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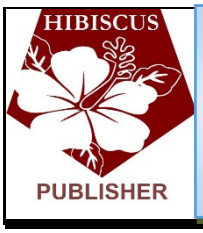

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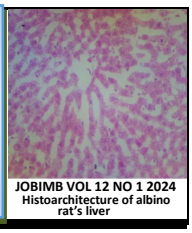

Optimization of Fermentative Parameters to Improve Gamma-Aminobutyric Acid (GABA) Production by *Lactiplantibacillus plantarum* B13

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ABSTRACT

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian brain, and it possesses several physiological properties, such as depression reduction and anxiety release. GABA production by microbial synthesis is favoured over other methods like enzymatic and chemical synthesis due to the ease of operation and less formation of harmful pollutants. Lactic acid bacteria are widely applied for GABA production because of high GABA yield and their Generally Recognized as Safe (GRAS) status , which is critical in food and ingredient safety. In this study, various fermentation conditions, including incubation time, pH, temperature, monosodium glutamate (MSG) concentration, pyridoxal-5'-phosphate (PLP) concentration and glucose concentration were screened by one-factor-at-a-time strategy to achieve the optimal GABA production by a potential probiotic strain, *Lactiplantibacillus plantarum* B13. The result revealed the strain exhibited the optimal GABA production of 19.073 ± 0.5214 g/L with the highest GABA productivity of 0.424 g/L/h under fixed conditions: incubation time of 66 hours, pH 5.5, temperature of 35°C, MSG concentration of 5% (w/v), PLP concentration of 0.7 mM PLP and glucose concentration of 60 g/L. The findings of this study show that fermentation parameters are dependent on species and strains due to the different properties of glutamic acid decarboxylase enzymes and optimization of single parameters is important as a preliminary step to identify the range of fermentation factors that affect GABA yield prior further research endeavours. This study also has great implications for GABA production by *L. plantarum* B13 and provides a prerequisite for developing new healthy products enriched with GABA as daily supplements to support relaxation and regulate mood, reduce stress and promote better sleep.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a popular apo-protein, bioactive nitrogenous compound, known as amino acid [1]. GABA is a non-protein amino acid that is biosynthesized by glutamic acid decarboxylase (GAD). GAD is a pyridoxal-5'-phosphate (PLP) dependent enzyme catalyses an enzymatic reaction known as irreversible α-decarboxylation that transforms the L-glutamic acid to GABA [2]. The commercial demand of GABA products is surging upward, propelled by its growing utilization in various sectors, including food and beverages, healthcare, pharmaceuticals, and animal feeds. GABA has been considered as a bioactive component in pharmaceutical and functional food products due to its vast biological and beneficial functions [3]. GABA is a bioactive compound with antioxidant, antidepressant, anti-insomnia, and pain-relieving effects. It is also used to treat autoimmune diseases, stroke, and neurological disorders. GABA is available from food sources such as spinach, sweet potato, broccoli, kale, kimchi, and tempeh; however, the foods that naturally consist of GABA are usually not able to fulfill the people's demands as they contain a limited or little amount of GABA [4]. Hence, GABA products are developed extensively as drugs or dietary supplements or as functional foods. As a result of rising public health and holistic awareness,

the GABA supplement market is expanding, and is anticipated to reach USD 76 million in 2033 [5].

Various biological and chemical synthesis and plant enrichment have been studied to satisfy the rising demand for GABA [6]. In recent years, research interest has also focused on the comprehensive investigations of the structure, function and significance of GABA molecules from animals, plants and microbes. In microorganisms, GABA helps to germinate spore and provide acid-resistant properties to the bacteria, whereas, in animals, GABA acts as an inhibitory neurotransmitter in peripheral tissue and central nervous system [1]. Only a small concentration of GABA is comprising in plants while the mechanism of synthesis is obscure. The productions of GABA by animals and plants are generally considered as complicated. Therefore, the production of GABA by microorganisms is widely studied mainly because it is easier to control the factors that affecting the yield of GABA. In addition, other advantages of using microorganisms to produce GABA include being more economical, eco-friendly, and safer to consume than chemically synthesized GABA.

Lactic acid bacteria (LAB) are among the most important GABA producers mainly because of their probiotic effects and generally recognized as safe (GRAS) status [1, 4]. Furthermore, LAB can produce higher amount of GABA and economically viable as the starter compared to other GABA producers such as yeasts and moulds. Essentially, the key sources for isolating the GABA-producing LAB are fermented foods such as cheese that are rich in L-glutamate. The health-promoting properties of GABA in fermented foods by LAB have been the subject of interest leading to the biosynthesis of GABA by the utilization of LAB [1]. Several LABs that are known as GABA-producing species include *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus plantarum* [7]. These LABs share a common characteristic: the presence of GAD enzyme activity, allowing them to synthesize GABA.

