

Article

Microbial production and enzymatic biosynthesis of γ -aminobutyric acid (GABA) using *Lactobacillus plantarum* FNCC 260 isolated from Indonesian fermented foods

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Abstract: In the present study, we isolated and screened thirty strains of GABA-producing lactic acid bacteria (LAB) from Indonesian traditional fermented foods. Two strains were able to convert monosodium glutamate (MSG) to GABA after 24 h of cultivation at 37°C based on thin layer chromatography (TLC) screening. 16S rDNA sequencing and proteomic identification using MALDI-TOF MS identified these two strains as *Lactobacillus plantarum* designated as *L. plantarum* FNCC 260 and *L. plantarum* FNCC 343. The highest yield of GABA production obtained from the fermentation of *L. plantarum* FNCC 260 was 809.2 mg/l of culture medium after 60 h of cultivation. Supplementation of 0.6 mM pyridoxal 5'-phosphate (PLP) and 0.1 mM pyridoxine led to the increase in GABA production to 945.3 mg/l and 969.5 mg/l, respectively. The highest GABA production of 1226.5 mg/l of culture medium was obtained with 100 mM initial concentration of MSG added in the cultivation medium. The open reading frame (ORF) of 1410 bp of the *gadB* gene from *L. plantarum* FNCC 260 encodes 469 amino acids with a calculated molecular mass of 53.57 kDa. The production of GABA via enzymatic conversion of monosodium glutamate (MSG) using purified recombinant glutamate decarboxylase (GAD) from *L. plantarum* FNCC 260 expressed in *Escherichia coli* was found to be more efficient (5-fold higher within 6 h) than the production obtained from fermentation. *L. plantarum* FNCC 260 could be of interest for the synthesis of GABA.

Keywords: GABA, Indonesian fermented foods, glutamate decarboxylase, lactic acid bacteria, *L. plantarum*.

1. Introduction

γ -aminobutyric acid (GABA), which is a four-carbon free amino acid and plays a major role as a suppressive neurotransmitter, is widely present in plants, microorganisms and the mammalian brain [1–3]. GABA has been extensively studied due to physiological and pharmacological effects including anti-depressant, hypotensive activity and anti-diabetic in humans [4–6]. Recently, GABA

administration in fluoride-exposed mice showed protective effects against hypothyroidism and maintained lipid and glucose level *in vivo* [4]. Furthermore, GABA-enriched foods have been developed [5–11]. GABA-rich chlorella has been shown to significantly reduce high blood pressure in hypertensive subjects [12]. A number of GABA-enriched foods such as soymilk [13], fermented milk [14], natto [15], green tea [16] and cheese [5], has been reported to depress the elevation of blood pressure in spontaneously hypertensive rats (SHR) and hypertensive subjects.

GABA can be synthesized using chemical or biochemical means, of which the latter involves enzymatic conversion, whole-cell biocatalysts or microbial fermentation. The chemical synthesis is considered hazardous due to corrosive nature of used reagents [17–19], hence its use in the food industry is limited. Moreover, the direct addition of synthetic GABA to food system is considered unnatural and unsafe [14]. Therefore, it is important to develop a natural and safe method to increase GABA in foods, since there are no side effects of natural GABA supplementation [16]. Recent studies showed that several strains of lactic acid bacteria (LAB) are promising candidates as GABA-producing bacteria [1–3,5] because of their GRAS (generally recognized as safe) status. A number of GABA-producing bacteria has been isolated from fermented foods such as, *L. brevis* (from kimchi) [20], *L. rhamnosus* (from fermented pickles) [21], *L. plantarum* (from fermented dairy products) [22], *L. helveticus* (from koumis fermented milk) [23], *L. buchneri* (from kimchi) [24] and *L. otakiensis* (from Pico cheese) [25], *L. casei* (from fermented milk) [11] and *L. paracasei* (from Italian cheese) [3]. These reports have shown that fermented foods are promising sources for GABA-producing bacteria. In addition, screening GABA-producing LAB from various fermented foods might bring the possibilities to obtain newly isolated strains for the use as functional starter cultures in the food industry.

