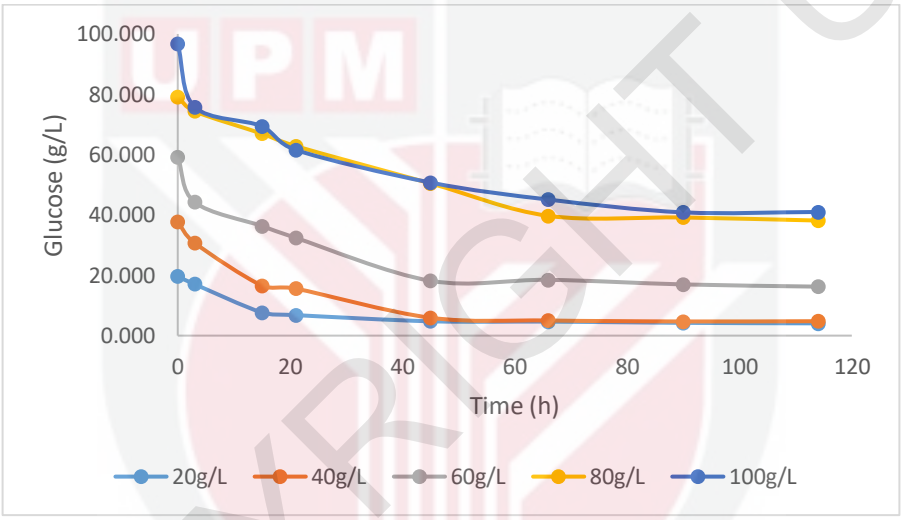

Figure 4.12 : Residual glucose concentration for fermentations by *L. plantarum* B13 in medium composed of different glucose concentrations (20, 40, 60, 80 and 100 g/L) within 114 hours incubation period. The fermentations conditions: initial pH: 5.5; temperature: 35°C; concentration of MSG: 5 % (w/v); and concentration of PLP: 0.7 mM. The error bars represent the standard deviations about the mean (n=3)

As depicted in **Figure 4.13**, an initial glucose concentration was found to affecting the GABA production by *L. plantarum* B13. Among the tested glucose concentrations, the highest GABA yield was attained in the fermentation with 40 g/L glucose that corresponding to 24.154 g/L. This was achieved during the stationary phase, at the fermentation time of 66 hours. Meanwhile, a maximum GABA production of 17.984 g/L was observed for the fermentation with 20 g/L glucose that was also reached at 66 hours. However, for *L. plantarum* B13 fermentations with medium containing 60 g/L, 80 g/L and 100 g/L, the maximum GABA productions were attained much earlier at 45 hours, corresponding to 19.073 g/L, 18.659 g/L and 18.407 g/L, respectively. The results showed that the initial concentration of glucose can affect the maximum yield of GABA. Too

high or too low initial glucose concentration may reduce the GABA production. Likewise, the GABA productions by *L. fermentum* were enhanced from 3.9 g/L to 4.74 g/L when glucose concentration was raised from 0.5 % to 1 % due to the improvement of cell growth (Rayavarapu et al., 2021). However, a declination in GABA production (0.79g/L) was observed when the glucose concentration was raised up to 2 %, due to the inhibitory effect from the high concentration of glucose.

According to **Table 4.6**, the maximum GABA yield (calculated as the amount of GABA produced per the amount of glucose consumed) by *L. plantarum* B13 was found at 20 g/L (1.171 g/g). Despite the maximum concentration of GABA (24.154 g/L) was attained from the fermentation with 40 g/L glucose, nonetheless the highest GABA productivity (0.424 g/L/h) was attained when 60 g/L glucose was supplemented in the MRS medium. Based on this kinetic data, therefore, the fermentation with 60 g/L glucose could be considered as the best concentration to accomplish higher efficiency of GABA production process during an industrial development. Likewise, based on the study demonstrated by Abdel-Rahman et al. (2019), they also have taken the productivity kinetic parameter to be used for the determination of optimum glucose concentration while obtaining the maximum production of targeted product (lactic acid) by a lactic acid bacterium (*Enterococcus hirae* BoM 1-2). Besides that, based on the study reported earlier by Abdel-Rahman et al. (2013), although the lactic acid concentration and yield were increased by approximately 5 to 6 % at 45 °C compared to those obtained at 43 °C, but the fermentation at 43 °C exhibited the highest lactic acid productivity of 1.63 g/L/h among all the tested temperatures (30 °C, 37 °C, 43 °C, 45 °C and 49 °C), and hence, the fermentation temperature of 43 °C was selected for further investigation. According to Clarke et al. (2011), the productivity is an important consideration in controlling the cost of the bioprocess. With good productivity, it can lead to higher production volume and improve profitability.

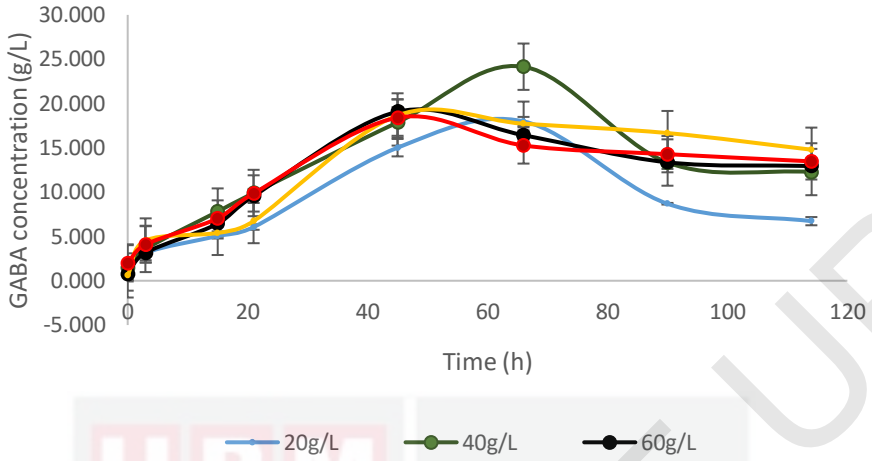

Figure 4.13 : GABA production profiles by *L. plantarum* B13 in the fermentations with different glucose concentrations (20, 40, 60, 80 and 100 g/L) within 114 hours incubation period. The fermentations conditions: initial pH: 5.5; temperature: 35°C; concentration of MSG: 5 % (w/v); and concentration of PLP: 0.7 mM. The error bars represent the standard deviations about the mean (n=3)

Table 4.6 : Cell growth, GABA production, residual glucose concentration and kinetic parameters of *L. plantarum* B13 for different glucose concentrations

Glucose conc. (g/L)	Time (h)	OD_{600}	log CFU/mL	Max. GABA conc. (g/L)	Residual glucose (g/L)	Y_{GABA} (g/g)	P_{GABA} (g/L/h)
20	66	2.732 ± 0.0015^{c}	9.297 ± 0.0012^{c}	17.984 ± 0.5071^{b}	4.648 ± 0.0081^{c}	1.171	0.272
40	66	2.976 ± 0.0248^{a}	9.487 ± 0.0193^{a}	24.154 ± 0.2200^{a}	5.016 ± 0.0093^{c}	0.690	0.366
60	45	2.924 ± 0.0056^{b}	9.447 ± 0.0043^{b}	19.073 ± 0.5214^{b}	18.171 ± 0.0233^{b}	0.456	0.424
80	45	2.903 ± 0.0015^{b}	9.431 ± 0.0012^{b}	18.659 ± 0.6411^{b}	50.454 ± 0.0466^{a}	0.632	0.415
100	45	2.760 ± 0.0026^{c}	9.319 ± 0.0021^{c}	18.407 ± 0.1470^{b}	50.736 ± 0.3024^{a}	0.374	0.409

Note: The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

4.6 Screening of natural supports for immobilization of *L. plantarum* B13

In this experiment, several vegetables including as okra (*Abelmoschus esculentus*), onion (*Allium cepa*), bell pepper (*Capsicum annuum*), sweet potato (*Ipomoea batatas*) and taro (*Colocasia esculenta*) were screened for selecting the most suitable natural support for cell immobilization of *L. plantarum* B13. These vegetables are known as potential prebiotic sources. Prebiotics are indigestible carbohydrates that cannot be absorbed by the small intestine and play an important role as the energy source for the gut microflora (Khangwal and Shukla, 2019). The most well-known sources of the prebiotics are fructooligosaccharides (FOS), inulin and galactooligosaccharides (GOS) from plants (Dwivedi et al., 2014). Prebiotics can either be produced from the enzymatic digestions or naturally contained in fruits (such as nectarine, pear, watermelon, blueberry, mulberry) and vegetables (such as cabbage, shallot, carrot, pepper, onion, okra, garlic, yam or taro, sweet potato, white radish, cassava, winter melon, bottle gourd, snake gourd, buttercup squash, bitter melon and mushrooms) (Jovanovic-Malinovska et al., 2014, Elok and Wilda, 2013, Sreenivas and Lele, 2013, Aida et al., 2009).

4.6.1 Cell retention on different natural supports

The initial viable cell counts of *L. plantarum* B13 that immobilised on different vegetables were recorded after 15 hours of immobilisation process (prior to application in the GABA fermentation). Among the five screened vegetables, the highest initial viable cell count, 8.071 log CFU/mL of *L. plantarum* B13 was recorded for bell pepper while the lowest cell retention of 7.724 log CFU/mL was observed for taro (**Figure 4.14 (A)**). The initial viable cell counts of immobilised cells on okra, onion, sweet potato, and bell pepper were non-significantly different from each other. However, the initial viable cell counts of immobilised cells on okra, onion, sweet potato, and bell pepper were significantly different from taro. Likewise, the immobilization of *L. casei* CSL3 on three different fruits (pineapple, guava, and kiwi), also shown different initial viable cell count despite using the same bacterial concentration for the immobilization process (Vitola et al., 2020). It was deduced that the presence of different undigested carbohydrate content in each fruit might increase the immobilization capacity. In addition, to increase the cells retention on natural supports, inoculum sized can be optimized in the future study. For example, increasing the inoculum size (in the range of 0.27 to 15 g dry cell) of *Pichia stipitis* was demonstrated to significantly increase the cell retention on pre-treated sugarcane bagasse (Yñiguez-Balderas et al., 2016).

The immobilized *L. plantarum* B13 cells were then applied in batch fermentations using the optimized parameters (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose, 35°C). The final viable cell counts of immobilized *L. plantarum* B13 were recorded after 45 hours of fermentation without agitation. Based on **Figure**

4.14 (B), the highest counted of final viable cell count (7.952 log CFU/mL) was found to be onion-immobilized *L. plantarum* B13 while the lowest (7.369 log CFU/mL) was attained by taro-immobilized *L. plantarum* B13. Despite having the highest initial viable cell counts, however bell pepper-immobilized *L. plantarum* B13 failed to retain its high cells viability after 45 hours of incubation. The final viable cell count for onion-immobilized *L. plantarum* B13 was significantly higher compared to the other four vegetables while there was a non-significant difference of the final viable cell counts between sweet potato and bell pepper and between bell pepper and taro. However, the final viable cell count of sweet potato-immobilized *L. plantarum* B13 was significantly different from taro-immobilized *L. plantarum* B13.

After 45 hours of fermentations, the highest (83 %) cell retention was measured for the onion-immobilized *L. plantarum* B13 while the lowest (23 %) was bell pepper-immobilized *L. plantarum* B13 (**Figure 4.15**). The cell retention of okra-immobilized *L. plantarum* B13 was significantly different from the other four vegetable supports immobilized cells, whereas it was a non-significantly different from the taro-immobilized *L. plantarum* B13. In addition, the cell retentions of immobilized *L. plantarum* B13 onto sweet potato and bell pepper were non-significantly different from each other, but they were significantly different from okra, onion, and taro.

The ability to retain high cell viability during applications is one of the important characteristics in selecting the most suitable support for immobilization of microorganisms (Mitropoulou et al., 2013, Vitola et al., 2020). The morphology and the biomolecules that composed on the inner epidermis of onions would enhance the adherence ability of microbial cells towards the surface of onion (Kumar & D'Souza, 2011). The morphology of onion provides a good support for immobilizing the microbial cells. In addition, the cell wall of the inner epidermis cells of onion consists of extracellular matrix. The extracellular matrix composed of two different phases, which are microfibrillar cellulose and matrix, that consists of many different types of polymers such as polygalacturonic acid (PGA), hemicelluloses, proteins, phenolic compounds and lignin (Carpita & Gibeaut , 1993; Brett & Waldron, 1996; Kumar & D'Souza, 2009). These biomolecules can provide a biocompatible environment for enhancing the metabolic activities of microbial cells. Therefore, onion was considered as the best natural support for the immobilisation of *L. plantarum* B13, as cells showed the greatest adhesive tendency towards onion compared to the other four tested vegetable supports.