Nowadays, diverse fermentation strategies have been explored to enhance GABA production by LAB such as two-stage fermentation, co-culturing technique, genetic engineering, immobilization technology and modes of fermentation [1, 6]. Nevertheless, optimizing various cultivation conditions is a prerequisite to studying the performance of LAB strains to produce GABA at the satisfactory yield prior to further research advancement. The parameters include pH, temperature, fermentation time, monosodium glutamate (MSG) and PLP concentrations [1]. The GABA-producing ability of different LAB strains is varied and significantly influenced by these cultivation parameters; thus, screening of the effects of fermentation conditions are required to obtain the optimal yield of GABA. For example, *Lactobacillus plantarum* FBT215 produced a maximum GABA concentration (121.76 µg/mL) at pH 7.5 of the fermentation medium, whereas increasing the pH to 8.5 resulted in a decrease of GABA concentration (114.75 µg/mL) [8]. Another work reported *L. plantarum* Y7 was able to produce the maximum GABA concentration (375.29 µg/mL) at pH 6.5 under the temperature of 37℃ but GABA was significantly reduced (79.86 µg/mL) when the temperature was below 30℃ [9]. Different LAB strains also have different optimum MSG concentrations to achieve their maximum GABA yield [4]. The MSG concentration was found to significantly affect the GABA production by *L. plantarum* CGMCC 1.234T [10]. Adding 100 mM L-MSG, 721.35 mM GABA was produced by L. plantarum CGMCC 1.234T, corresponding to 7.7 folds higher than the fermentation process without adding MSG. In contrast, the maximum GABA yield (17.6 mM) by *P. pentosaceus* MN12 was obtained at only 60 mM MSG while the yields were decreased at higher concentrations of MSG ranging from 90 to 120 mM [11]. The supplementation of PLP was reported to produce the highest yield of GABA (909.2 mg/L) after 60 hours of fermentation by *L. plantarum* FNCC 260 [12]. 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. In the meantime, another study revealed that *L. plantarum* subsp. *plantarum* IBRC10817 produced the highest yield of GABA (295. 04 mg/L) within a shorter incubation time of 30.9 hours [13].

As different culture conditions would influence the GABA yield of certain LAB, screening the effects of various fermentation conditions is a prerequisite for more efficient GABA production. In this study, the effects of several culture conditions including fermentation time, pH, temperature, MSG, PLP, and glucose concentration were screened to achieve an enhanced yield of GABA production by a newly isolated *Lactiplantibacillus plantarum* (previously known *Lactobacillus plantarum*) B13, a potential probiotic locally isolated strain from budu (fermented anchovy sauce).

MATERIALS AND METHODS

Glycerol stocks of *L. plantarum* B13 strain

L. plantarum B13 strain (locally isolated from fermented anchovy sauce (or known as budu) used in this research was obtained from Bioprocessing and Biomanufacturing Research Complex (BBRC), Universiti Putra Malaysia. The whole-genome sequence of this bacterium has been deposited in NCBI under GenBank accession number of ON482178.1 which belongs to *Lactobacillus plantarum*. The broth culture was prepared by transferring 100 µL of the stock culture into 10 mL de Man Rogosa-Sharpe (MRS) media. After 48 hours of incubation at 37°C in an incubator (SI-50D, Protech, Malaysia), the culture was transferred on MRS agar plate and continued for 48 hours of incubation at 37°C. The single colony was picked and transferred into new MRS broth and then incubated for 48 hours at 37°C. Glycerol stock culture preparation was done by mixing 80% (v/v) glycerol with the cell culture in the ratio of 1:1. The glycerol stock culture was stored at -80°C for further use.

Preliminary study of growth profile

The culture of *L. plantarum* B13 (10% v/v) was inoculated into MRS broth that was supplemented with 1% (w/v) MSG and 0.5 mM PLP for the total volume of 80 mL in 250 mL shake flask. The fermentation medium was adjusted to pH 6.0. The flask was incubated at 37°C, 150 rpm for 96 hours. Sampling was done to measure cell growth, residual glucose and GABA concentrations at every 3-hour time intervals at the first 24 hours, followed by every 24-hour time intervals until 96 hours. The effects of various conditions, including pH, temperature, incubation time, MSG concentration, PLP concentration and glucose concentration were investigated to determine the optimum conditions for the GABA production.

Effects of incubation time

To evaluate the effect of incubation time, fixed fermentation conditions for *L. plantarum* B13 was used (initial pH 6; 37°C; 1% (w/v) MSG; and 0.5 mM PLP) under 150 rpm within the optimum range (from 48 to 72 hours) of fermentation time determined based on the results obtained earlier (in the experiment of preliminary study of growth profile). GABA

production by LAB begins in the late logarithmic growth phase and is most effective during the stationary phase [14]. The cell growth and GABA yield concentration was then measured for every 6 hours-intervals sampling.