Several Indonesian fermented foods such as *gatot*, *growol*, *tape ubi*, *bekasam* and *tempoyak* are spontaneously fermented by LAB, which involve mainly the strains of the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* [26,27]. However, the potential of these LAB strains from Indonesian fermented foods to be used as GABA-producing bacteria as well as their relevant enzymes have not yet been studied. Therefore, the development of GABA-enriched foods using suitable LAB is a promising strategy, to bring new functional food to the market. In addition, biosynthesis of GABA using LAB also provides advantageous effects including probiotic activity and extension of the shelf-life of food products [9].

The biosynthesis of GABA involves the one-step reaction of decarboxylation of glutamate to GABA and carbon dioxide catalyzed by glutamate decarboxylase (GAD). GAD (EC 4.1.1.15) is a major enzyme for GABA production and it requires pyridoxal 5'-phosphate (PLP) as cofactor [28–30]. The GAD genes from various sources have been cloned, expressed and their biochemical properties have been characterized [20,31–34]. The use of purified GAD for the biosynthesis of GABA is also of interest because only simple downstream purification of GABA is required and yet, the process could overcome the limitation of microbial fermentation (i.e GABA catabolism). In the present study, we describe the screening of GABA-producing LAB from Indonesian fermented foods (fermented soy beans, *growol*, *gatot*, *tempeh* and *bekasam*) and GABA productions using microbial fermentation of the isolated strain and the purified GAD of this strain for the conversion of glutamate to GABA.

2. Materials and Methods

2.1. Screening of GABA-producing LAB

Thirty strains of *Lactobacillus* spp. were previously isolated from Indonesian fermented foods such as fermented soybeans, *growol*, *gatot*, *tempeh* and *bekasam* (fermented fish) [35]. *Lactobacillus* spp. were the predominant genus according to cell morphology, Gram reactions and catalase tests. All strains were obtained and stored in the Food and Nutrition Culture Collection, Gadjah Mada University (Yogyakarta, Indonesia). Prior to screening, all strains were grown in MRS broth containing 118 mM monosodium glutamate (MSG) (Ajinomoto, Tokyo, Japan) for 24–48 h at 37°C under microaerophilic conditions. The cultures broth was then centrifuged at 8000 × g for 5 min at 4°C. GABA formation in the supernatant was analyzed using thin layer chromatography (TLC).

Briefly, 0.5 – 1.0 µl of supernatants were spotted onto TLC plates Silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany). The mobile phase consists of a mixture of 1-butanol: acetic acid: distilled water (5:2:2). Subsequently, the plates were sprayed with 0.5% ninhydrin and heated at 105°C for 5 min to visualize the spots. GABA (Sigma Aldrich, MO, USA) was used as a standard, and the R_f values were calculated. LAB cultures showing the same R_f values as GABA standard were considered as positive GABA-producers. Positive GABA-producing strains were identified using proteomic and genotype techniques. Furthermore, the amount of GABA produced was determined by the GABase assay [36]. All chemicals were of the highest grade.

2.2. Identification of GABA-producing LAB

GABA-producing LAB was identified using proteomic and genotype techniques. Genomic DNA of GABA-producing LAB was extracted using peqGOLD Bacterial DNA Mini Kit (PeqLab, Erlangen, Germany) according to manufacturer's instructions. The extracted DNA was used as a template for 16S rDNA amplification. The amplifications of 16S rDNA were performed using forward primer bak4 (5'-AGGAGGTCATCCARCCGCA-3') and reverse primer bak11w (5'-AGTTTGATCMTGGCTCAG-3'). The PCR reaction mixtures consisted of 10x PCR buffer (Dynazyme buffer 10x Thermo scientific, Waltham, MA, USA), 10 nmol /µl dNTP mix (GE Healthcare Buckinghamshire, UK), 2 U/µl DNA polymerase (Dynazyme II, Thermo scientific) and high-quality sterile water to a total volume of 25 µl. The conditions for PCR amplification were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension 72°C for 2 min, and a final extension at 72°C for 7 min. After PCR amplification, the amplified products were visualized by gel electrophoresis. The gel was stained with GelRed Nucleic Acid (Biotium, CA, USA) and subsequently visualized with a ultraviolet transilluminator (BioRad, CA, USA).