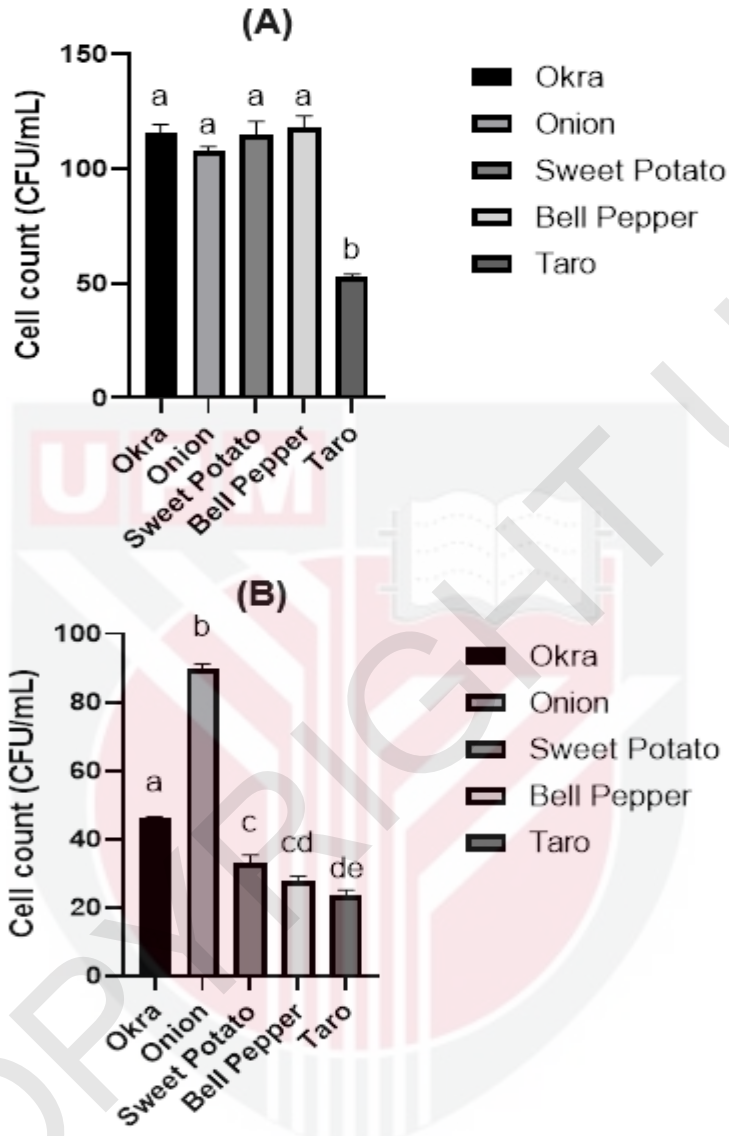

Figure 4.14 : Initial viable cell counts of immobilized *L. plantarum* B13 on different vegetables (okra, onion, sweet potato, bell pepper and taro) as natural supports after 15 hours immobilization process (A) and final viable cell count of immobilized *L. plantarum* B13 after 45 hours of fermentation (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose) at 35°C, without agitation (B). The error bars represent the standard deviations about the mean (n=3). Different superscript letters indicate significant different based on Tukey's test at $p < 0.05$. The results were expressed as mean (n = 3) ± SD

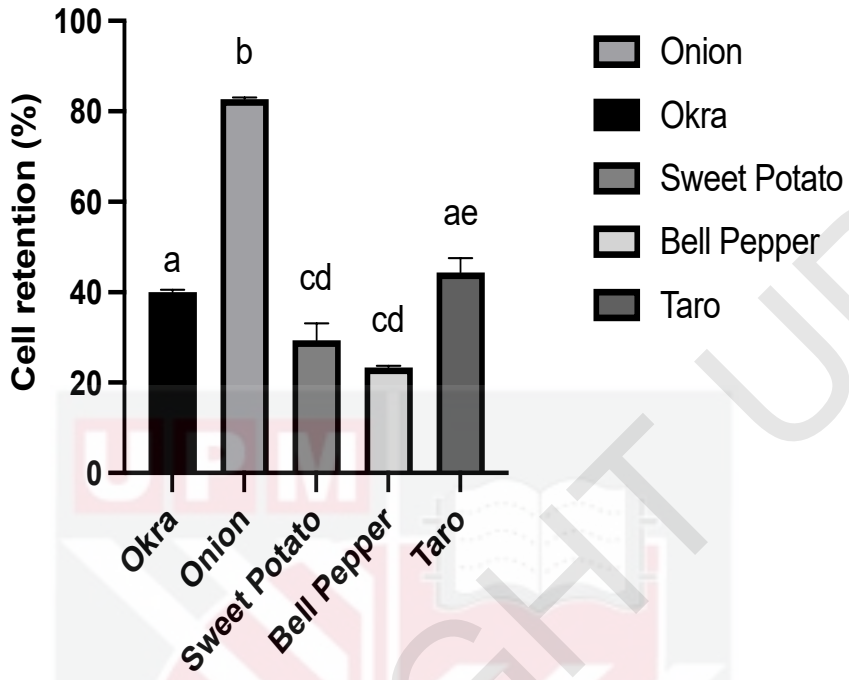

Figure 4.15 : Percentage cell retention of immobilized *L. plantarum* B13 on different natural supports (okra, onion, sweet potato, bell pepper and taro) after 45 hours of fermentation (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose) at 35°C, without agitation. The error bars represent the standard deviations about the mean (n=3). Different superscript letters indicate significant different based on Tukey's test at $p < 0.05$

4.6.2 GABA production by immobilized *L. plantarum* B13

The production of GABA by immobilized *L. plantarum* B13 were obtained after 45 hours of fermentation. Based on **Figure 4.16**, the highest GABA concentration (18.854 g/L) was recorded for the fermentation by onion-immobilized *L. plantarum* B13, whereas taro-*L. plantarum* B13 produced the lowest GABA (12.967 g/L). The amounts of GABA produced by the immobilised *L. plantarum* B13 on okra (16.325 g/L GABA), sweet potato (16.569 g/L GABA) and bell pepper (16.220 g/L GABA) were not significantly different with each other, however they were significantly different from onion and taro.

Onion was determined as the best natural immobilisation support due its highest cell retention (**Section 4.6.1**) and GABA yield compared to the other tested vegetable supports (okra, sweet potato, bell pepper and taro) and hence onion-immobilized *L. plantarum* B13 was selected to be used in the subsequent experiments. Onion is a stronger and good natural polymer for the immobilisation of microbial cells or enzymes due to its morphology and the biomolecules that

composed on its inner epidermis (Kumar & D'Souza, 2011). The cell wall of the inner epidermis cells of onion consists of extracellular matrix composed of two different phases, which are microfibrillar cellulose and matrix, that consists of many different types of polymers such as polygalacturonic acid (PGA), hemicelluloses, proteins, phenolic compounds, and lignin (Carpita & Gibeaut, 1993; Brett & Waldron, 1996; Kumar & D'Souza, 2009). The morphology of onion provides a good support for immobilising microbial cells or enzymes, meanwhile, biomolecules that composed on the inner epidermis of onion provide a biocompatible environment for enhancing the metabolic activities of microbial cells or the performance of enzymes. Previously, the inner epidermis of onion was used as a natural support for the immobilization of *Sphingomonas* sp. via adsorption and cross-linking methods (Kumar & D'Souza, 2011). The onion-immobilized cells were shown to be stable and reusable in nature for microbial biosensor applications.

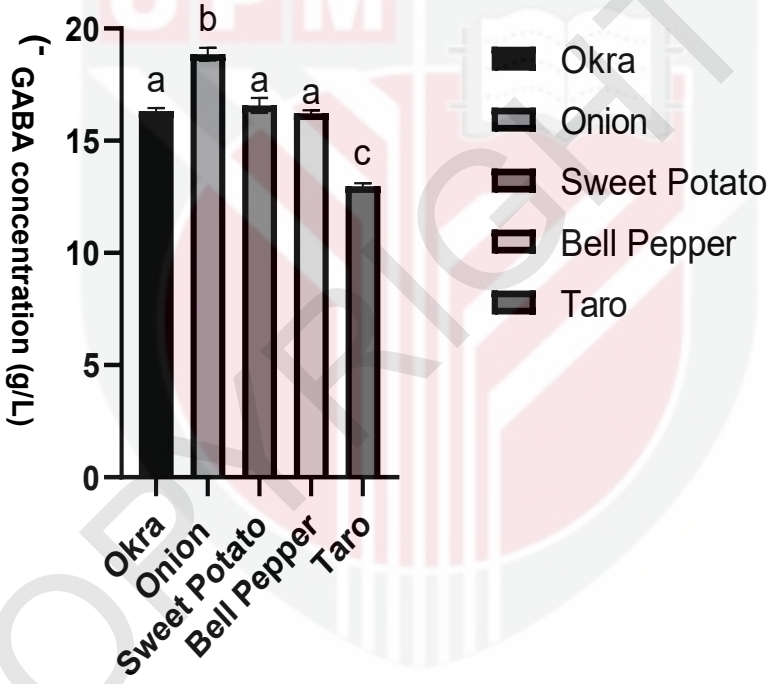

Figure 4.16 : GABA production by immobilized *L. plantarum* B13 on different natural supports (okra, onion, sweet potato, bell pepper and taro) after 45 hours of fermentation (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose) at 35°C, without agitation. The error bars represent the standard deviations about the mean (n=3). Different superscript letters indicate significant different based on Tukey's test at $p < 0.05$

4.7 Repeated batch fermentation by onion-immobilized *L. plantarum* B13

Batch mode of fermentation is the most used method to produce various bioactive compounds such as GABA (Thuy et al., 2020) and lactic acid (Litchfield et al., 2009) by lactic acid bacteria. However, batch fermentation is facing some limitations such as low productivity and low concentration of cells due to the elongation of fermentation time as well as the substrate and end-product inhibitions (Abdel-Rahman et al., 2013a). Thus, to cope with these problems, beside conventional fed-batch, and continuous modes of fermentation (Hsueh et al., 2021), repeated batch fermentation utilizing immobilized cells can be explored as the alternatives. Repeated fermentation can be operated using batch or fed-batch mode, that involves the repeatedly use of cells from the previous run into a subsequent run (Zhao et al., 2010). The advantages of repeated fermentation include enhancing the productivity of targeted product, high cell concentration, time saving, elimination of cleaning and sterilisation process of the bioreactor, exclusion of the step for seed preparation, and short fermentation time as the starting inoculation volume is high (Abdel-Rahman et al., 2013a; Gao et al., 2012; Wu et al., 2011). Hence, in this study, a combination of cell immobilisation method and repeated batch fermentation was used to enhance the production of GABA by onion-immobilized *L. plantarum* B13, which was also compared with the free cells *L. plantarum* B13. Besides GABA production, viable cell count, lactic acid production and changes of pH were also evaluated.

4.7.1 GABA production

The production of GABA by free cells and onion-immobilized *L. plantarum* B13 in repeated batch fermentations were obtained after 45 hours of fermentation. As depicted in **Figure 4.17**, the results showed that the concentrations of GABA by *L. plantarum* B13 were constantly reduced when the free cells and immobilized cells were repeatedly used in consecutive batches. The highest GABA yield was obtained from the first batch of fermentations by both free cells and onion-immobilized cells that corresponding to 19.073 g/L and 18.463 g/L, respectively. Despite attained a slightly lower GABA production in the first batch, however, the onion-immobilized *L. plantarum* B13 managed to be reused for more successive fermentation batches than that observed for the free cells *L. plantarum* B13. The onion-immobilized cells were managed to be reused for at least 10^{th} successive fermentation batches with the lowest GABA of 4.228 g/L (attained at the final 10^{th} batch). Meanwhile, the GABA production from the recycling of free cells *L. plantarum* B13 was obtained for only 8 successive fermentation batches with the lowest GABA concentration of 2.909 g/L (attained at the final 8^{th} batch).

The total amount of GABA production throughout the 10 successive fermentation batches by onion-immobilized *L. plantarum* B13 was 123.634 g/L, whereas for free cells, a total GABA of 95.545 g/L was attained during 8

successive fermentation batches. In comparison, during the 8 successive fermentation batches, the production of GABA by onion-immobilized *L. plantarum* B13 was approximately 30 % higher (equivalent to 123.634 g/L GABA) than that attained by the free cells. Based on **Table 4.7**, if a threshold limit to be deemed as a successful production cycle is considered to be 50 %, it can be seen for the onion-immobilized *L. plantarum* B13, the production of GABA was reduced to 50 % of the initial GABA concentration after the 8th batch, whereas, for free cells, after the 6th batch.

The results of this study demonstrated that the cell immobilisation technology and repeated batch fermentation are capable to significantly improve the GABA production than the convention method of utilizing free cells. The enhanced reusability of immobilized cells highlights an important trait which is crucial for reducing the costs of potential industrial applications (Zotta et al., 2022). Previously, the immobilizations of *L. casei* on apple and quince pieces in repeated batch fermentations of whey medium were also able to produce a high amount of lactic acid, due to high activity of lactose conversion (Kourkoutas et al., 2005). The immobilised cells were able to be reused for 15 successive fermentation batches, whereas the free cells were only able to be reused for 6 successive fermentation batches resulting in higher (ranged between 10.9 g/L and 13 g/L) lactic acid productivity than the free cells (ranged between 7.2 g/L and 10.3 g/L). The enhanced productivity may be due to the enhanced cell viability of the bacteria by the immobilisation method (Shah, 2000). Similarly, the yoghurt production by immobilised cells of *L. casei* ATCC393 and *L. bulgaricus* DSM20081 on wheat bran possessed higher cell viabilities and higher survival rates during the exposure to gastric juice at pH3.0 compared to free cells (Terpou et al., 2017). The viability of immobilised cells might be enhanced due to the prebiotic effect of the delignified wheat bran. However, the cell viability and productivity of the immobilised cells were significantly reduced after going through the successive fermentation batches. Furthermore, the repeated fermentation method was demonstrated to significantly improved the lactic acid production by *L. casei* subsp. *rhamnosus* ATCC11443 (Velázquez et al., 2001).

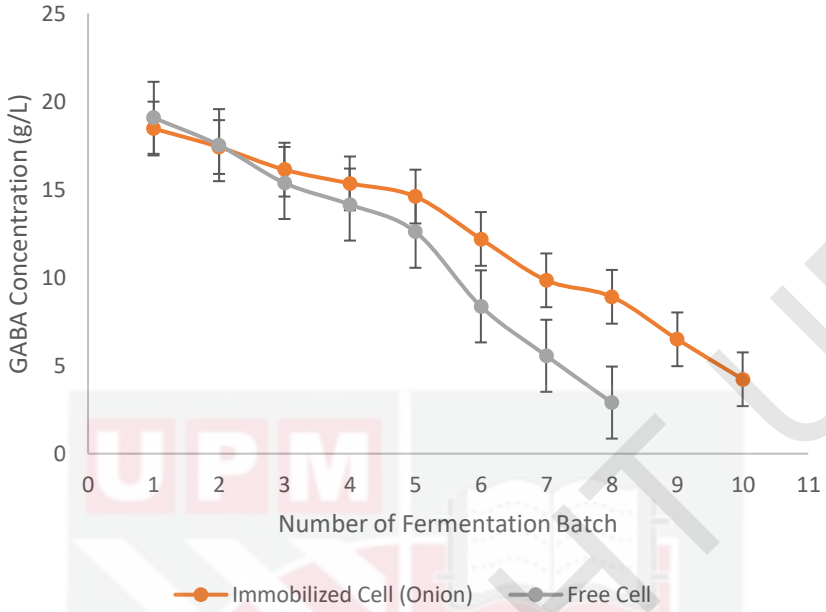

Figure 4.17 : GABA production by free cells and onion-immobilized *L. plantarum* B13 in repeated batch fermentations (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose) at 35°C, without agitation. For each number of fermentation batch, GABA was measured after 45 hours of fermentation. The error bars represent the standard deviations about the mean (n=3)

Table 4.7 : Total successful GABA production by free-cell and immobilized *L. plantarum* B13

Sample	Successful Cycles	Total successful GABA production (g/L)
Free cells	6	87.081 ± 0.246^{a}
Onion-immobilized cells	8	112.911 ± 0.231^{b}

Note: Each data represents the mean ± SEM of three replicates. Different lowercase letters within the same column represent the statistically significant differences ($p < 0.05$) of the results between the two different samples.