Effects of initial pH

Hence, to determine the effect of initial pH, fixed fermentation conditions for *L. plantarum* B13 were used (37°C; 1% (w/v) MSG; 0.5 mM PLP) under 150 rpm after the optimum fermentation time obtained, but at different initial pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 [15]. GAD in LAB is active under acidic conditions with the highest GABA production often observed in the range of pH 4.0 to 6.0 while at higher pH, it may lose its activity or denature [16]. After the optimum time, the cell growth and GABA yield were then measured for each sampling.

Effects of temperature

To determine the effect of temperature, fixed fermentation conditions for *L. plantarum* B13 were used (initial pH 6; 1% (w/v) MSG; and 0.5 mM PLP) under 150 rpm after the optimum fermentation time obtained, but at different temperatures: 33, 35, 37, 39, and 41°C [15]. The optimal temperature for GABA production by LAB is generally between 30 and 37˚C while above 40˚C will adversely affect cell growth, and further limit their ability to produce GABA [17]. The cell growth and GABA yield were then measured for each sampling after the optimum time.

Effects of MSG concentration

To determine the effect of MSG concentration, fixed fermentation conditions for *L. plantarum* B13 were used (initial pH 6; 37°C; and 0.5 mM PLP) under 150 rpm after the optimum fermentation time obtained, but at different MSG concentrations (1, 3, 5, 7 and 9% (w/v)) [15]. MSG concentration in the range up to 5 % (w/v) could promote increased GABA accumulation in MRS broth [18]. After the optimum time, the cell growth and GABA yield were then measured for each sampling.

Effects of PLP concentration

To determine the effect of PLP concentration, fixed fermentation conditions for *L. plantarum* B13 were used (initial pH 6; 37°C; and 1% (w/v) MSG) under 150 rpm after the optimum fermentation time obtained, but at different PLP concentrations: 0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 mM. The addition of PLP (as a cofactor) in the range of 0.2 to 0.6 mM could increase GAD activity and GABA production by LAB [12]. The cell growth and GABA yield were then measured for each sampling after the optimum time.

Effect of glucose concentration

To determine the effect of glucose concentration, the optimized fermentation conditions obtained from the results earlier was used under 150 rpm after 114 hours but at different glucose concentrations (20, 40, 60, 80 and 100 g/L). According to Abdel-Rahman et al. [19], some LAB strains can withstand high tolerance to glucose concentrations up to 100 g/L. The cell growth, GABA yield and reducing sugar concentration were then measured for each sampling at time intervals and the kinetic parameters were calculated to determine optimal glucose concentration. The yield of GABA (YGABA, g/g) was calculated as the ratio of the maximum amount of GABA produced to the amount of glucose consumed. GABA productivity (P_{GABA}, g/L/h) was defined as the ratio of maximum GABA amount produced (g/L) to the fermentation time (h).

Analysis procedure

Bacterial growth by optical density

A volume of 1 mL sample was collected from the fermentation medium and used for the measurement of absorbance under the wavelength of 600 nm using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany) to determine the cell growth.

Bacterial growth by colony forming unit

Serial dilution was initially carried out for the samples to spread plate the culture on the MRS agar. The plates were then incubated in 37°C for 48 hours in an incubator (SI-50D, Protech, Malaysia) to determine the viable cells. Valid cell counts were ranged between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are considered as "too numerous to count" (TNTC), while plates with less than 30 colonies are considered as "too few to count" (TFTC). The number of cells per mL of original cell culture (CFUs/mL) was measured as below:

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

GABA analysis

GABA production was analysed through a thin layer chromatography (TLC) and colorimetric estimation methods. The samples collected from the shake flask culture were centrifuged at 9000×g for 10 minutes by using a benchtop centrifuge (Microfuge® 16, Beckman Coulter, USA) and the supernatant was collected for further analysis.

Thin layer chromatography

TLC procedure was done based on the method described by Rayavarapu et al. [17], which was used for the qualitative analysis of the GABA production. Initially, 2 µL of the supernatant was taken and spotted on TLC 60F254 aluminium sheets (Merck, Darmstadt, Germany) which were immersed in the solution of n-butanol, acetic acid, and water (5:3:2) as the mobile phase. The observation of a red colour spot on TLC detected GABA presence. The spot was compared with the standard GABA after spraying 1.0% of ninhydrin reagent and the incubation at 60°C for 30 minutes in a dryer (FDD-720, Protech, Malaysia).

Colorimetric estimation of GABA

GABA was further analysed through a colorimetric estimation [17]. After running the TLC, the GABA spots were identified, scraped into the powder, and placed into a glass tube containing 3 mL borate buffer (pH 7) and 0.5 mL ninhydrin reagent (0.8% dissolved in acetone). After that, it was vortexed and incubated in a water bath (WB-22, WiseBath®, South Korea) at 70°C for 20 minutes. The absorbance was then measured at 570 nm by using the UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany). Standard curve was prepared by using the standard GABA compound with different concentrations (0 -10 µg/µL), which was used for the estimation of GABA concentration of the samples. The same amount of borate buffer and ninhydrin reagent without samples was used as the blank.