Proteomic identification was performed using matrix-assisted laser desorption/ionizing time-of-flight mass spectrometry (MALDI-TOF MS). GABA-producing bacteria were identified by the extended direct transfer method. A single colony was directly spread onto a MALDI target plate. The spot was overlayed with 1 µl of 70% formic acid and allowed to dry at room temperature. Furthermore, 1 µl of 10 mg/ µl HCCA (α-cyano-4-hydroxycinnamic acid) solution was then added to the spot and allowed to dry at room temperature. The target plate was immediately applied to MALDI-TOF MS and analyzed using Microflex LT bench-top mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with the FlexControl 3.4 software. A mass spectrum was processed using BioTyper software (version 3.0, Bruker Daltonics, Bremen, Germany). MALDI-TOF MS profiles were obtained from bacteria isolates and matched with a database containing 8223 reference MALDI-TOF MS profiles.

2.3. Determination of GABA production and GAD assay

The GABase method was performed to determine GABA concentration in culture supernatants. Briefly, culture broth was centrifuged at 8000 x g for 5 min at 4°C. 10 µl of supernatants were mixed with 140 µl of 100 mM K₄P₂O₇ buffer (pH 8.6), 30 µl of 4 mM NADP⁺, 10 µl of 1 U/ml GABase (Sigma-Aldrich, MO, USA). The mixtures were dispensed into each well of 96-well plate. The initial absorbance was read at 340 nm in PerkinElmer plate reader (PerkinElmer, Buckinghamshire, UK). After initial reading, 10 µl of 20 mM α-ketoglutarate were added and the mixtures were incubated for 1 h. The final absorbance was read after 1 h at the same wavelength. GABA concentrations were determined based on the difference of A₃₄₀ values and the standard curve of GABA.

The GAD assay was carried out using colorimetric method [37]. The reaction mixtures consist of 200 mM Na₂HPO₄-citric acid buffer (pH 5.0), 20 mM L-MSG, 0.2 mM PLP and 20 µl of purified GAD. The mixtures were thoroughly mixed and incubated at 37°C for 1 h and then inactivated by boiling for 3 min. The reaction mixtures were used to determined GAD activity using the Berthelot reaction method which was composed of 100 µl of reaction sample, 250 µl of H₂O, 50 µl of 200 mM sodium borate (pH 9.0), 250 µl of 6% phenol and 200 µl of 5% (w/v) sodium hypochlorite. Subsequently, the reaction mixtures were thoroughly mixed and boiled for 10 min until the blue color developed, then

immediately placed on ice for 15 min. The mixtures were analyzed colorimetric at 630 nm to determine absorption value. One unit of GAD activity was defined as the amount of enzyme that liberates 1 μ mol of GABA per minute under activity assay condition.

Concentrations of GABA formed in supernatants and after enzymatic conversions were confirmed using Ultra Performance Liquid Chromatography (UPLC Acquity H- Class, Waters Corporation, Milford, MA, USA) equipped with a PDA detector and an AccQ. Tag Ultra C18 column (1.7 μ m particle, 2.1 x 100 mm). The samples were hydrolyzed using 6 N HCL and followed by derivatization of the samples and the GABA standard using AccQ-Tag ultra-derivatization kit (Waters, Milford, MA, USA) according to manufacture instructions. For UPLC analysis, the derivatives samples were injected to Acquity UPLC H class [38]. The system was operated at a flow rate of 0.5 mL/min at 49°C with a wavelength of 260 nm. The mobile phase used were AccQ. Tag Ultra Eluent A 100%; Accq. Tag Ultra Eluent B (Aquabides 90:10); Aquabides Eluent C; AccQ. Tag Ultra Eluent B 100%.

2.4. GABA production

The MRS medium was inoculated with 5% inoculum of GABA-producing LAB and incubated at 37°C for 108 h. The optical densities (OD₆₀₀) of the cultures were measured every 12 h. Various MSG concentrations (25 - 100 mM) (Sigma Aldrich, MO, USA) and various concentrations of pyridoxal 5-phosphate (PLP, 0 – 0.6 mM) and pyridoxine (vitamin B6, 0 – 0.3 mM) were added to the MRS medium and GABA production under these conditions was investigated subsequently.