4.7.2 Lactic acid production and changes of pH

According to **Figure 4.18**, the lactic acid production (measured after 45 hours fermentation) was reduced for both free cells and onion-immobilised *L. plantarum* B13 after going through the successive fermentation batches. It could be seen that the lactic acid production for each single batch of free cells were higher than the immobilized cells up to the 5th batch. Afterward, the concentration of lactic acid produced by free cells was drastically declined to lower than that

attained by the immobilized cells. Throughout the 8 successive batches, the total amount of lactic acid produced by the free cells was 199.612 g/L, whereas the total amount of lactic acid produced by the immobilised cells was higher corresponding to 205.201 g/L within 10 successive batches. Based on **Table 4.8**, if a threshold limit to be deemed as a successful production cycle is considered to be 50 % of the initial lactic acid attained in the first batch, the total production of lactic acid by free cells was 161.945 g/L after the 5th batch while for 188.362 g/L after 8th batch for immobilized cells.

To date, many reports have also demonstrated the advantages of utilizing immobilized cells in repeated batch fermentations for achieving higher lactic acid production. For example, the polyurethane immobilised *R. oryzae* MTCC 8784 by direct hydrolysis of starch and sugar bagasse was observed to produce higher amount of lactic acid compared to free cells (Ranjit and Srividya, 2016). The immobilized *R. oryzae* MTCC 8784 was also shown to be highly stable within four repeated batch fermentation producing higher lactic acid than the free cells. The same trend was reported for the production of lactic acid by polyurethane immobilised *Rhizopus oryzae* (Tanyıldızı et al., 2012). Under optimum fermentation conditions, immobilized cells produced 55 % higher lactic acid than the free cells. Meanwhile, immobilised *L. Lactis* ATCC19435 in a fibrous bed bioreactor system was able to produce higher amount of lactic acid and its productivity compared to the free cells (Shi et al., 2012). Furthermore, the immobilised *L. lactis* IO-1 either by entrapment in beads or encapsulation in alginate membrane, both were feasible to be reused for more fermentation cycles and resulting in high lactic acid productivity (Sirisansaneeyakul et al., 2007).

Besides that, based on **Figure 4.18**, the first five batches of fermentation by using the free *L. plantarum* B13 produced more lactic acid than onion-immobilized *L. plantarum* B13. According to Radovich (1985), the immobilization matrix might cause the mass transfer limitation that was attributed to the transport resistance. Hence, it affect the activity of immobilized cells. This caused the viability of immobilized cells to be lesser than free cells in the first batch of fermentation and resulted in lesser amount of lactic acid was produced by immobilized cells than free cells during the first five batches of fermentation. Based on **Figure 4.20**, the free cells had higher viability compared to the immobilized cells in the first batch of fermentation. This indicated there were higher amount of free cells consumed the glucose and produced higher amount of lactic acid. However, the stability of free cells was lower compared to the immobilized cells because the total successful cycle for lactic acid production by using free cells was 5, whereas, the total successful cycle for lactic acid production by using immobilized cells was 8. According to Shi et al. (2012), utilization of the immobilized cells in a repeated batch fermentation further exhibited the persistence and stability of the system within extended period and producing higher yield of lactic acid.

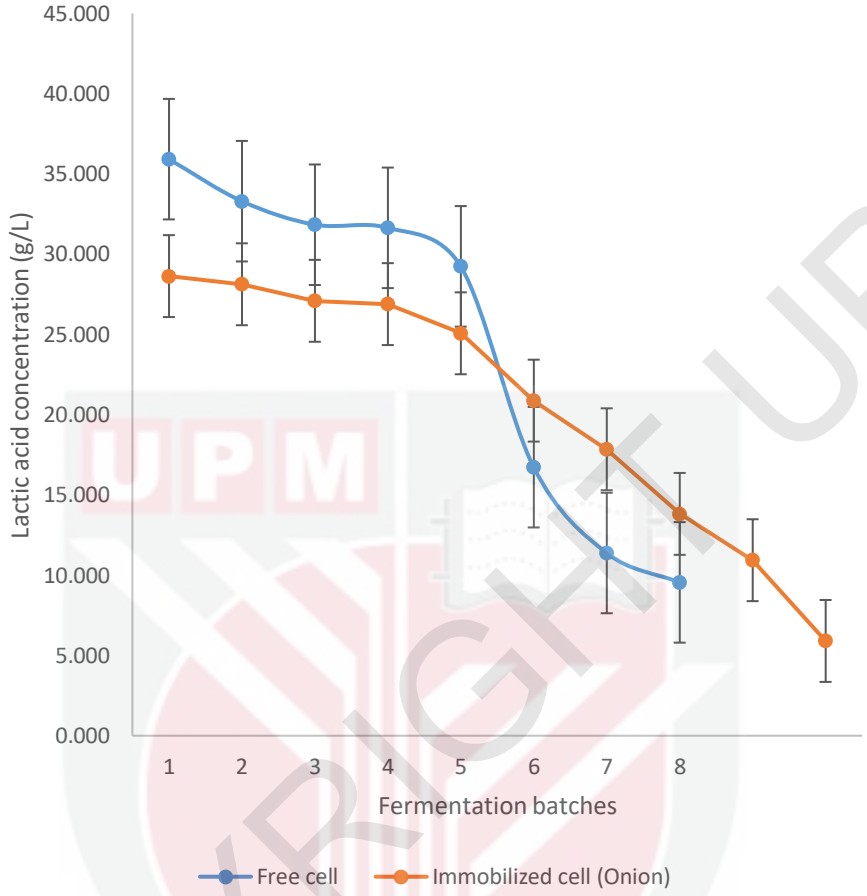

Figure 4.18 : The lactic acid production by free cells and onion-immobilized *L. plantarum* B13 in repeated batch fermentations (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose) at 35°C, without agitation. For each number of fermentation batch, lactic acid was measured after 45 hours of fermentation. The error bars represent the standard deviations about the mean (n=3)

Table 4.8 : Total successful lactic acid production by free-cell and immobilized *L. plantarum* B13

Sample	Successful Cycles	Total successful lactic acid production (g/L)
Free cells	5	161.945 ± 0.093[a]
Immobilized cells	8	188.362 ± 0.160[b]

Note: Each data represents the mean ± SEM of three replicates. Different lowercase letters within the same column represent the statistically significant differences ($p < 0.05$) of the results between the two different samples.

Generally, lactic acid (pKa value of 3.86) is a type of organic acid which has a low molecular weight and is mostly synthesised by lactic acid bacteria throughout the fermentation process (Abd Alsaheb et al., 2015). The production and accumulation of both lactic acid and GABA will give a major impact on the changes of pH values of the fermentative medium. Glutamate decarboxylase is responsible for producing GABA (a neutral compound) by decarboxylating of glutamate, which is acidic in nature through the incorporation of H^+ (Lee et al., 2015). According to **Figure 4.19**, after 45 hours of incubation, increasing trends of pH values of the medium were observed for both repeated batch fermentations of free cells and onion-immobilized *L. plantarum* B13. These results are correlated to the production of organic acids by-products by *L. plantarum* B13, in particularly lactic acid which was found to be decreasing when cells were reused in consecutive batches (**Figure 4.18**). It could be observed that the pH of the medium for the first batch of fermentation by free cells *L. plantarum* B13 was 3.85, whereas the pH of the medium for the final batch (8^{th} batch) was increased to pH 4.37 (**Figure 4.19(A)**). Meanwhile, the pH of the medium of the first fermentation batch by onion-immobilized *L. plantarum* B13 was 3.94 and the last batch (10^{th} batch) was measured to be at pH 4.48 (**Figure 4.19 (B)**). This showed that the end pH of medium (final batch) was still maintained at the optimum range of pH (pH 4.0 to pH 5.5) for glutamate decarboxylase to catalyse the production of GABA (Lee et al., 2015). Hence, it could be speculated that pH is not the major factor contributing to the decreasing trend of GABA production in the repeated batch fermentation by both free cells and immobilized cells.

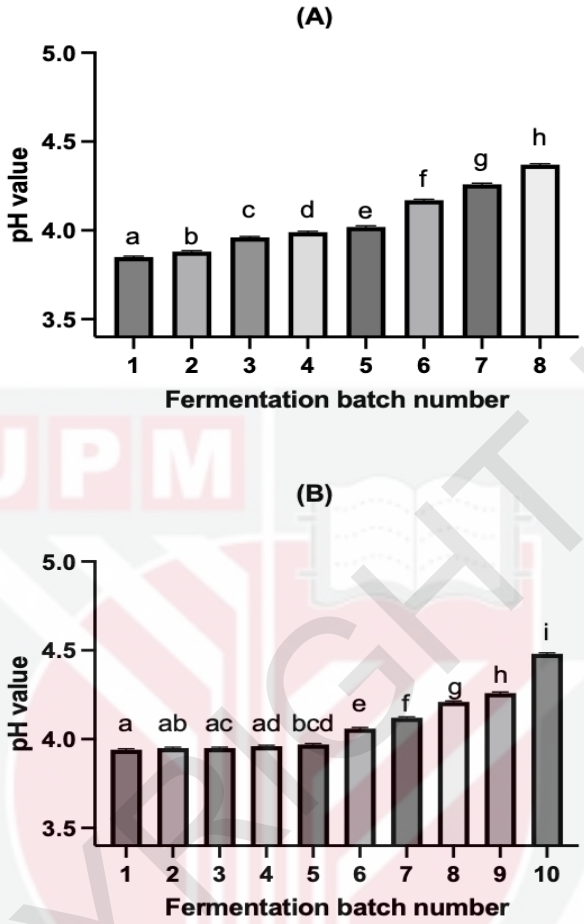

Figure 4.19 : The profile of pH changes of the medium during the repeated batch fermentation (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose, 35°C, without agitation) by free cells of *L. plantarum* B13 (A) and onion-immobilized *L. plantarum* B13 (B). For each number of fermentation batch, pH was measured after 45 hours of fermentation. The error bars represent the standard deviations about the mean (n=3)

4.7.3 Cell viability of free cells and onion-immobilized cells

For comparison of cell viabilities during the repeated batch fermentations by free cells and onion-immobilized *L. plantarum* B13, the viable cell counts were measured for the initial batch (before the fermentation), first batch of fermentation (sampling at 45 hours) and final batch of fermentation which were the 8th batch for free cells and at the 10th batch for the immobilized cells (sampling at 45 hours). According to **Figure 4.20**, the results showed that the viable cell counts for each sampling points (initial point, first batch and final batch) were significantly different with each other for both free cell and

immobilized cells. For free cells *L. plantarum* B13 (FC), the viable cell count for the initial batch (before fermentation) was 7.244 log CFU/mL. After the first batch of fermentation, the free cells grew up to 9.524 log CFU/mL. However, after it was consecutively reused by inoculating the free cells from the previous batch into the next batch, the viable cells count started to decline to 4.035 log CFU/mL after the 8th batch of fermentation. The same trend was observed for the viable cell counts of onion-immobilized cells (IC). The viable cell count for the initial batch (before fermentation) was measured to be 7.045 log CFU/mL. After the first batch of fermentation, the onion-immobilised *L. plantarum* B13 grew up to 8.952 log CFU/mL. However, after the 10th batch of fermentation, the viable cell count for the immobilised cells was significantly reduced to 5.186 log CFU/mL. The results are in accordance with the study disclosed by Li et al. (2020), reporting the enhanced production of *L-tyrosine* by *E. coli* GHLTYR-168 in a repeated batch fermentation within 5 consecutive batches. The results also showed that there was an increment of viable cell count from the first to the third batch, however, the viable cell count was reduced afterwards.

The decreased of viable cells after its repetitive usage might be due to the accumulation of toxic metabolic by-products that made the culture medium as no longer suitable for cells to divide, proliferate and grow. This effect is especially crucial for free cells as proven by the lower viable cell counts than the immobilized cells. This is supported by the results reported by Li et al. (2020), that discovered when the accumulation of acetic acid in medium exceeded 2 g/L, the cell growth would be suppressed, as well as the cell metabolism would be obstructed resulting in a declination of cell viability, productivity, and yield. Wang et al. (2012) also showed that the viable cell counts, lipid production and dry cell mass were decreased after the cells were going through 5 consecutive fermentation batches. According to Wan et al. (2016), the efficiency of the repeated batch fermentation for the production of exopolysaccharide was becoming lower after several consecutive fermentation batches due to the accumulation of toxic metabolic by-products that led to cell death. In the meantime, Yang et al. (2017), reported on the reduction of cell viability after cells were reused for several consecutive batches that might be due to the inhibitory effect of the metabolic products such as organic acid and ethanol. The concentrations of both the organic acid and ethanol were increased after several consecutive batches of fermentation, followed by impeding and suppressing the cell proliferation and growth.

In comparison, the immobilization technique significantly helped to maintain the viability of *L. plantarum* B13, hence resulting in higher viable cell count at the final batch of repeated fermentation, even though it was reused for another two extra batches compared to free cells. This proved that the immobilised cells possessed a greater cell viability and stability after going through several consecutive batches of fermentation compared to free cells (Min-tian et al., 2004; Øyaas et al., 1996; Panesar et al., 2007; Tong et al., 2004). Furthermore, the higher cell viability of immobilized *L. plantarum* B13 compared to free cells could also be influenced by the prebiotic effect exerted by the onion. Onion can produce prebiotic effects and is a good source of bioactive compounds (such as phenolic acids, flavonoids and thiosulfinates) with various therapeutic properties

(Desjardins, 2008). The use of prebiotic as the immobilizer carrier can increase the cells survival rate and improve the stability of cells during processing and storage (Terpou et al., 2017; Charalampopoulos et al., 2002). The cell immobilisation technology will also be more practical and advantageous when it is applied in a bioreactor system for further improving the concentration of cells and targeted metabolic products (Abdel-Rahman et al., 2013a).