Glucose determination by reducing sugar analysis

Glucose consumption was determined by using 3, 5-dinitrosalicylic acid (DNS) [3]. To prepare a standard curve, glucose was mixed with distilled water at different volumes, forming a mixture with a total volume of 200 µL. Then the diluted glucose solutions were added with 1 mL of DNS solution and mixed well.

The mixture was then put into boiling water bath for 5 minutes. After cooling down for 5 minutes, the absorbance of 540 nm was taken by using UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany). For DNS analysis of samples, similar procedure was repeated by replacing the glucose with the supernatant. The concentration of glucose was then calculated according to the equation of the standard curve.

Statistical analysis

Data of triplicates was expressed as the means ± standard deviation (SD). The statistical significance of the differences between the two means was evaluated by the one-way analysis of variance (one-way ANOVA) using IBM SPSS Statistics 26. Duncan's multiple range test was performed to determine the significance of the difference within treatments for each analysis at probability level $p < 0.05$. Values of $p < 0.05$ is considered significant. The figures were made using the program Sigmaplot version 11.0.

RESULTS AND DISCUSSION

Preliminary study of cell growth profile and GABA production

L. plantarum B13 was grown in MRS medium with 1% (w/v) MSG and 0.5 mM PLP under the pH of 6.0. The time course of cell growth, residual glucose concentration and GABA production of the strain for 96 hours incubation times was shown in **Fig. 1**. The cells were in a lag phase for the first 3 hours before started to exponentially grow until 21 hours of cultivation. After that, there was a maximum cell growth in which glucose was almost completely consumed and the cells reached a stationary phase. GABA started to be produced at the late logarithmic phase until the maximum GABA production was observed within the stationary phase that range between 48 hours (7.317 g/L) and 72 hours (7.740 g/L).

The result was comparable with several reported literatures. For instant, *L. plantarum* B7 reached the highest GABA yield at the stationary phase that ranged between 24 to 64 hours of cultivation [3]. Similarly, the highest GABA yield (4.83 mM) by *L. plantarum* DSM19463 was recorded at 72 hours and started to decrease when the fermentation time was extended to 96 hours of cultivation [20]. This may be a result of the GABA transaminase enzyme's activity, which broke down GABA [21]. This enzyme used either pyruvate or α-ketoglutarate as the amino acceptor to catalyse the breakdown of GABA into succinic semialdehyde. During the process, succinic semialdehyde was irreversibly converted to succinate by succinic semialdehyde dehydrogenase.

Effects of fermentation conditions on GABA production

Effect of incubation time

Optimization method by One-Factor-At-Time (OFAT) was applied to determine the accurate optimum incubation time for the GABA production that was observed within the 48 to 72 hours from the preliminary study. Incubation time has a significant effect on GABA production because GABA is started to be produced by LAB during the late logarithmic growth phase and maximally during the stationary phase [1].

In this phase, the high accumulation of organic acid by-products will cause a decrease in pH which stimulates the GAD enzyme to initiate the production of GABA [14].

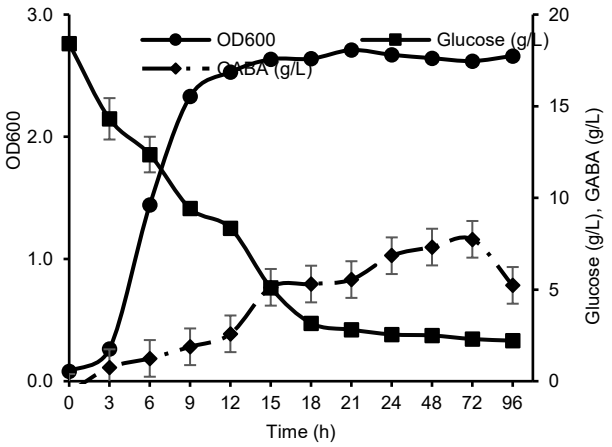

Fig. 1. The cell profile of *L. plantarum* B13 shake flask culture based on OD_{600}, residual glucose concentration (g/L) and GABA concentration (g/L). The results were expressed as mean (n = 3) ± SD.

In this study, the incubation time was varied (48, 54, 60, 66, and 72 hours), whereas the other fermentation parameters including initial pH, temperature, MSG and PLP concentrations were fixed at initial pH 6.0, 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively. According to **Fig. 2**, the results showed that the GABA yield was gradually increased from 7.317 g/L (at 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} 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 [22]. 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 utilized the resources to produce the amino acids for enhancing their survivability rather than for the growth. As observed earlier, the GABA yield was started to decline when the incubation time was extended to 72 hours. This trend was in accordance with a study by Yogeswara et al. [12] for the GABA synthesis by *L. plantarum* FNCC 260. They reported that GABA (450 mg/L) began to be rapidly synthesized during the stationary phase after 48 hours and reached the maximum yield (809.2 mg/L) after 60 hours. Likewise, the highest GABA yield (497.97 mM) attained by *L. plantarum* Taj-Apis362 was also reported at 60 hours [15].