2.5. Cloning of *gadB* gene

The glutamate decarboxylase gene (*gad*) from *L. plantarum* FNCC260 was amplified using degenerated primers *gad_FwdNdeI* (5'-CATATGATGGCAATGTTTAYGGTAAAC-3') and *gad_RevEcoRI* (5'-GAATTCCAGTGTGTGAATMSGTATTTC-3'), which were designed based on the sequences of the *gad* genes of *Lactobacillus* spp. available in GenBank (Accession numbers JN248358.1, KU214639.1, AB986192.1, CP029349.1, AL935263.1, CP018209.1, CP028977.1, GU987102.1, JX545343.1).

The primers were supplied by VBC-Biotech Service (Vienna, Austria) and the appropriate endonuclease restriction sites were introduced in the forward and reverse primers (underlined sequences). The conditions for PCR reactions were as follows: initial denaturation at 98°C for 20 sec; 30 cycles of denaturation at 98°C for 20 sec, annealing at 58°C for 20 sec, extension at 72°C for 1 min 45 sec; and final extension at 72°C for 2 min. The amplified PCR products were purified using the Monarch DNA Gel Extraction Kit (New England Biolabs), digested with *NdeI* and *EcoRI* and cloned into the pET 21(+a) vector (Novagen, Merck KGaA, Darmstadt, Germany) resulting in the plasmid pET21GAD. *E. coli* NEB5 α was used as a host for obtaining the plasmids in sufficient amounts. The sequence of the insert was confirmed by DNA sequencing performed by a commercial provider (Microsynth, Vienna, Austria). The alignment tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for the alignment of the nucleotide sequence of the *gad* gene from *L. plantarum* FNCC 260 with the available *gad* sequences from LAB. The comparison of glutamate decarboxylases (GAD) from different LAB species was carried out using the program Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) [31,33].

2.6. Overexpression of GADLbFNCC260 in *E. coli* and protein purification

The expression vector of pET21GAD harboring the *gad* gene from *L. plantarum* FNCC 260 was transformed into *E. coli* T7 Express GRO carrying the plasmid pGRO7, which encodes the chaperones GroEL and GroES (Takara, Shiga, Japan). Subsequently, *E. coli* T7 Express GRO carrying the plasmid pET21GAD was cultivated in LB broth medium supplemented with 100 μ g/mL ampicillin, 20 μ g/mL chloramphenicol, and 1 mg/mL arabinose until OD_{600nm} of 0.6 was reached. Thereafter, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM for induction. The culture was further incubated at 18°C for 20 h with shaking at 180 rpm. The cells were harvested,

washed twice with 50 mM sodium phosphate buffer (pH 6.5) and resuspended in buffer A (50 mM NaH_2PO_4 , 300 mM NaCl, and 10 mM imidazole, pH 7.0). The resuspended cells were disrupted using a French press (Aminco, Silver Spring, MD, USA) and centrifuged at $15000 \times g$ for 20 min at 4°C . The cell-free extracts were collected and loaded to a prepacked 1 ml HisTrap HP Ni-immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with buffer A (50 mM NaH_2PO_4 , 300 mM NaCl, and 10 mM imidazole, pH 7.0). The His-tagged protein was eluted at a rate of 1 mL/min with a 15 mL linear gradient from 0 to 100% buffer B (20 mM NaH_2PO_4 , 500 mM imidazole, 500 mM NaCl, pH 6.5). Active fractions were pooled, desalted and concentrated by ultrafiltration using an Amicon Ultra centrifugal filter unit with a 30 kDa cut-off membrane (Millipore, MA, USA). The purified enzyme was stored in 50 mM citrate-phosphate buffer (pH 5.0) for further characterization and enzymatic conversion. The molecular masses of purified GAD was determined by SDS-PAGE and Native PAGE. Protein bands were visualized by staining with Bio-safe Coomassie (Bio-Rad). The determination of protein mass was carried out using Unstained Precision plus Protein Standard (Bio-Rad).

The size of the protein was also confirmed by LC-ESI-MS analysis. The proteins were S-alkylated with iodoacetamide and digested with Trypsin (Promega). The digested proteins were directly injected to LC-ESI-MS (LC: Dionex Ultimate 3000 LC). A gradient from 10 to 80% acetonitrile in 0.05% trifluoroacetic acid (using a Thermo ProSwiftTM RP-4H column (0.2×250 mm)) at a flow rate of 8 $\mu\text{L}/\text{min}$ was applied (30 min gradient time). Detection was performed with a Q-TOF instrument (Bruker maxis 4G) equipped with standard ESI source in positive ion, MS mode (range: 400-3000 Da). Instrument calibration was performed using ESI calibration mixture (Agilent). Data were processed using Data analysis 4.0 (Bruker) and the spectrum was deconvoluted by MaxEnt.