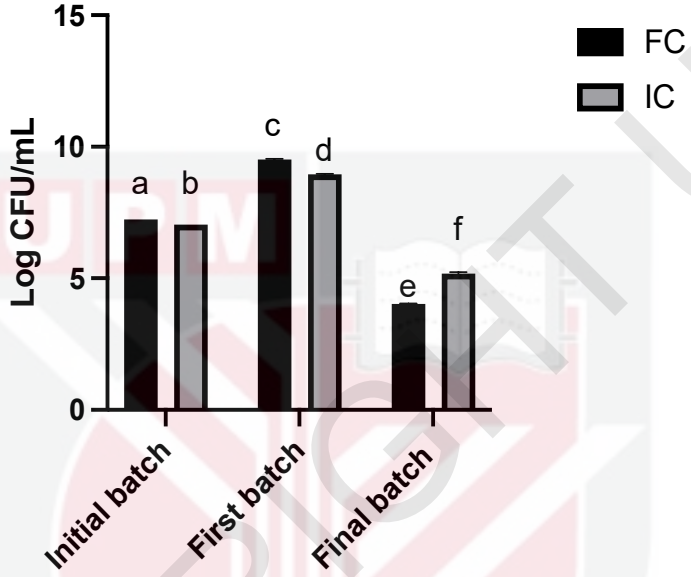

Figure 4.20 : Viable cell count (log CFU/mL) free cell *L. plantarum* B13 (FC) and for the onion-immobilized *L. plantarum* B13 (IC) during the repeated batch fermentations (initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose, 35°C, without agitation). Initial batch: cells before used in the fermentation; First batch: sampling at 45 hours of the first cycle of fermentation; and Final batch: sampling at 45 hours of the 8th batch for free cells (FC), and at the 10th batch for immobilized cells (IC). The error bars represent the standard deviations about the mean (n=3). Different superscript letters indicate significant different based on Tukey's test at p < 0.05

L. plantarum B13 cells that naturally retained on the surface of onion can be seen from the scanning electron microscopic (SEM) images (**Figure 4.21**). Natural adsorption of microorganisms that is also defined as passive immobilizations can be observed for most natural supports including plants, fruits, and organic compounds (Teh et al., 2009). For some microorganisms, the formation of biofilm that allows cells to adhere to a surface and attached to each other may assist the immobilization on natural supports (Junter and Jouenne, 2017). Immobilization of cells in the optimum conditions will lead to greater cells attachment to the surface of supports followed by cell colonisation that triggers the formation of biofilms. Furthermore, the attachment of cells on the surfaces

of natural supports could also occurred via electrostatic or covalent binding. From the SEM images, an obvious reduction in number of cells attachment on the surface of onion can be observed after the immobilized cells were recycled for 10 successive fermentation batches (**Figure 4.21 (B)**) compared to the immobilized cells sample before used in the repeated batch fermentation (**Figure 4.21 (A)**).

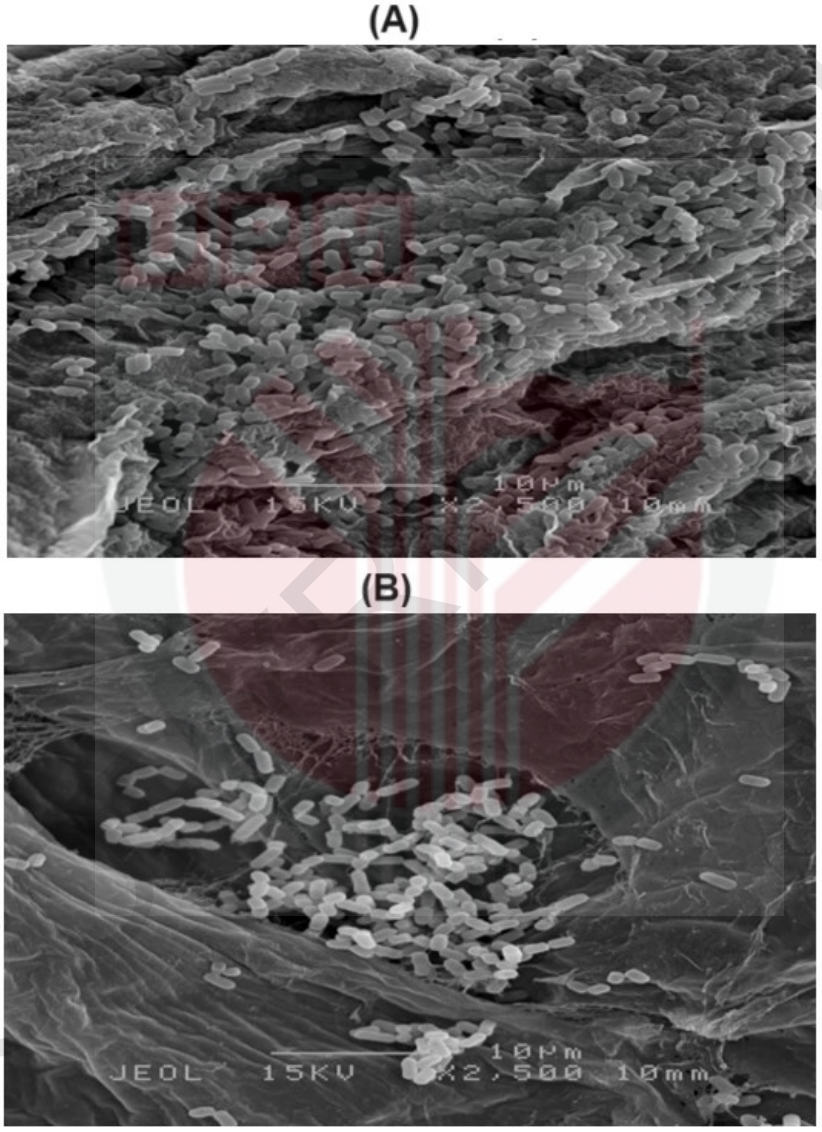

Figure 4.21 : Scanning electron microscopic (SEM) images of onion-immobilized *L. plantarum* B13 before fermentation (A) and after the 10th cycle of repeated batch fermentation (B)

4.8 Preparation and storage study of freeze-dried probiotic and postbiotic

Nowadays, probiotic (live bacteria of at least 10^6 CFU/g) and postbiotic (metabolites or by-products of probiotics including GABA) preparations come in various forms such as powders, suspensions, capsules, and inclusion in functional food products (Kiepś and Dembczyński, 2022). All these forms of preparation often share the same main issue of loss of cells viability and reduced activity and stability of beneficial metabolic compounds during processing and storage (Santivarangkna et al., 2007). In comparison, the preparations of probiotics in solid forms by various drying technologies can produce more stable products that can be stored for a longer shelf-life than the liquid suspension forms (Kiepś and Dembczyński, 2022). The solids or fluid suspension forms of probiotics can then be applied as ready-to-use ingredients that could be directly incorporated in the formulations of various probiotics enriched products such as in foods, creams, lotions, and cosmetics (Prapa et al., 2020; Jokicevic et al., 2021; Blanchet-Réthoré et al., 2017). Hence, in this study, freeze drying technique was applied to prepare solid forms of the potential probiotic *L. plantarum* B13 and its postbiotics (including GABA). Three different freeze-dried samples were prepared for comparison which were (i) free cells *L. plantarum* B13 with postbiotic only **(FCP)**; (ii) free cells *L. plantarum* B13 with postbiotic and cryoprotectant **(FC)**; and (iii) onion-immobilised *L. plantarum* B13 with postbiotic and cryoprotectant **(IC)**. The cryoprotectant used in this study were composed of a mixture of 10% (w/v) skim milk and 10% (w/v) sucrose. All samples were stored for 8 weeks at 4°C for comparison of cell viability, cell stability on GABA production and moisture content.

4.8.1 Cell viability of freeze-dried free cells and onion-immobilized cells

According to **Figure 4.22(A)**, after the freeze-drying process (labelled as week 0), a significant declination of cell viability was observed for all three potential probiotic-postbiotic solid preparation samples (FCP, FC and IC). The lowest cell viability (7.404 log CFU/mL) was observed for FCP, whereas the cell highest cell viability (8.250 log CFU/mL) was FC. The cell viability of IC (8.179 log CFU/mL) right after the freeze-drying process was only slightly lower than the FC. During the storage at 4°C, all the three samples showed a declination of cell viability from week 0 to week 8^{th}. In week 8^{th}, the cell viability of IC was the highest (7.288 log CFU/mL), followed by FC (6.880 log CFU/mL), and FCP (5.860 log CFU/mL).

Based on **Figure 4.22(B)**, the survival rate for IC in week 0 (right after the freeze-drying process) was the highest (91.49 %), followed by FC (87.29 %) and FCP at the lowest (78.34 %). The survival rate for each sample was significantly reduced from week 0 to week 8^{th}. In the final storage of week 8^{th}, the same trend was observed in which the highest survival rate was IC (81.52 %) followed by FC (72.82 %) and FCP (62 %). The results of this study also highlighted the importance of cryoprotectant in protecting cells during storage. Among various

cryoprotectant agents, skimmed milk (a complex substrate type) and sucrose (a carbohydrate) or their combination are among the frequently used cryoprotectants due to their excellent capability in protecting bacterial cells from the damage caused by the freezing process (Yuste et al., 2021;). Skimmed milk can prevent cell damage by stabilizing the cell membrane and hence providing a protective layer to the cells while sucrose can prevent the harmful eutectic fluid cell freezing (Hubalek, 2003). Sucrose was also shown to be able to prevent membrane damage and maintain the structure of proteins and biomolecules during the freezing stages (Leslie at al., 1995). The synergies effects between skimmed milk and sucrose in helping to maintain the viability of *L. plantarum* during the freeze-drying process and storage was also previously demonstrated from the cryoprotectants optimization study via Response Surface Methodology (Gisela et al., 2014). The results of this study are also in accordance with the study reported by Oluwatosin et al. (2022), showing that the viable cell count and survival rate of freeze-dried *L. plantarum* topical probiotic was reduced during the storage time of up to 12 weeks at 4°C and room temperature. Among the tested cryoprotective agents (10 % (m/v) of skimmed milk, sucrose, maltodextrin, and inulin), they discovered skimmed milk demonstrated the highest cell survival rate of 91% while inulin was the lowest of less than 1 %. Suitable cryoprotectants will help to stabilise the cell membrane contents and contribute a protective coating to the cells (Carvalho et al., 2004). In addition, another study stated that the freeze-dried lactic acid bacteria were becoming inactive during storage due to chemical reactions such as oxidation and denaturation of protein (Passot et al., 2012). The cells would die during storage, predominantly due to the lipid oxidation that occurred on the membrane fatty acids, and thereby, reducing the cell viability as the storage period is prolonged. Moreover, the lipid oxidation may also form free radicals that would impair the DNA and cell membrane during storage (Albadran et al., 2015).

The result of this study also showed that the used of an immobilization technique was positively influenced the cell viability and survival rate after the freeze-drying process and during cold storage. This is proven from the higher cell viability and survival rate attained by IC than the FC. Similarly, the survival rates of freeze-dried immobilised *L. plantarum* B282 on zea flakes and pistachios (ranged between 92.6 % and 98.8 %) were reported to be higher compared to the freeze-dried free cells *L. plantarum* B282 (87.8 %) within 6 months of storage at 4°C (Prapa et al., 2023). This proved the beneficial effect of cell immobilization for preserving and maintaining the cell viability undergoing freeze drying process and during storage.

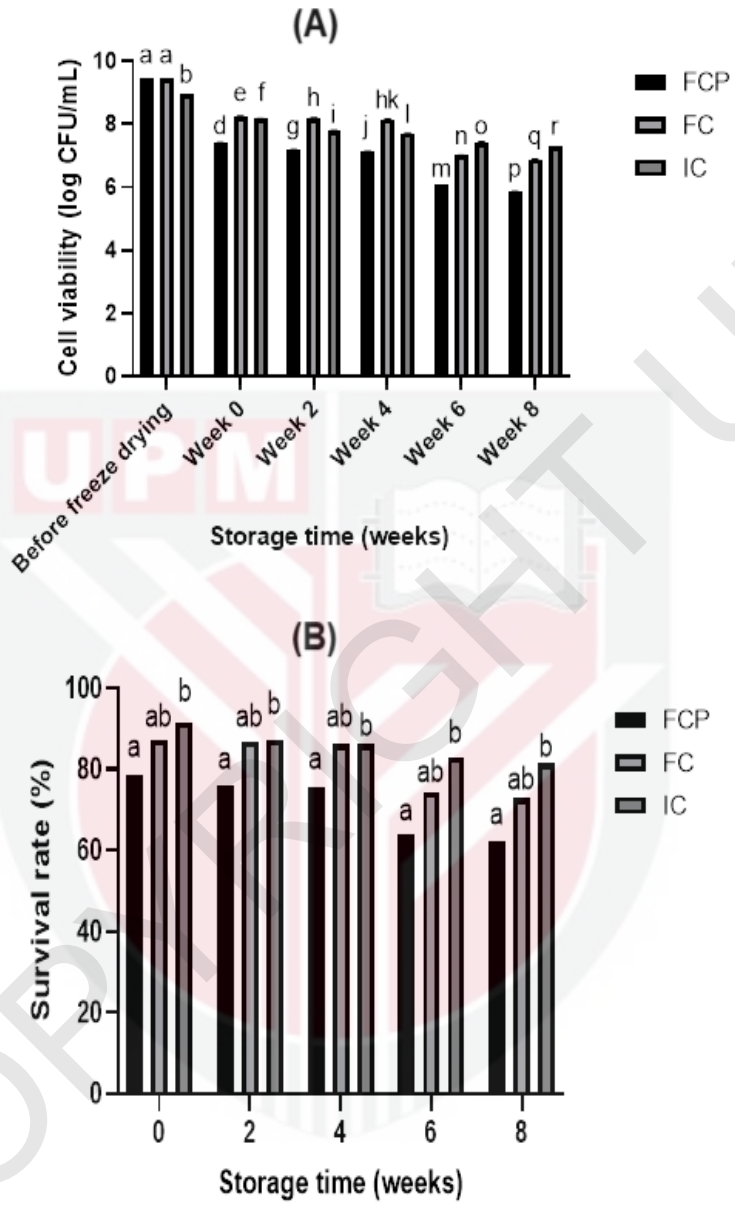

Figure 4.22 : The cell viability (log CFU/mL) (A) and the survival rate (%) (B) of three freeze-dried probiotic-postbiotic preparation samples: free cells *L. plantarum* B13 with postbiotic only (FCP); free cells *L. plantarum* B13 with postbiotic and cryoprotectant (FC); and onion-immobilised *L. plantarum* B13 with postbiotic and cryoprotectant (IC), after freeze drying process for 8 weeks storage at 4°C. The error bars represent the standard deviations about the mean (n=3). Different superscript letters indicate significant different based on Tukey's test at $p < 0.05$

4.8.2 GABA production by freeze-dried preserved *L. plantarum* B13

The results showed that the ability of freeze-dried preserved *L. plantarum* B13 to produce GABA dependent on the protective medium that affecting the cell viability during storage. As depicted in Figure **4.23**, after the cells' revival process, the ability of FCP, FC and IC to produce GABA were decreasing with the prolonged storage period up to 8 weeks. After the freeze-drying process (week 0), the lowest production of GABA that was determined after 45 hours of fermentation was observed for FCP (14.447 g/L) which was significantly lower than the GABA concentration obtained from the free cell before the freeze drying (19.073 g/L). Whereas FC and IC managed to retain its capability in producing GABA, resulting almost similar GABA concentrations (19.569 g/L and 19.122 g/L, respectively) as before they were undergoing the freeze-drying process (19.073 g/L and 18.463 g/L, respectively). During the final week of storage study, the GABA production by IC was the highest (11.309 g/L), followed by FC (8.707 g/L) and FCP (4.894 g/L). This might be due to the declination of cell viability and cell survival rate as shown in **Figure 4.22**. Similarly, the abilities of *P. pentosaceus* GS4, *P. pentosaceus* GS17 and *L. gasseri* ATCC 19992 to produce lactic acid were shown to be affected by different formulations of cryoprotectant, freeze drying process as well as the storage period (Bagad et al., 2017). Based on the study by Kumar et al. (2007), high cell viability could result in the maximum production of the desired products. Besides that, regulating cell proliferation for obtaining a high cell viability could improve the productivity and yields (Oguchi et al., 2006).