Meanwhile, *L. plantarum* BC114 was able to produce the maximum concentration of GABA (3.45 g/L) at 72 hours, while prolonged the incubation time reduced the GABA yield [23].

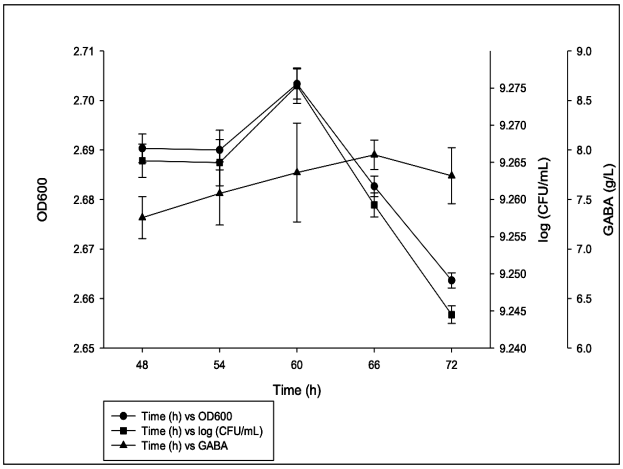

Fig. 2. Cell growth (OD_{600} and CFU/mL) and GABA production of *L. plantarum* B13 at different incubation times (48, 54, 60, 66, and 72 hours). The other fermentation parameters: 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively. The error bars represent the standard deviations about the mean (n=3).

Effect of pH

GABA synthesis is highly correlated with the pH of the fermentation medium [14]. 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 [14]. 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 **Fig. 3**, the results showed that the initial pH value of the culture medium was significantly affecting the GABA production. The OD_{600} and log CFU/mL for the maximum cell growth at initial pH 5.5 were 2.660 and 9.242, respectively. When the initial pHs 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. Adjusting the fermentation medium higher than pH 5.5 also did not favor the GABA production despite supporting the cell growth of *L. plantarum* B13.

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. Meanwhile, the GABA yields for fermentations with initial pH 6.0, 6.5, 7.0 and 7.5 were 7.268 g/L, 4.715 g/L, 3.967 g/L and 3.805 g/L, respectively. From the screening, it could be deduced that the optimum GABA production (9.268 g/L) was obtained when the culture medium was slightly acidic at pH 5.5. Nonetheless, this study contradicted with the study reported earlier by Lu et al. [24]. They observed 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 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 in the maximum amount of GABA of 7.2 g/L. This indicated that the optimum pH for GABA production was strain dependent. pH regulation is very crucial LAB to produce GABA as the initial pH value would affect the activity of GAD [25]. GAD 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 GAD will be partially lost if the pH is too high (highly alkaline) or too low (highly acidic). According to Tajabadi et al. [15], the highest GABA (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* [17] and *L. futsaii* CS3 [26].

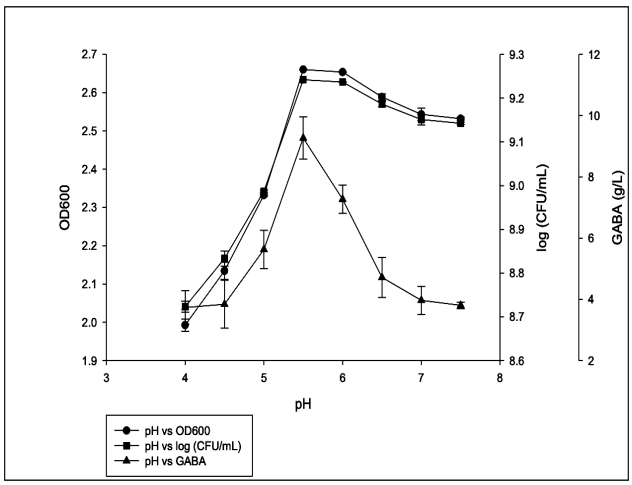

Fig. 3. Cell growth and GABA production of *L. plantarum* B13 with different pH values (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) within 66 hours cultivation. The other fermentation parameters: 37°C, 1 % (w/v) MSG and 0.5 mM PLP, respectively. The error bars represent the standard deviations about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

Effect of 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 [1]. Generally, most LAB possesses high rate of GABA biosynthesis at the optimum temperature that ranged between 30 to 50°C. 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 GAD and cell. 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, 35, 37, 39 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 **Fig. 4**, 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 to 35°C. The maximum cell growth (9.272 log CFU/mL; OD_{600} 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 GAD leading to the enhancement of GABA production [23]. The result of this study was in line with the study for *L. plantarum* NDC75017 that resulted with a maximum concentration of GABA (314. 56 mg/100 g) at 36°C [28]. However, *L. plantarum* NDC75017 attained the maximum number of viable cells at a lower temperature of 30°C. Meanwhile, *L. plantarum* BC114 [23] and *L. plantarum* DSM19463 [20] were able to attain the highest amount of GABA corresponding to 3.45 g/L and 0.497 g/L, respectively at the temperature of 30°C. Based on extensive reported works on LAB, the optimal temperature for yielding the maximum amount of GABA is species dependent. For example, *L. brevis* CD0817 [29], *Streptococcus thermophilus* QYW-LYS1 [30], and *Lactococcus lactis* 311 [31] attained the maximum GABA yields at the incubation temperature of 45°C, 34°C and 30°C, respectively.