2.7 Enzymatic synthesis of GABA

Batch conversion reactions were carried out in 2 ml scale with 0.64 U/mL purified GAD using 200 mM MSG in 50 mM citrate-phosphate buffer (pH 4.5) containing 0.2 mM of PLP as cofactor. Decarboxylation reactions were performed at 30°C with 300 rpm agitation using a Thermomixer (Eppendorf, Hamburg, Germany). Samples were withdrawn at time intervals and the enzyme GAD was inactivated at 100°C for 5 min. The samples were stored at -20°C for subsequent analysis. GABA content in the reaction mixtures was determined using the GABase assay and confirmed with UPLC analysis as described in 2.3.

3. Results and Discussion

3.1. Screening and Identification of GABA-producing LAB

Thirty isolates of *Lactobacillus* spp. from Indonesian fermented foods were screened for the formation of GABA in the culture medium using the TLC method and only two isolates showed clear spots on TLC plate (Figure 1), which have similar R_f value (0,78) as GABA standard. These two isolates were FNCC 260 and FNCC 343 isolated from fermented cassava and fermented fish, respectively. The two strains FNCC 260 and FNCC 343 were cultivated in MRS broth containing 118 mM MSG for 48 h to determine the GABA production in the culture medium, which was analysed to be 352 mg and 328 mg of GABA per liter of culture medium, respectively. Based on morphological observation, these two strains were Gram positive, rod-shape, microaerophilic and catalase negative.

Subsequently, these two GABA-producing LAB were identified using 16S rDNA and MALDI-TOF MS (Bruker Biotyper). Proteomic identification by MALDI-TOF MS was performed since this technique is very effective in identifying species and subspecies of LAB. A number of species and subspecies of LAB have been successfully identified using proteomic-based identification technique [39–42]. Based on MALDI-TOF MS identification, both strains FNCC 260 (2,15 log score) and FNCC 343 (2,12 log score) were identified to be *L. plantarum*, which matches with the strain in a reference database. The log scores also indicate accuracy and reliability of MALDI-TOF MS identification. A

log score between 2.00 and 2.30 indicates accurate identification to the genus and species level [43,44]. Subsequently, GABA-producing LAB were subjected to 16S rDNA sequencing.

Based on 16S rDNA sequencing, both strains belong to the species *L. plantarum*, with 99.81% sequence identity with *L. plantarum* strain CIP 103151 (accession number, NR_104573.1) for the strain FNCC 260, and also 99.81 % sequence identity with *L. plantarum* strain NBRC 15891 (accession number, NR_113338.1) for the strain FNCC 343, respectively. The results confirmed that both strains FNCC 260 and FNCC 343 are indeed *L. plantarum*.