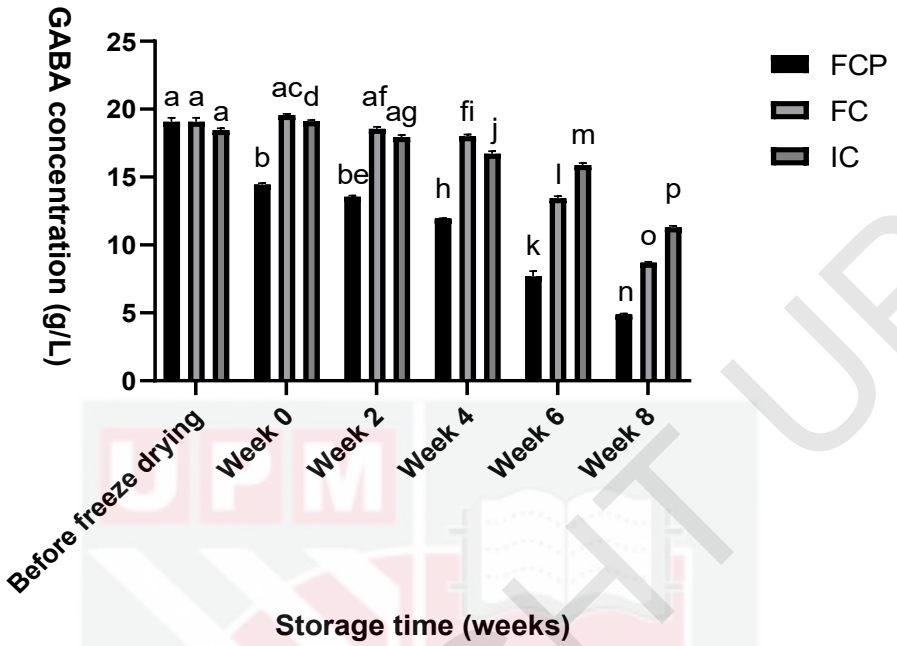

Figure 4.23 : The GABA productions by freeze-dried *L. plantarum* B13 cells (free cells with postbiotic only (FCP); free cells with postbiotic and cryoprotectant (FC); and onion-immobilised cells with postbiotic and cryoprotectant (IC)) that preserved within 8 weeks at 4°C. The fermentations were conducted for 45 hours, based on optimum conditions of initial pH 5.5, 5 % (w/v) MSG, 0.7 mM PLP, 60 g/L glucose, 35°C, without agitation. The error bars represent the standard deviations about the mean (n=3). Different superscript letters indicate significant different based on Tukey's test at $p < 0.05$

4.8.3 Moisture content

Long term stability of probiotics can be affected by the moisture content and storage temperature (Bagad et al., 2017, Wang et al., 2004; Saarela et al., 2005; Abe et al., 2009). As shown in **Figure 4.24**, the moisture contents of FCP, FC and IC were increased throughout the storage period from week 0 to week 8. Likewise, the moisture content of L. plantarum TISTR 2083, was increased up to 18 % during the storage of 90 days and at the temperature of 4°C (Al Mamun et al., 2021). The moisture content was increased during storage because of the storage relative humidity and the movement of water that was adsorbed into the samples to achieve the equilibrium state (Razak et al., 2020; Al-Muhtaseb et al., 2002). Among the three freeze-dried *L. plantarum* B13 cells, the moisture contents of FCP were observed to be the highest, from 21.82 % in week 0 increased to 38.71 % in the week 8. In comparison, after the freeze-drying process (week 0), the moisture content of FC (7.23 %) was slightly lower than the moisture content of IC (8.39%). However, in the final week 8, the moisture content for FC (18.52 %) was recorded to be higher than the moisture content

of IC (15.69 %). In general, bacterial cells tend to survive in the environment with low water activity and moisture content (Shamekhi et al., 2012). The results of this study reflected the results obtained for the cell viability and percentage of survival rate as depicted earlier in **Figure 4.22**, showing higher cell viability and survival rate for IC and FC than the FCP.

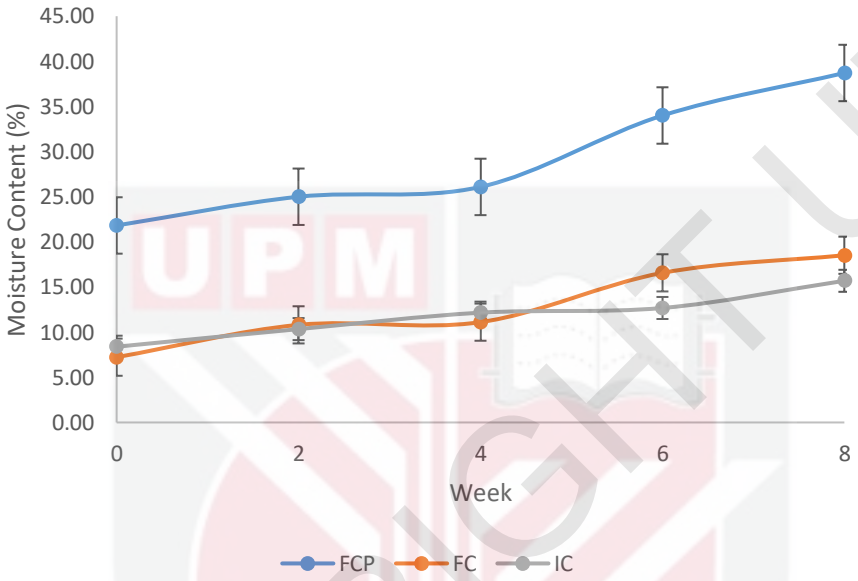

Figure 4.24 : The moisture content of freeze-dried *L. plantarum* B13 cells (free cells with postbiotic only (FCP); free cells with postbiotic and cryoprotectant (FC); and onion-immobilised cells with postbiotic and cryoprotectant (IC)) during 8 weeks of storage at 4°C. The error bars represent the standard deviations about the mean (n=3)

CHAPTER 5

SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Summary and conclusion

L. plantarum B13 possessed the characteristics of lactic acid bacteria which included Gram positive, catalase-negative and oxidase-negative. The strain also showed the probiotic potential based on the results of several probiotic characterization assessments. It was proved that the *L. plantarum* B13 was able to survive in the gastrointestinal tract due to its high survival rate in acidic and bile salt conditions, as well as high number of viable cell counts in the presence of phenol. *L. plantarum* B13 showed a high adhesion activity reflecting to its high autoaggregation activity (73.03 %) even though it had a hydrophilic nature because of the higher adherence towards chloroform (72.48 %) than the other solvents. Besides that, *L. plantarum* B13 also exhibited the antimicrobial effect towards several microbial pathogens including *E. coli*, *S. aureus*, *S. typhimurium*, and *B. subtilis*. The cell free supernatant of *L. plantarum* B13 also possessed considerably high antioxidant activity of 77.27 %. However, *L. plantarum* B13 did not produce EPS.

The GABA production by *L. plantarum* B13 was significantly affected by the fermentative parameters such as incubation time, temperature, pH, concentration of monosodium glutamate (MSG), concentration of pyridoxal-5-phosphate (PLP) and concentration of glucose. Based on the kinetic data, the optimum fermentative parameters for producing GABA were incubation time of 45 hours, pH 5.5, temperature of 35°C, MSG concentration of 5 % (w/v), PLP concentration of 0.7 mM and glucose concentration of 60 g/L resulting GABA concentration of up to 19.073 ± 0.521 g/L with the highest GABA productivity of 0.424 g/L/h.

Onion was determined as the best natural support for immobilizing *L. plantarum* B13 compared to the other tested natural supports (okra, sweet potato, bell pepper and taro) as it possessed the best cell retention (83 %) and produced the highest GABA concentration of 18.854 g/L. Meanwhile, the onion-immobilized *L. plantarum* B13 was able to be reused with a greater number of fermentation batches compared to the free cell. The GABA yield was reduced to below 50 % of the initial concentration after the 8^{th} and 6^{th} batch of repeated batch fermentation for the onion-immobilized *L. plantarum* B13 and free-cells *L. plantarum* B13, respectively.

Moreover, the effects of freeze drying process on cell viability and survival rate during cold storage as well as the capability of the freeze-dried onion-immobilized and free-cells *L. plantarum* B13 for producing GABA were investigated. Three different samples (FCP: free cell and postbiotic; FC: free cell

and postbiotic and cryoprotectant; IC: immobilized cell and postbiotic and cryoprotectant) were prepared. The mixture of 10 % (w/v) skim milk and 10 % (w/v) sucrose was used as the cryoprotective agent. After 8 weeks of storage at 4ºC, the IC retained the highest cell viability and showing the best GABA producing capability (7.29 log CFU/mL, survival rate, 81.52 %, and 11.31 g/L GABA) than the FC (7.02 log CFU/mL, 72.82 % survival rate, 8.71 g/L GABA) and FCP (6.06 log CFU/mL, 62.00% survival rate, 4.89 g/L GABA).

In the nutshell, the probiotic characteristics and the optimal culture conditions determined in this study demonstrated that *L. plantarum* B13 possess high potential to bring probiotic health effects and it is also a good producer of GABA. Furthermore, the onion-immobilized *L. plantarum* B13 was more effective compared to the free-cells for maintaining high cell viability, survivability and GABA yield. Therefore, this strain is suitable to be used for commercial applications such as in the development of naturally GABA-enriched foods and beverage products.

5.2 Future recommendations

Overall, all the objectives of this study have been successfully achieved. However, future research can be expanded to further improve the screening and optimization process, the immobilization technique, freeze drying process as well as the storage study. Some recommendations for further research are as follows:

1. In this study, *L. plantarum* B13 was being characterized as a lactic acid bacteria (LAB) based on several assessments. However, further identification of *L. plantarum* B13 as LAB should be done for obtaining more precise results. Macroscopic and microscopic examinations and other biochemical tests such as anaerobic tests can be conducted to further examine the characteristics of LAB (Sulmiyati et al., 2018).
2. *L. plantarum* B13 was being characterized as a potential probiotic strain after carrying out a few assessments. However, more characterisation analysis can be conducted to further evaluate the safety and probiotic potential of *L. plantarum* B13. For instance, *in-vitro* assays such as pancreatin tolerance test, sodium chloride tolerance test, lysozyme, pepsin and pancreatin tolerance can be done for probiotic characterization, whereas, for evaluating the safety aspect of the strain, assessments such as haemolytic activity, antibiotic susceptibility test and *in-vivo* in a mouse model can be conducted (Nath et al., 2020; Abid et al., 2022).
3. Several fermentative parameters (pH, incubation time, temperature, MSG concentration, PLP concentration and glucose concentration) were screened for optimizing the GABA production by *L. plantarum* B13 via OFAT method. Beside the selected parameters, there are other factors that may also affect the GABA yield and productivity including types of carbon sources, nitrogen sources and growth factors

(Wu et al., 2018). Furthermore, to study the interactions between the fermentative factors and enhancing the yield of GABA, optimization tools such as response surface methodology (RSM) (Kim et al., 2022) and artificial neural networks (ANN) (Huang et al., 2007a) can be applied.

4. In this study, several vegetables including okra, onion, taro, bell pepper and sweet potato were being selected as the natural supports for whole-cell immobilizations. Other natural supports can be screened to examine their potential as natural cell immobilizers and to evaluate their effects towards GABA production. Besides vegetables, fruits and edible agricultural wastes can be explored as the potential immobilizers. For examples, grape skin was being successfully used to immobilize *Saccharomyces cerevisiae* (Mallouchos et al., 2002), while, quince pieces were applied for immobilizing *L. casei* (Kourkoutas et al., (2005).

5. Commonly used cryoprotectant composed of a mixture of 10 % (w/v) skim milk and 10 % (w/v) sucrose was applied to protect the *L. plantarum* B13 during the freeze drying process. To further improve the cell viability and rate of survival after freeze drying and during storage period, many other types of cryoprotectants can be screened. For instance, according to Savedboworn et al. (2017), by combining protein with trehalose and protein with maltodextrin, the cell viability of *L. plantarum* managed to be significantly improved with the survival rate of 98.13 % and 97.58 %, respectively.

6. In this research, the storage study was conducted for only 8 weeks. For commercial feasibility, the storage study can be extended up to at least 24 weeks, which is equal to 6 months in order to determine the cell viability and stability more accurately. For example, in accordance to the study performed by Savedboworn et al. (2017), the specific cell death rate of the *L. plantarum* was identified within a storage period of 168 days (or equal to 6 months) at the temperature of 4°C.