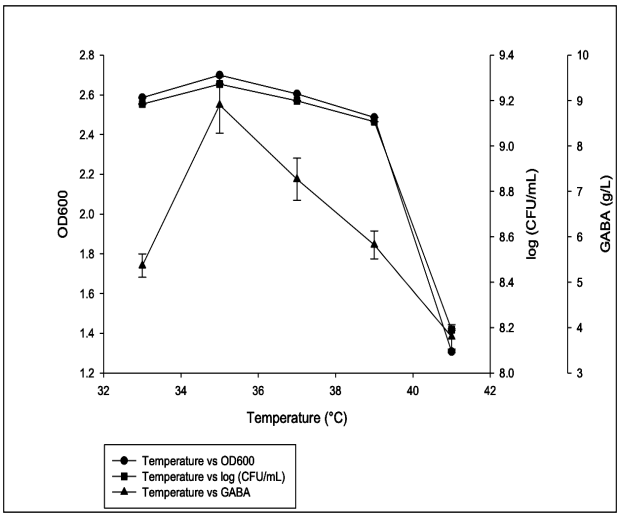

Fig. 4. Cell growth and GABA production of *L. plantarum* B13 with different temperatures (33, 35, 37, 39 and 41°C) within 66 hours cultivation. The other fermentation parameters: initial pH of 6.0, 1 % (w/v) MSG and 0.5 mM PLP. The error bars represent the standard deviations about the mean (n=3).

Effect of monosodium glutamate (MSG) concentration

GABA synthesis begins once the MSG supplied to the fermentation medium enters the cells through their antiporter mechanism in the cell membrane [14]. Theoretically, the yield of GABA is affected by the amount of MSG that has been decarboxylated by the GAD enzyme [32]. Nevertheless, it is crucial to provide an appropriate initial level of MSG because excess glutamate can suppress LAB growth of LAB and subsequently reduce GABA production [33]. Various studies have reported that the supplementation of MSG onto the culture medium would affect the activity of GADs, hence influencing the GABA production by *L. plantarum* strains [1,15, 28]. 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, 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 **Fig. 5**, 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) favored the *L. plantarum* B13 to obtain higher cell growth and GABA yield. The results of this study demonstrated that cell growth was stimulated by adding glutamate to the culture medium. The presence of glutamate promotes the counterreaction that raises the pH back to protect the cells from acidic conditions while GABA is being generated [34]. It is a coupling reaction process. 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, however, it reduced the long diameter of cells [35].

This study also suggested that the addition of MSG up to 6%, may protect *L. plantarum* FL-664 cells from the osmotic stress by increasing the expression of genes involved in the cell wall synthesis and cell division. Meanwhile, the cell growth of *L. 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 [36]. The addition of MSG into the culture medium also 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 can be acidic due to the accumulation of high concentration of organic acids by-products produced by the LAB.

In this study, it was discovered that the supplementation of 9 % (w/v) of MSG did not favor the GABA production and cell growth of *L. plantarum* B13. The cells growth of LAB tends to be inhibited by high concentrations of glutamate [15]. Gandhi and Shah [37] stated that the addition of salt instigated the osmotic pressure to the LAB, resulting in a defective formation of cell wall and cell extension. In this study, within the MSG concentrations 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). Although the cell growth showed a decreasing trend with MSG concentrations of 3 %, 5 % and 7 % (w/v), however the GABA yield was considerably higher 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. [35], in which the ability of LAB 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 [38]. These results show MSG can help to improve the cell growth but at certain concentrations, it may reduce the efficiency of GAD and would increase the osmotic pressure of the cells thus inhibiting the metabolic activities and cell growth [39].

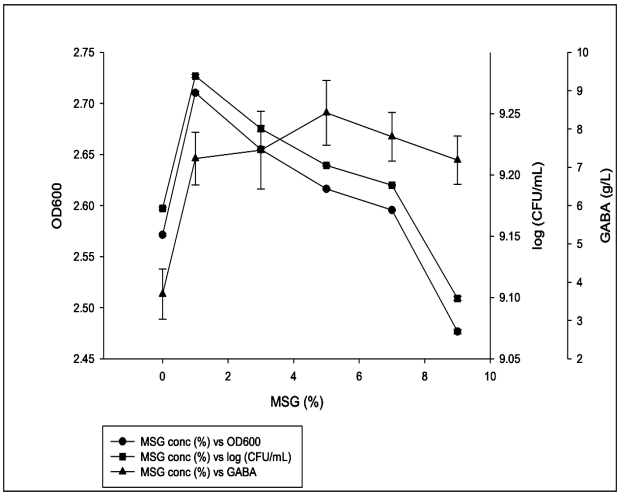

Fig. 5. Cell growth and GABA production of *L. plantarum* B13 with different MSG concentrations (0 (control), 1, 3, 5, 7, and 9 % (w/v)) within 66 hours cultivation. The other fermentation parameters: initial pH of 6.0, 37°C and 0.5 mM PLP. The error bars represent the standard deviations about the mean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