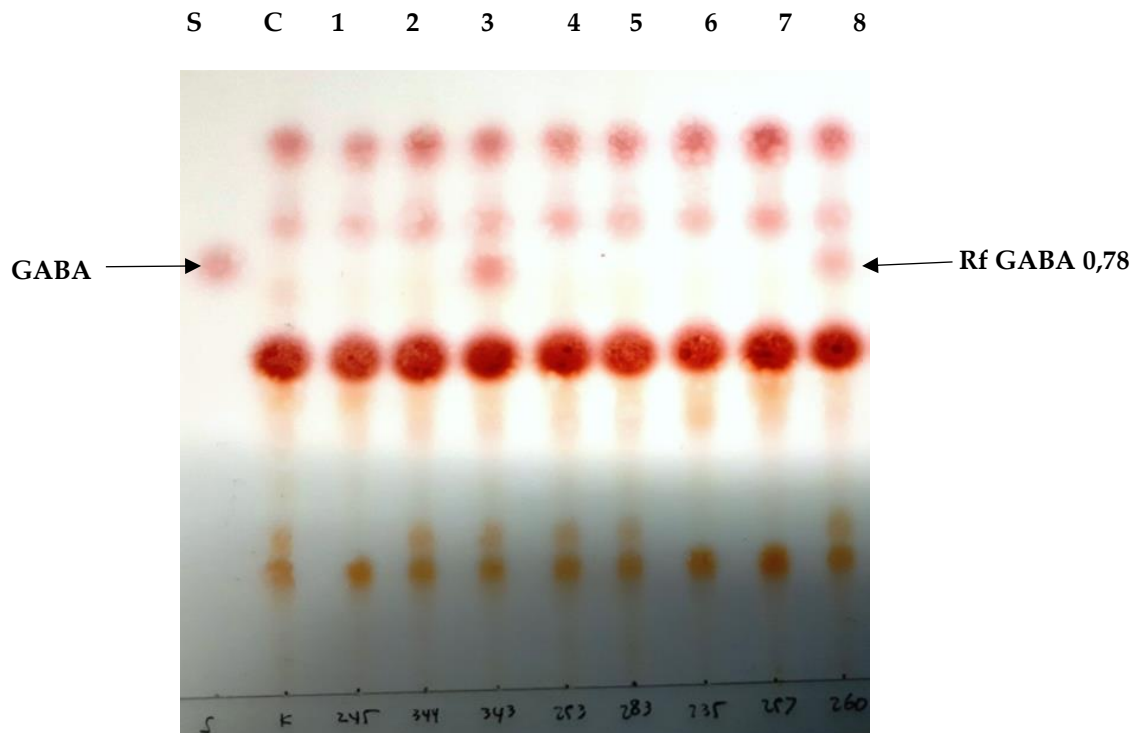


Figure 1. Thin layer chromatography (TLC) screening of GABA-producing LAB. Lane S: GABA standard, Lane C: MRS with 118 mM MSG, Lane 1-8: the strains FNCC 245, FNCC 344, FNCC 343, FNCC 253, FNCC 283, FNCC 235, FNCC 257, FNCC 260, respectively. All strains were cultivated in MRS broth supplemented with 118 mM MSG and incubated at 37°C for 48 h.

3.2. Time-course of GABA Production by *L. plantarum* FNCC 260

L. plantarum FNCC 260 was cultivated in MRS medium supplemented with 118 mM MSG at 37°C. the time courses of GABA production, the pH value and the growth profile of *L. plantarum* FNCC 260 are shown in Figure 2. GABA production started when cell growth reached the stationary phase after 12 h of cultivation. Slightly higher GABA production was obtained after 48 h (450 mg/L) compared to the GABA production mentioned above in the screening experiment (352 mg/L). It was due two different MSG used in these two experiments (see Materials and Methods). The highest GABA production was 809.2 mg/L of cultivation medium after 60 h of cultivation, at which cell growth is still in the stationary phase. This observation is in agreement with the previous reports in the literature. Maximum GABA production of *L. brevis* L-32 was observed between 36 to 72 h of cultivation and GABA was mainly produced during the stationary growth phase [45-47]. However, GABA production decreased when the cultivation time was prolonged further. This might be due to the activity of the enzyme GABA transaminase (GABA-T), which degrades GABA. This enzyme catalyzes the degradation of GABA to succinic semialdehyde using either pyruvate or α -ketoglutarate as the amino acceptors and succinic semialdehyde is irreversibly oxidized to succinate by succinic semialdehyde dehydrogenase [45,48,49]. Interestingly, we observed that the cell growth did not show a decreasing trend when cultivation time was extended up to 108 h since GABA is utilized as a nutrient during prolonged cultivation [48]. Ko et al. (2013) reported a similar observation of cell growth of *L. brevis* FPA 3709 during GABA synthesis when GABA production decreased [48].