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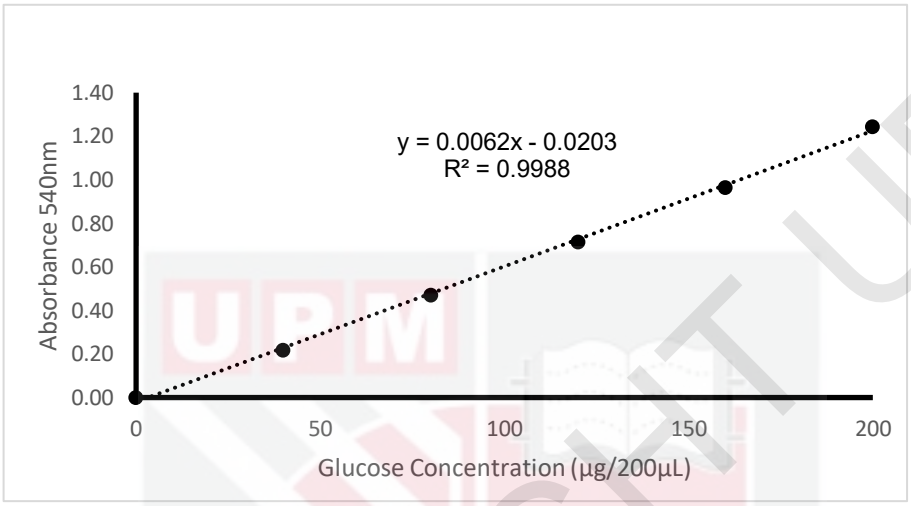
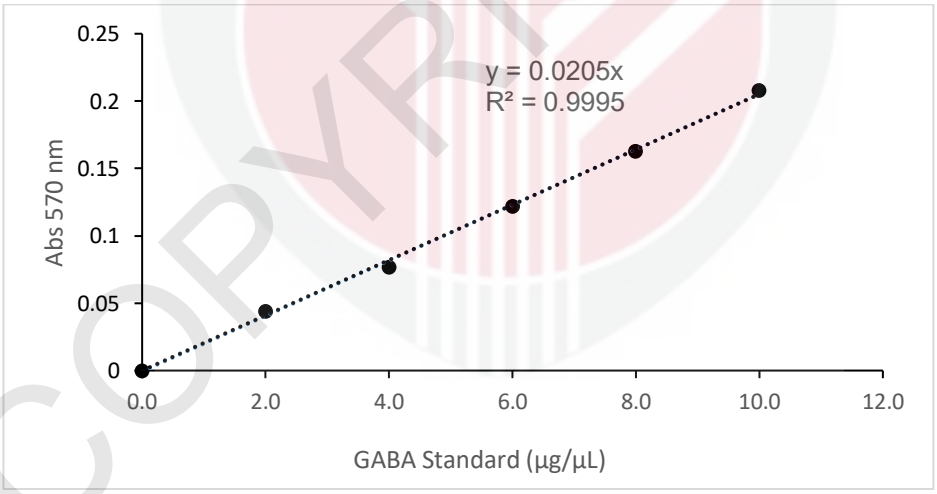
APPENDICES

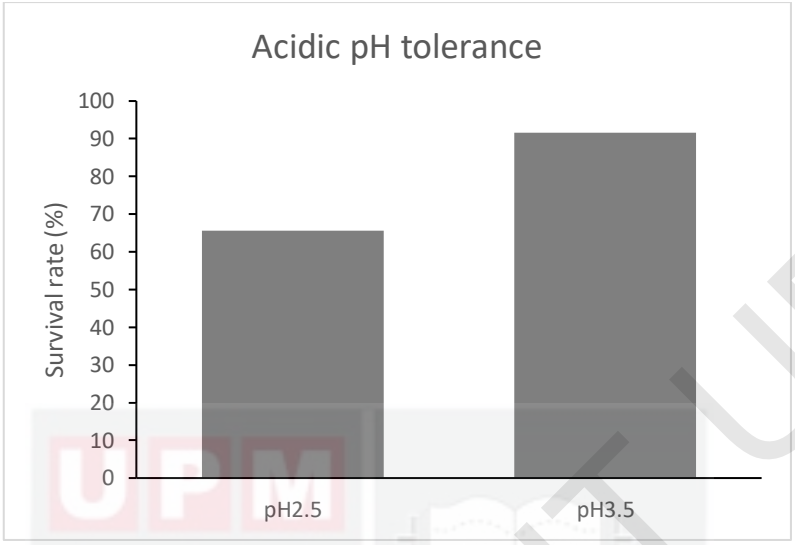
Appendix A

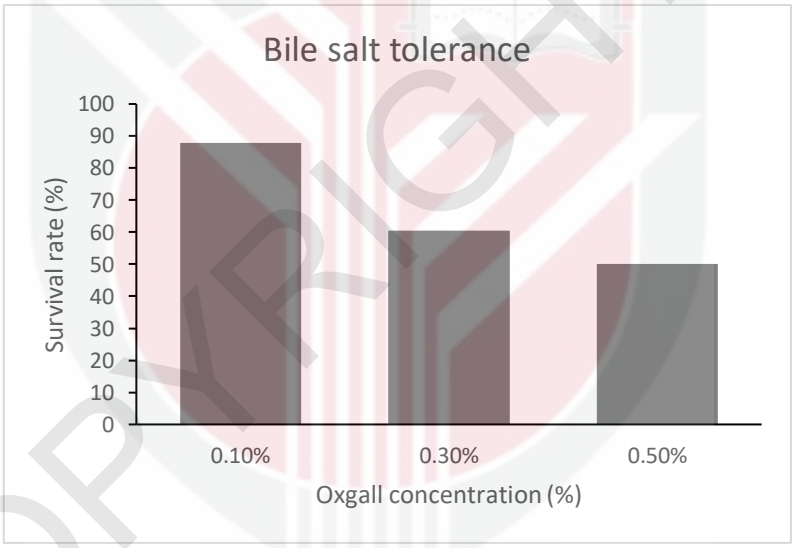


Appendix A1 : Standard curve for different standard glucose concentration

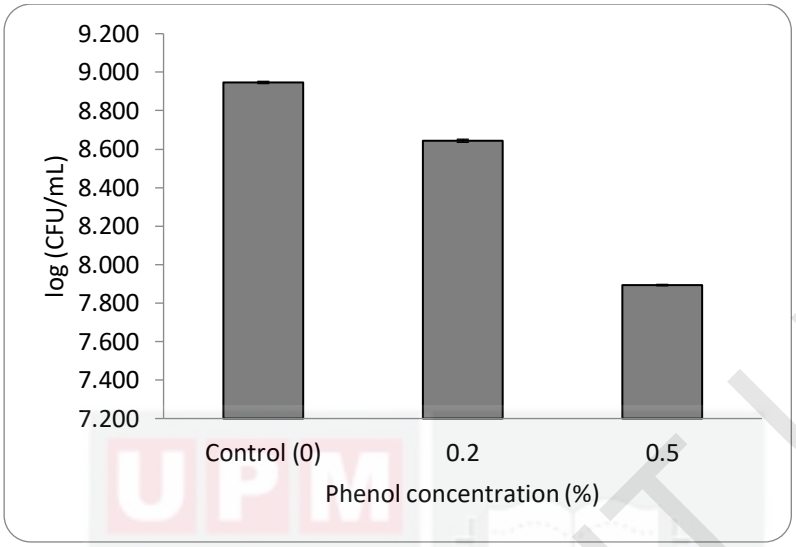


Appendix A2 : Standard curve for different standard GABA concentration

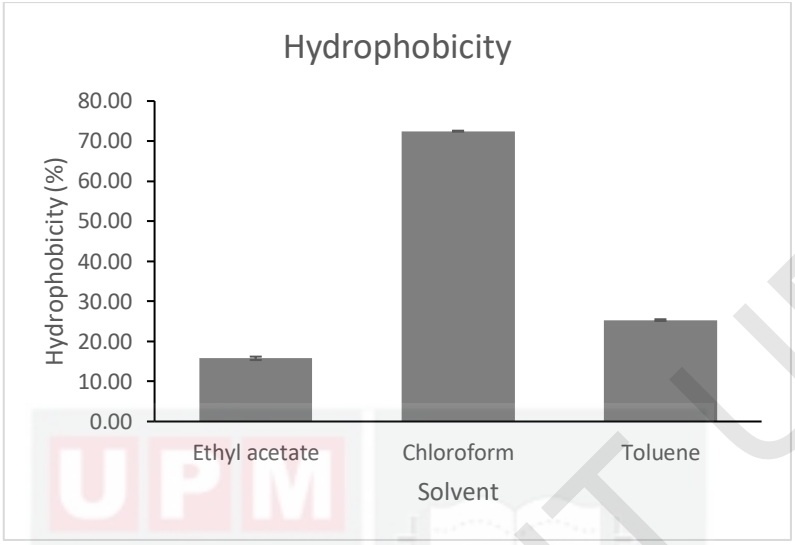


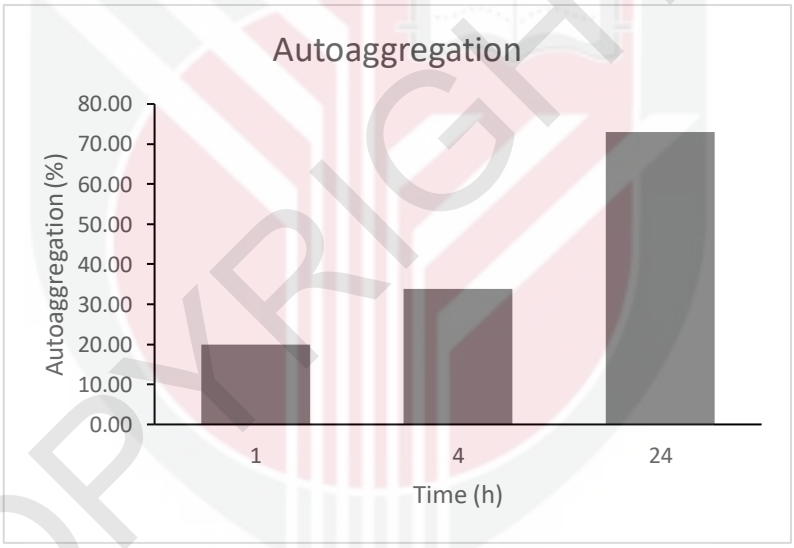


Appendix A3 : Acidic pH and bile salt tolerance of *Lb. plantarum* B13 strain after incubated at 37 °C for 0 hour and 4 hours

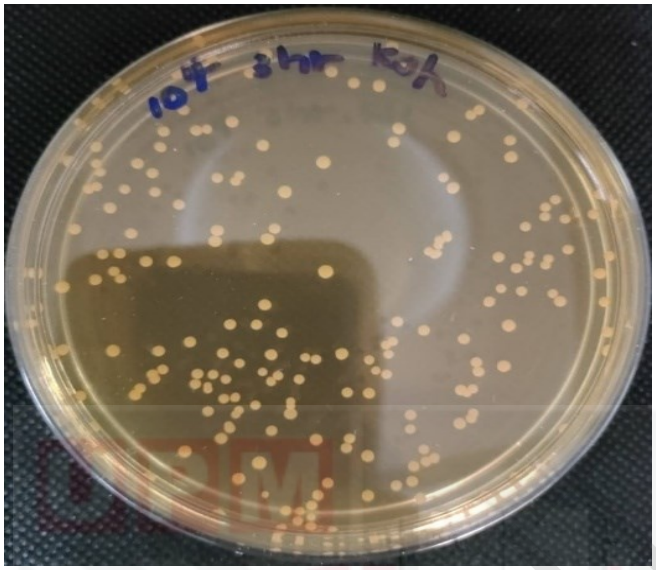


Appendix A4 : Phenol tolerance of *Lb. plantarum* B13 after incubated at 37 °C for 24h. The results were expressed as mean (n = 3) ± SD

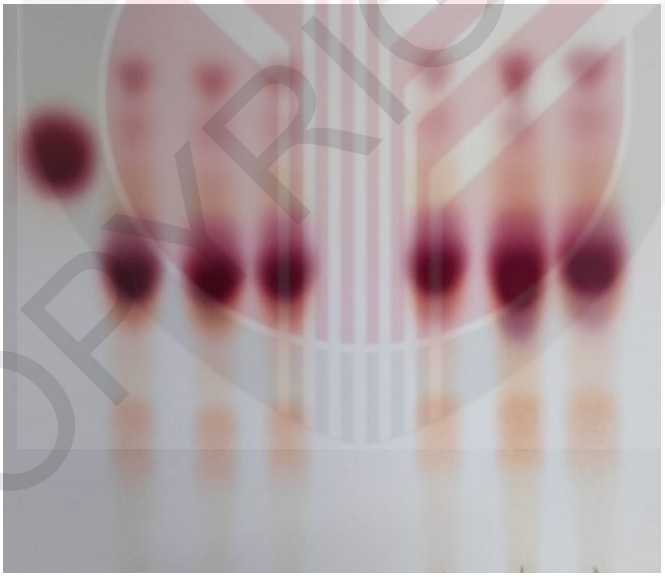




Appendix A5 : Cell surface hydrophobicity against different solvents and cellular autoaggregation of *Lb. plantarum* B13 strain after incubated at 37 °C for 0h, 4h and 24h. Data were expressed as mean (n = 3) ± SD



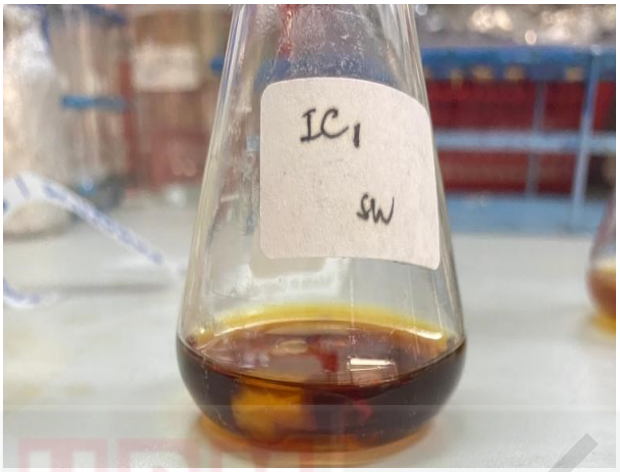
Appendix A6 : Colonies of *Lb. plantarum* B13 culture on MRS agar by spread plate



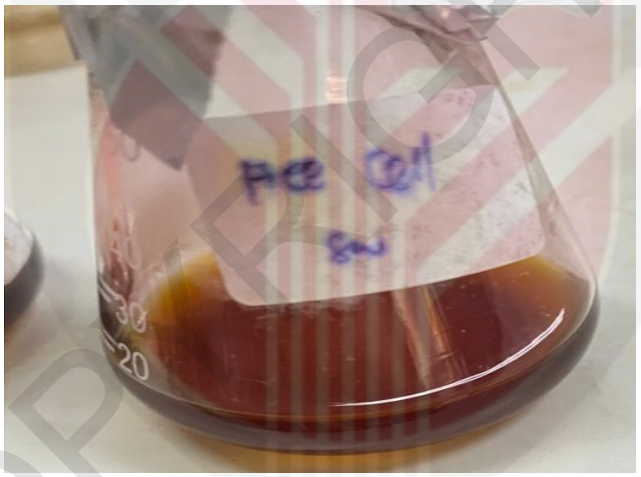
Appendix A7 : Qualitative detection of GABA produced by *Lb. plantarum* B13 by thin layer chromatography