Effect of pyridoxal-5-phosphate (PLP) concentration

PLP is an essential co-factor for enhancing the GABA yield by LAB [1]. Generally, GAD 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. PLP may recover the activity of GAD especially during the later stages of bacterial growth [40]. 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, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 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 **Fig. 6**, 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 [12]. 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 trend obtained by *L. plantarum* B13 is similar to the trend observed for *L. plantarum* NDC75017 as reported earlier by Shan et al. [28]. A maximum GAD 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 by *L. plantarum* NDC75017. Nevertheless, when the PLP concentration supplied exceeded 20 mM, the cell growth, activity of GAD and GABA yield were all significantly reduced. Meanwhile, in a study reported by Santos-Espinosa et al. [41], *Lactococcus lactis* L-571 and L-572 produced more GABA with the addition of 100µm PLP compared to 200µm PLP. These showed that the effect of PLP is strain-dependent. PLP plays its role as a cofactor of GAD, which helped to improve the enzyme catalytic activity, however an excessive concentration of PLP will cause a negative effect to both cell growth and GABA yield [12]. However, the the dose–effect relationship between PLP concentration and GABA production needs to be further studied to elucidate the effect and gain a better understanding.

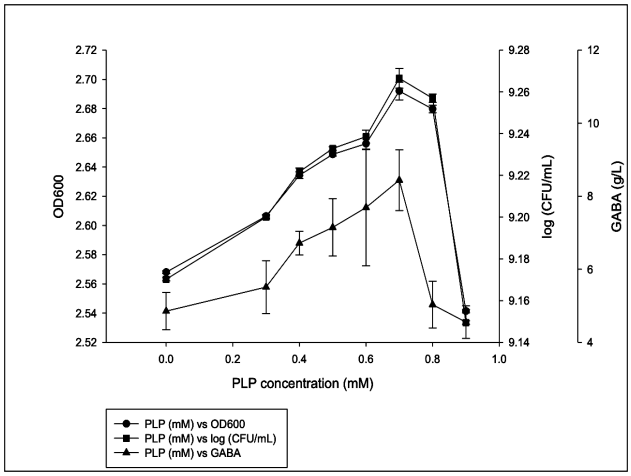

Fig. 6. Cell growth and GABA production of *L. plantarum* B13 with different PLP concentrations (0 (control), 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mM) within 66 hours cultivation. The other fermentation parameters: initial pH of 6.0, 37°C and 1 % (w/v) MSG. The error bars represent the standard deviations about the mean (n=3).

Effect of 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 beside it may reduce the production cost [1]. 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, 40, 60, 80, 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; 35°C; 5 % (w/v) MSG; and 0.7 mM PLP) based on the results that obtained earlier.

Based on **Fig. 7**, *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 to 100 g/L corresponding to cell growth that ranged from OD_{600} 0.075 to 0.223, and log CFU/mL of 7.233 to 7.349, 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 [42]. This is because cells will be concentrating on the

synthesis of carbon source utilization genes instead of cell growth and proliferation.

Subsequently, *L. plantarum.* B13 started to enter the exponential growth phase until the 45 hours of incubation period for all tested glucose concentrations except for 20 g/L that reached the stationary phase after 21 hours. The range of the maximum OD_{600} reading for all the tested glucose concentrations was between OD_{600} 2.7 to 3.0 (**Fig. 7 (A)**) while the range of viable cells count is between 9.3 to 9.5 log CFU/mL (**Fig. 7 (B)**). These observations showed that the initial concentrations of glucose affect the biomass density beside influencing the duration of exponential phase. Among the tested glucose concentrations, the highest maximum cell growth (9.526 log CFU/mL and OD_{600} 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 favorable for the enhancement of the cell growth of *L. plantarum* B13. This might be due to the inhibition effect resulting from the excessive glucose concentration in the culture medium. According to Abdel-Rahman et al. [43], high concentration of glucose will increase the osmotic pressure towards cells leading in higher rate of cell death. For example, 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 [17]. 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.