Appendix A8 : The immobilizers (Onions) after washing and autoclaving

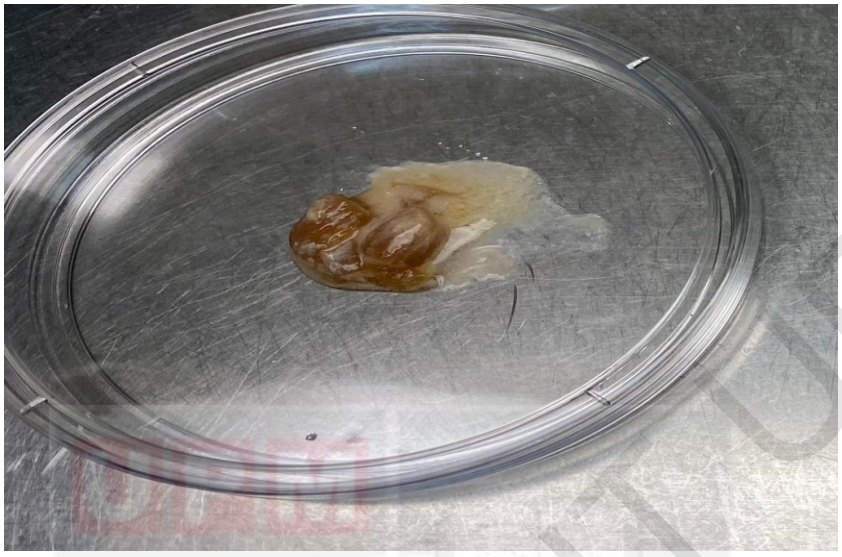
Appendix A9 : The weight of an immobilizer (onion) after cutting into the size of 1cm^3



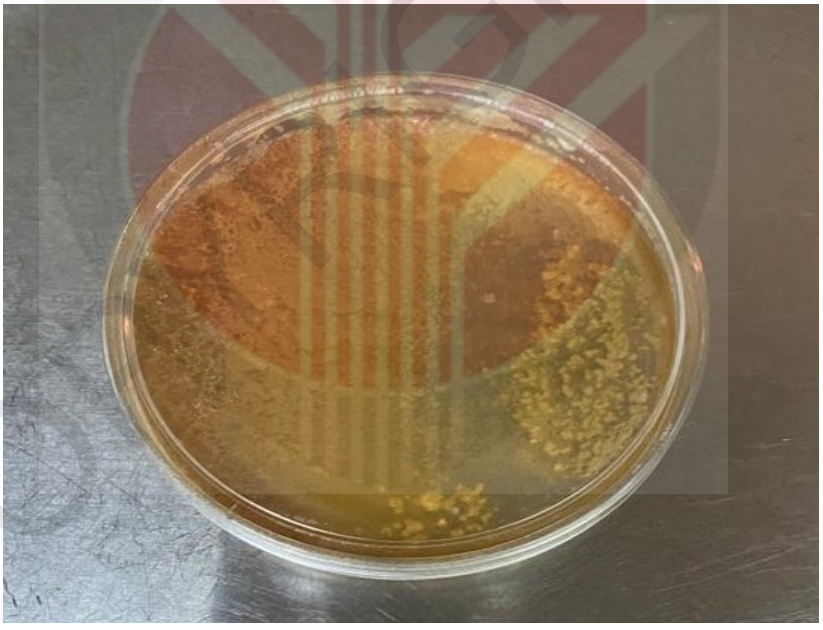
Appendix A10 : The first batch of the fermentation for the immobilized *Lb. plantarum B13* during the repeated batch fermentation



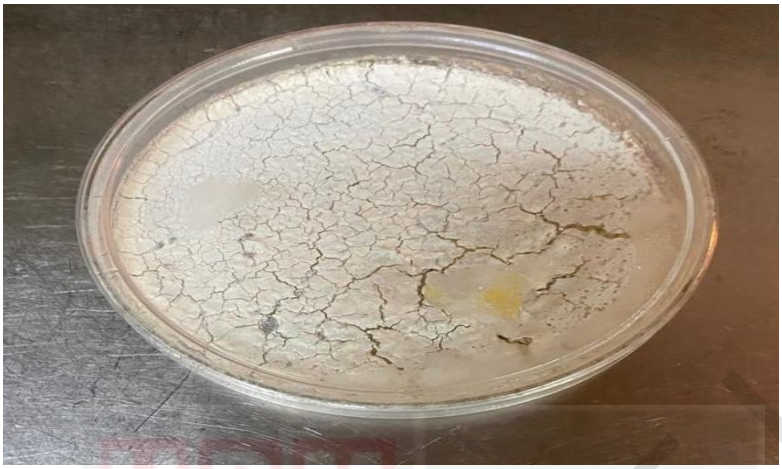
Appendix A11 : The first batch of the fermentation for the free *Lb. plantarum B13* during the repeated batch fermentation



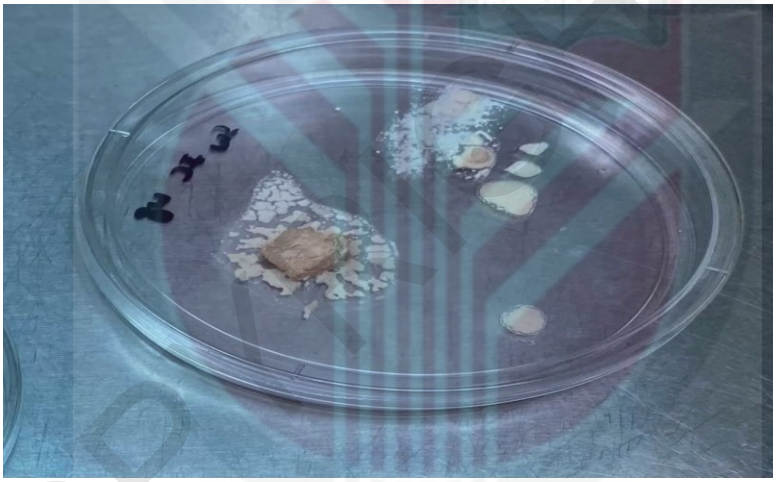
Appendix A12 : The immobilizer (onion) after 10th fermentation batches



Appendix A13 : The FCP after freeze drying

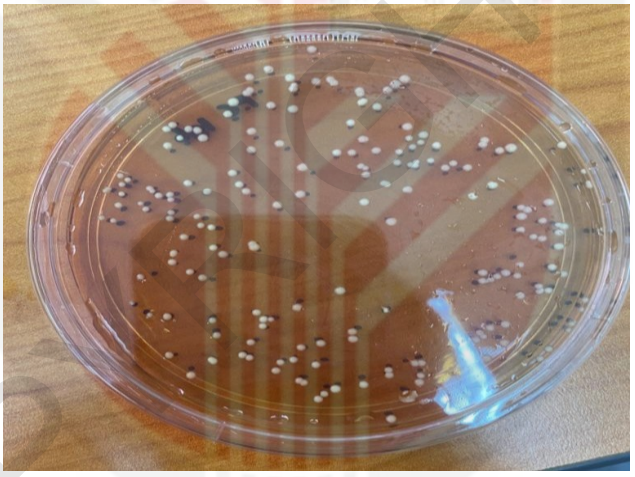


Appendix A14 : The FC after freeze drying



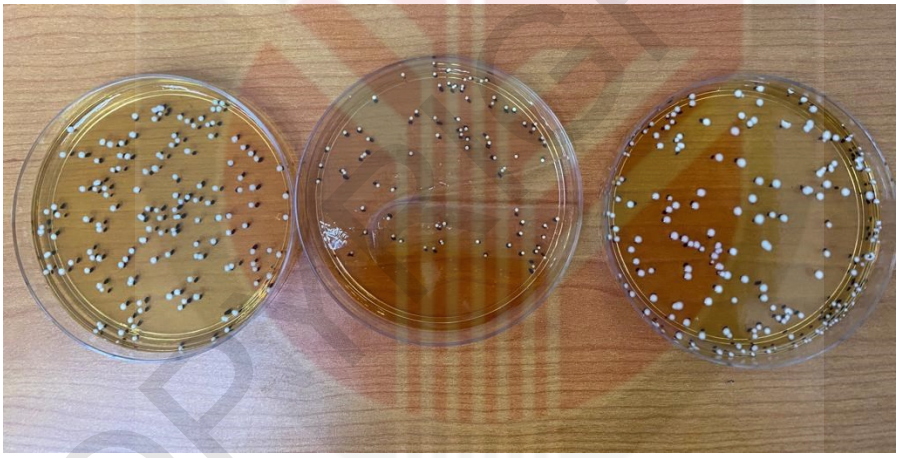
Appendix A15 : The IC after freeze drying

Appendix A16 : Viable cells count for FCP in week 0 (DF=10^5)



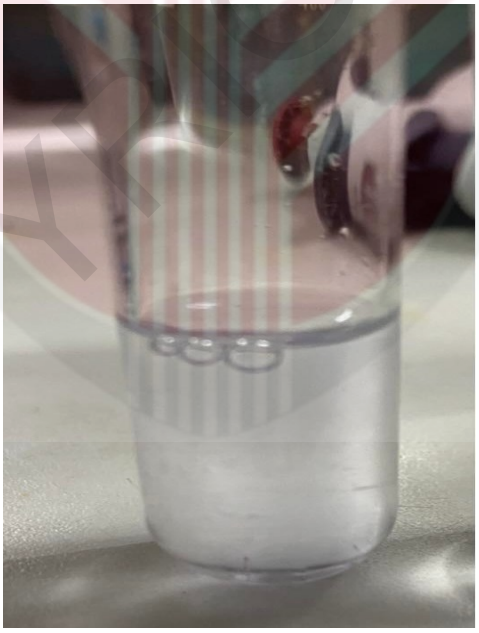
Appendix A17 : Viable cells count for FC in week 0 (DF=10^6)

Appendix A18 : Viable cells count for IC in week 0 ($DF=10^6$)

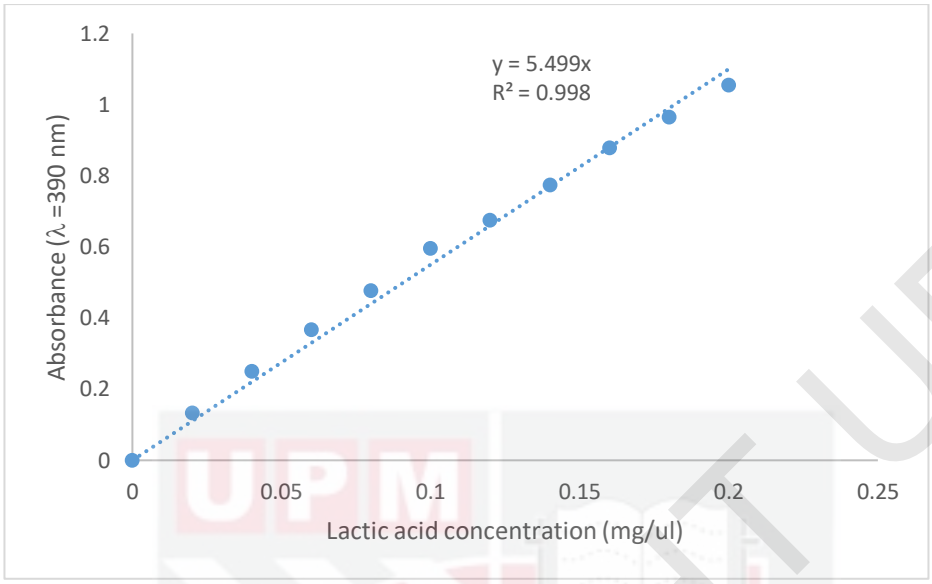


Appendix A19 : Viable cells count for FC ($DF=10^4$); FCP ($DF=10^3$); and IC ($DF=10^5$) from left to the right in week 8

Appendix A20 : Sample of FCP, IC and FC (from left to the right) after drying at 105 °C for 4 hours



Appendix A21 : Mixture of sample with 3 mL of borate buffer and 0.5 mL of ninhydrin



Appendix A22 : Standard curve for different standard lactic acid concentration

Appendix A23 : Preparation of borate buffer (pH 7.0)

Borate buffer was prepared through mixing 3.093 g of boric acid, 2.515 g of sodium tetraborate and 2.193 g of sodium chloride with 400 mL of distilled water. After that, the volume of the mixture was added with distilled water until it was 500 mL. Stirring was applied by using a stir bar along with low heat to ensure the boric acid was completely dissolved. Then, the pH was adjusted to the value of 7.0 by using hydrochloric acid and sodium hydroxide.