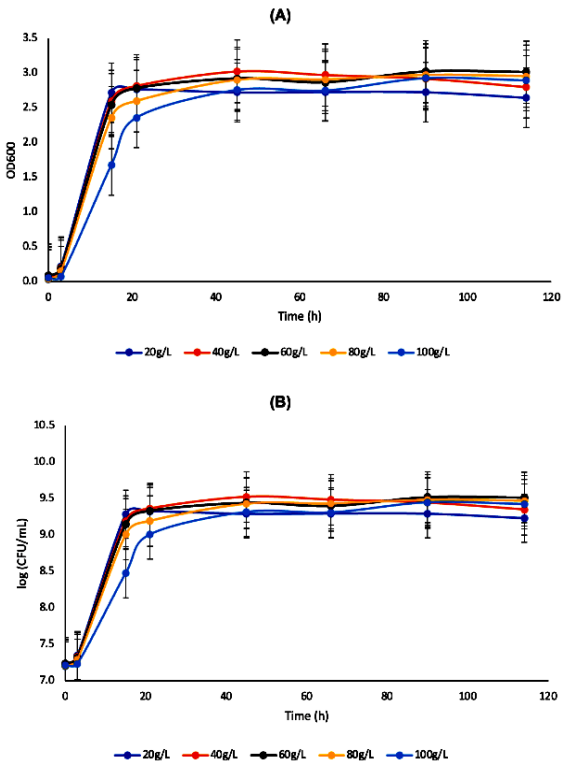

Fig. 7. OD_{600} readings **(A)** and log CFU/mL **(B)** for the cell growth of *L. plantarum* B13 in medium composed of different glucose concentrations (20, 40, 60, 80 and 100 g/L) within 114 hours incubation period. The fermentations conditions: initial pH: 5.5; temperature: 35°C; concentration of 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 (**Fig. 8 (A)**). 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. As depicted in **Fig. 8(B)**, 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 [17]. However, a declination in GABA production (0.79 g/L) was observed when the glucose concentration was raised up to 2 %, due to the inhibitory effect from the high concentration of glucose.

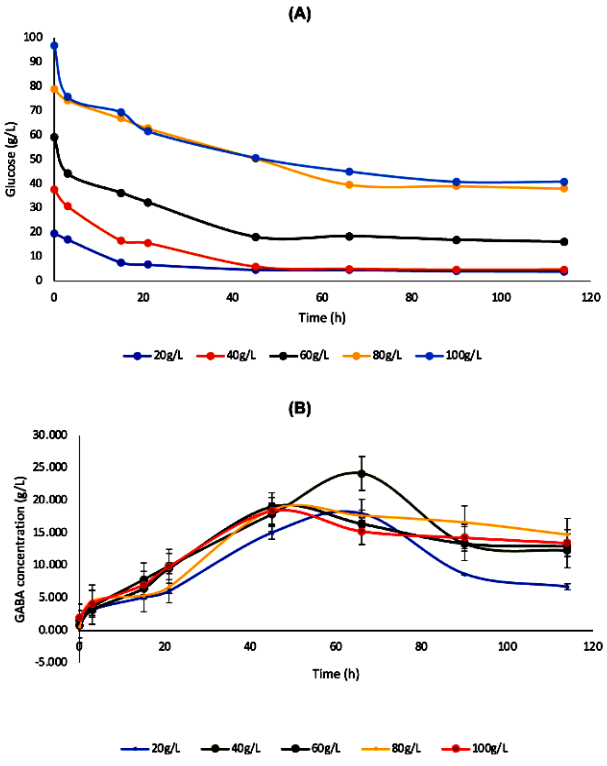

Fig. 8. Residual glucose concentration **(A)** and GABA production **(B)** profiles for fermentations by *L. plantarum* B13 in medium composed of different glucose concentrations (20, 40, 60, 80 and 100 g/L) within 114 hours incubation period. The fermentations conditions: initial pH: 5.5; temperature: 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).

According to **Table 1**, the maximum GABA yield 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, Abdel-Rahman et al. [19], 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 *Enterococcus hirae* BoM 1-2. Besides that, based on a study reported by Abdel-Rahman et al. [43], 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, 37, 43, 45 and 49°C), and hence, the fermentation temperature of 43°C was selected for further investigation. Generally, 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.

Table 1. 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^{d}	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

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 $p < 0.05$.

CONCLUSION

L. plantarum B13 was significantly affected by various fermentation conditions including incubation time, pH, temperature, MSG concentration, PLP concentration and glucose concentration. The optimal factors for GABA production were incubation time of 66 hours, pH 5.5, temperature of 35°C, MSG concentration of 5% (w/v), PLP concentration of 0.7 mM PLP and glucose concentration of 60 g/L that resulted in the optimal GABA of 19.073 ± 0.521 g/L with the highest GABA productivity of 0.424 g/L/h. Beside the selected parameters, there are other factors that may affect the yield and productivity of GABA including the type of carbon sources, nitrogen source and growth factor. Furthermore, optimization tools such as response surface methodology (RSM) and artificial neural networks (ANN) can be used to study the interactions between fermentative factors and the yield of GABA. These results may support the potential use of *L. plantarum* B13 as a starter culture for the manufacture of naturally GABA-enriched food and dietary supplement formulations. However, future studies on the comprehensive safety evaluation of GABA are needed to access the functional applicability for human consumptions.

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