Appendix B

Appendix B1 : The cell profile of *Lb. plantarum* B13 shake flask culture after 96 hours incubation times based on OD_{600}, residual glucose concentration (g/L) and GABA concentration (g/L)

Time (h)	OD_{600}	Glucose (g/L)	GABA (g/L)
0	0.081 ± 0.0010^{i}	18.413 ± 0.1416^{a}	0.244 ± 0.0422^{g}
3	0.265 ± 0.0006^{h}	14.313 ± 1.1293^{b}	0.740 ± 0.0508^{fg}
6	1.443 ± 0.0029^{g}	12.364 ± 0.9714^{c}	1.244 ± 0.3518^{ef}
9	2.330 ± 0.0150^{f}	9.421 ± 0.0931^{d}	1.878 ± 0.1118^{de}
12	2.530 ± 0.0021^{e}	8.345 ± 0.0233^{d}	2.585 ± 0.3593^{d}
15	2.634 ± 0.0029^{c}	5.102 ± 0.0047^{e}	5.122 ± 0.2327^{c}
18	2.638 ± 0.0040^{c}	3.166 ± 0.0047^{f}	5.301 ± 0.4843^{c}
21	2.710 ± 0.0025^{a}	2.801 ± 0.0081^{f}	5.545 ± 0.0784^{c}
24	2.671 ± 0.0015^{b}	2.551 ± 0.0856^{f}	6.846 ± 0.1798^{b}
48	2.643 ± 0.0015^{c}	2.494 ± 0.0084^{f}	7.317 ± 0.2126^{ab}
72	2.620 ± 0.0031^{d}	2.300 ± 0.0734^{f}	7.740 ± 0.2827^{a}
96	2.661 ± 0.0012^{b}	2.212 ± 0.0023^{f}	5.228 ± 0.2848^{c}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B2 : Cell growth of shake flask culture of *Lb. plantarum* B13 after incubation of 96 hours in terms of OD_{600} and log CFU/mL

Time (h)	OD_{600}	log CFU/mL
0	0.081 ± 0.0010^{i}	7.169 ± 0.0871^{d}
3	0.265 ± 0.0006^{h}	7.389 ± 0.0226^{d}
6	1.443 ± 0.0029^{g}	8.417 ± 0.1639^{c}
9	2.330 ± 0.0150^{f}	8.898 ± 0.1198^{b}
12	2.530 ± 0.0021^{e}	9.165 ± 0.0357^{ab}
15	2.634 ± 0.0029^{c}	9.209 ± 0.0152^{a}
18	2.638 ± 0.0040^{c}	9.230 ± 0.0108^{a}
21	2.710 ± 0.0025^{a}	9.247 ± 0.0452^{a}
24	2.671 ± 0.0015^{b}	9.282 ± 0.0449^{a}
48	2.643 ± 0.0015^{c}	9.228 ± 0.0036^{a}
72	2.620 ± 0.0031^{d}	9.208 ± 0.0019^{a}
96	2.661 ± 0.0012^{b}	9.241 ± 0.0035^{a}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B3 : Cell growth and GABA production of *Lb. plantarum* B13 at different incubation times

Time (h)	OD_{600}	log CFU/mL	GABA (g/L)
48	2.690 ± 0.0029^{b}	9.265 ± 0.0022^{b}	7.317 ± 0.2126^{a}
54	2.690 ± 0.0040^{bc}	9.265 ± 0.0031^{bc}	7.561 ± 0.3189^{a}
60	2.703 ± 0.0031^{a}	9.275 ± 0.0024^{a}	7.772 ± 0.4983^{a}
66	2.683 ± 0.0021^{c}	9.259 ± 0.0016^{c}	7.951 ± 0.1484^{a}
72	2.664 ± 0.0015^{d}	9.245 ± 0.0012^{d}	7.740 ± 0.2827^{a}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B4 : Cell growth and GABA production of *Lb. plantarum* B13 at different pH after incubation of 66 hours

pH	OD_{600}	log (CFU/mL)	GABA (g/L)
4	1.993 ± 0.0159^{f}	8.723 ± 0.0124^{f}	3.732 ± 0.5492^{d}
4.5	2.134 ± 0.0222^{e}	8.833 ± 0.0172^{e}	3.837 ± 0.7799^{d}
5	2.332 ± 0.0032^{d}	8.987 ± 0.0025^{d}	5.626 ± 0.6268^{c}
5.5	2.660 ± 0.0006^{a}	9.242 ± 0.0004^{a}	9.268 ± 0.6916^{a}
6	2.654 ± 0.0006^{a}	9.237 ± 0.0004^{a}	7.268 ± 0.4634^{b}
6.5	2.589 ± 0.0081^{b}	9.186 ± 0.0063^{b}	4.715 ± 0.6501^{cd}
7	2.543 ± 0.0162^{c}	9.151 ± 0.0126^{c}	3.967 ± 0.4578^{d}
7.5	2.532 ± 0.0036^{c}	9.142 ± 0.0028^{c}	3.805 ± 0.1063^{d}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B5 : Cell growth and GABA production of *Lb. plantarum* B13 at different temperature after incubation of 66 hours

Temperature (°C)	OD_{600}	log (CFU/mL)	GABA (g/L)
33	2.586 ± 0.0025^{c}	9.184 ± 0.0020^{c}	5.366 ± 0.2581^{c}
35	2.700 ± 0.0015^{a}	9.272 ± 0.0012^{a}	8.902 ± 0.6185^{a}
37	2.605 ± 0.0017^{b}	9.199 ± 0.0013^{b}	7.268 ± 0.4634^{b}
39	2.487 ± 0.0012^{d}	9.107 ± 0.0009^{d}	5.821 ± 0.3088^{c}
41	1.307 ± 0.0049^{e}	8.191 ± 0.0038^{e}	3.797 ± 0.2687^{d}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B6 : Cell growth and GABA production of *Lb. plantarum* B13 at different MSG concentration after incubation of 66 hours

MSG concentration (%)	OD_{600}	log (CFU/mL)	GABA (g/L)
0	2.571 ± 0.0012^{e}	9.173 ± 0.0009^{e}	3.691 ± 0.6551^{b}
1	2.710 ± 0.0015^{a}	9.281 ± 0.0012^{a}	7.228 ± 0.6900^{a}
3	2.655 ± 0.0030^{b}	9.238 ± 0.0023^{b}	7.447 ± 1.0181^{a}
5	2.616 ± 0.0006^{c}	9.208 ± 0.0004^{c}	8.423 ± 0.8436^{a}
7	2.596 ± 0.0006^{d}	9.192 ± 0.0004^{d}	7.797 ± 0.6380^{a}
9	2.477 ± 0.0021^{f}	9.099 ± 0.0016^{f}	7.187 ± 0.6272^{a}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B7 : Cell growth and GABA production of *Lb. plantarum* B13 at different PLP concentration after incubation of 66 hours

PLP concentration (mM)	OD_{600}	log CFU/mL	GABA (g/L)
0	2.568 ± 0.0010^{f}	9.170 ± 0.0008^{f}	4.854 ± 0.5093^{cd}
0.3	2.606 ± 0.0012^{e}	9.200 ± 0.0009^{e}	5.512 ± 0.7248^{bcd}
0.4	2.634 ± 0.0023^{d}	9.222 ± 0.0018^{d}	6.715 ± 0.3239^{abcd}
0.5	2.649 ± 0.0012^{c}	9.233 ± 0.0009^{c}	7.146 ± 0.7839^{abc}
0.6	2.656 ± 0.0040^{c}	9.239 ± 0.0031^{c}	7.691 ± 1.5994^{ab}
0.7	2.692 ± 0.0061^{a}	9.267 ± 0.0047^{a}	8.439 ± 0.8311^{a}
0.8	2.680 ± 0.0025^{b}	9.257 ± 0.0020^{b}	5.033 ± 0.6408^{cd}
0.9	2.541 ± 0.0012^{g}	9.149 ± 0.0009^{g}	4.553 ± 0.4446^{d}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B8 : OD_{600} of *Lb. plantarum* B13 for each time interval at different glucose concentration after incubation of 114 hours

Time (h)	OD_{600}				
	20g/L	40g/L	60g/L	80g/L	100g/L
0	0.039 ± 0.0006^{f}	0.062 ± 0.0006^{g}	0.091 ± 0.0006^{g}	0.047 ± 0.0006^{g}	0.061 ± 0.0020^{g}
3	0.223 ± 0.0006^{e}	0.203 ± 0.0006^{f}	0.186 ± 0.0010^{f}	0.155 ± 0.0010^{f}	0.075 ± 0.0006^{f}
15	2.719 ± 0.0012^{c}	2.598 ± 0.0029^{e}	2.543 ± 0.0029^{e}	2.361 ± 0.0036^{e}	1.679 ± 0.0083^{e}
21	2.769 ± 0.0047^{a}	2.818 ± 0.0020^{d}	2.783 ± 0.0038^{d}	2.599 ± 0.0015^{d}	2.361 ± 0.0059^{d}
45	2.725 ± 0.0020^{bc}	3.026 ± 0.0182^{a}	2.924 ± 0.0056^{b}	2.903 ± 0.0015^{c}	2.760 ± 0.0026^{c}
66	2.732 ± 0.0015^{b}	2.976 ± 0.0248^{b}	2.869 ± 0.0047^{c}	2.908 ± 0.0139^{c}	2.750 ± 0.0049^{c}
90	2.728 ± 0.0050^{b}	2.923 ± 0.0055^{c}	3.024 ± 0.0030^{a}	2.975 ± 0.0015^{a}	2.930 ± 0.0032^{a}
114	2.648 ± 0.0035^{d}	2.802 ± 0.0070^{d}	3.016 ± 0.0047^{a}	2.956 ± 0.0057^{b}	2.897 ± 0.0023^{b}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B9 : log CFU/mL of *Lb. plantarum* B13 for each time interval at different glucose concentration after incubation of 114 hours

Time (h)	log CFU/mL				
	20g/L	40g/L	60g/L	80g/L	100g/L
0	7.205 ± 0.0004^{f}	7.223 ± 0.0004^{g}	7.246 ± 0.0004^{g}	7.212 ± 0.0004^{g}	7.222 ± 0.0016^{g}
3	7.349 ± 0.0004^{e}	7.333 ± 0.0004^{f}	7.320 ± 0.0008^{f}	7.296 ± 0.0008^{f}	7.233 ± 0.0004^{f}
15	9.288 ± 0.0009^{c}	9.193 ± 0.0022^{e}	9.150 ± 0.0022^{e}	9.009 ± 0.0028^{e}	8.480 ± 0.0065^{e}
21	9.327 ± 0.0037^{a}	9.364 ± 0.0016^{d}	9.337 ± 0.0029^{d}	9.195 ± 0.0012^{d}	9.010 ± 0.0046^{d}
45	9.292 ± 0.0016^{bc}	9.526 ± 0.0141^{a}	9.447 ± 0.0043^{b}	9.431 ± 0.0012^{c}	9.319 ± 0.0021^{c}
66	9.297 ± 0.0012^{b}	9.487 ± 0.0193^{b}	9.404 ± 0.0037^{c}	9.434 ± 0.0108^{c}	9.312 ± 0.0038^{c}
90	9.294 ± 0.0039^{b}	9.446 ± 0.0043^{c}	9.524 ± 0.0023^{a}	9.486 ± 0.0012^{a}	9.451 ± 0.0025^{a}
114	9.232 ± 0.0027^{d}	9.352 ± 0.0054^{d}	9.518 ± 0.0037^{a}	9.472 ± 0.0044^{b}	9.426 ± 0.0018^{b}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B10 : GABA yield of *Lb. plantarum* B13 for each time interval at different glucose concentration after incubation of 114 hours

Time (h)	GABA (g/L)				
	20g/L	40g/L	60g/L	80g/L	100g/L
0	0.756 ± 0.6799^{g}	1.496 ± 0.2981^{g}	0.764 ± 0.6839^{g}	0.602 ± 0.4156^{f}	1.967 ± 0.1387^{g}
3	3.146 ± 0.1690^{f}	3.593 ± 0.4149^{f}	3.171 ± 0.8557^{f}	4.545 ± 0.4910^{e}	4.106 ± 0.9127^{f}
15	5.049 ± 0.2813^{e}	7.805 ± 0.8784^{e}	6.439 ± 0.8661^{e}	5.398 ± 0.4473^{e}	7.024 ± 0.0732^{e}
21	6.057 ± 0.3069^{de}	9.902 ± 0.5626^{d}	9.569 ± 0.7950^{d}	6.732 ± 0.1936^{d}	9.854 ± 0.0732^{d}
45	15.016 ± 0.9824^{b}	17.870 ± 0.1863^{b}	19.073 ± 0.5214^{a}	18.659 ± 0.6411^{a}	18.407 ± 0.1470^{a}
66	17.984 ± 0.5071^{a}	24.154 ± 0.2200^{a}	16.431 ± 0.5357^{b}	17.724 ± 0.1624^{ab}	15.268 ± 0.6829^{b}
90	8.691 ± 0.0923^{c}	13.341 ± 0.7426^{c}	13.366 ± 0.7474^{c}	16.667 ± 0.5256^{b}	14.285 ± 0.0745^{bc}
114	6.724 ± 0.4643^{d}	12.268 ± 0.2168^{c}	12.951 ± 0.5513^{c}	14.789 ± 0.4941^{c}	13.472 ± 0.7108^{c}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Appendix B11 : Residual glucose concentration of *Lb. plantarum* B13 for each time interval at different glucose concentration after incubation of 114 hours

Time (h)	Residual glucose concentration (g/L)				
	20g/L	40g/L	60g/L	80g/L	100g/L
0	19.582 ± 0.0616^{a}	37.712 ± 0.2832^{a}	59.272 ± 0.3053^{a}	79.134 ± 0.7273^{a}	96.823 ± 0.4267^{a}
3	17.055 ± 0.4844^{b}	30.750 ± 0.0806^{b}	44.245 ± 0.1232^{b}	74.349 ± 0.6518^{b}	75.801 ± 0.9451^{b}
15	7.672 ± 0.0246^{c}	16.569 ± 0.0323^{c}	36.329 ± 0.0616^{c}	66.933 ± 0.0931^{c}	69.419 ± 0.0582^{c}
21	6.801 ± 0.0566^{d}	15.580 ± 0.0336^{d}	32.405 ± 0.1818^{d}	62.820 ± 0.0466^{d}	61.556 ± 0.1540^{d}
45	4.750 ± 0.0093^{e}	5.924 ± 0.0161^{e}	18.171 ± 0.0233^{e}	50.454 ± 0.0466^{e}	50.736 ± 0.3024^{e}
66	4.648 ± 0.0081^{ef}	5.016 ± 0.0093^{f}	18.493 ± 0.0233^{e}	39.702 ± 0.1613^{f}	45.091 ± 0.2016^{f}
90	4.228 ± 0.0161^{fg}	4.698 ± 0.0323^{fg}	16.974 ± 0.0233^{f}	39.164 ± 0.0466^{f}	40.857 ± 0.3024^{g}
114	4.072 ± 0.0047^{g}	4.774 ± 0.0093^{g}	16.235 ± 0.0233^{g}	38.142 ± 0.1232^{g}	40.991 ± 0.0582^{g}

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

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This student was born on June 16th, 1997 in Mentakab, Pahang. He completed his secondary education at Sekolah Menengah Kebangsaan Triang, Triang in year 2014. He continued his education at Kolej Matrikulasi Pahang in year 2015 and completed his 1 year pre-university course in science stream which included Biology, Physics, Chemistry and Additional Mathematics. In year 2016, he pursued his tertiary education in Universiti Putra Malaysia, Selangor in the field of Biotechnology and obtained his Bachelor's Degree of Science In Biotechnology in year 2020. In March 2022, he further studied his Master's Degree of Science in the field of Industrial Biotechnology at University Putra Malaysia, Selangor.

PUBLICATION

Tan SW, Zu Koh Y, Siva S, Wasoh H, Mohamed MS, Sobri ZM, Halim M. (2024). Optimization of fermentative parameters to improve gamma-aminobutyric acid (GABA) production by *Lactiplantibacillus plantarum* B13. Journal of Biochemistry, Microbiology and Biotechnology, 12 (1), 7-16.

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