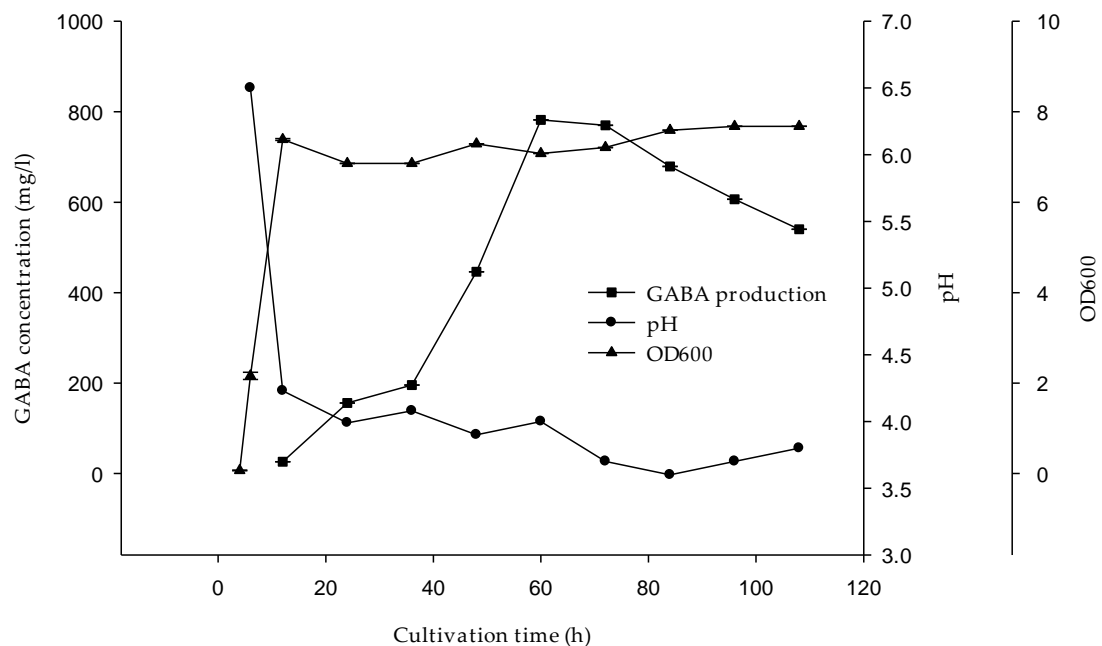


Figure 2. Time course of GABA production, bacterial growth and change in pH during cultivation of *L. plantarum* FNCC 260 strain in MRS broth supplemented with 118 mM MSG at 37°C. Data shown as mean \pm SD with SD less than 5%. The experiments were conducted at least as duplicates.

During GABA production, a decrease in pH of MRS medium was observed. The pH of the cultivation medium rapidly decreased from the initial pH 6.5 to pH 4.1 after 12 h of cultivation. The decrease in pH was due to lactic acid and acetic acid formation during the cultivation of the organism [49]. GABA production involves cytoplasmic decarboxylation, which results in extracellular proton consumption after the uptake of glutamate by its specific transporter [28]. This may lead to the removal of hydrogen ions and an increase of pH in the cytoplasm [50]. Apparently, we observed that the decrease in pH of the cultivation medium (to below pH 4.0) correlated with the increase in GABA production during cultivation of *L. plantarum* FNCC 260. Similarly, maximum GABA production of *L. buchnerii* was achieved when the pH of the cultivation medium dropped to pH 5.0 [24]. In this study, although GABA production started to decrease after 60 h of cultivation, which might be due to the activity of the enzyme GABA transaminase, the pH of the cultivation medium kept decreasing until 84 h of cultivation indicating that decarboxylation of glutamate still occurred.

3.3. The Effect of Cofactors on GABA Production and Cell Growth

Glutamate decarboxylase is a pyridoxal 5'-phosphate (PLP) dependent enzyme. Theoretically, the addition of PLP to the medium could increase GAD activity and GABA production [2, 21, 51, 52]. PLP and pyridoxine were added into the medium at various concentrations. As expected, the addition of 0.2 mM and 0.6 mM PLP led to the increase in GABA production, reaching 903.0 mg/L and 945.3 mg/L after 108 h of cultivation, respectively (Figure 3a). Furthermore, GABA was still produced when cultivation time was prolonged to 108 h. In contrast, GABA production in a medium without PLP rapidly decreased after 72 h. The addition of PLP to the medium did not inhibit cell growth during the cultivations (Figure 3b). Previous studies by Komatsuzaki et al.[2] and Yang et al.[52] reported that the addition of 0.1 mM and 0.02 mM PLP significantly enhanced GABA production and GAD activity in the culture media of *L. paracasei* NFRI 7451 and *S. thermophilus* Y2, respectively.

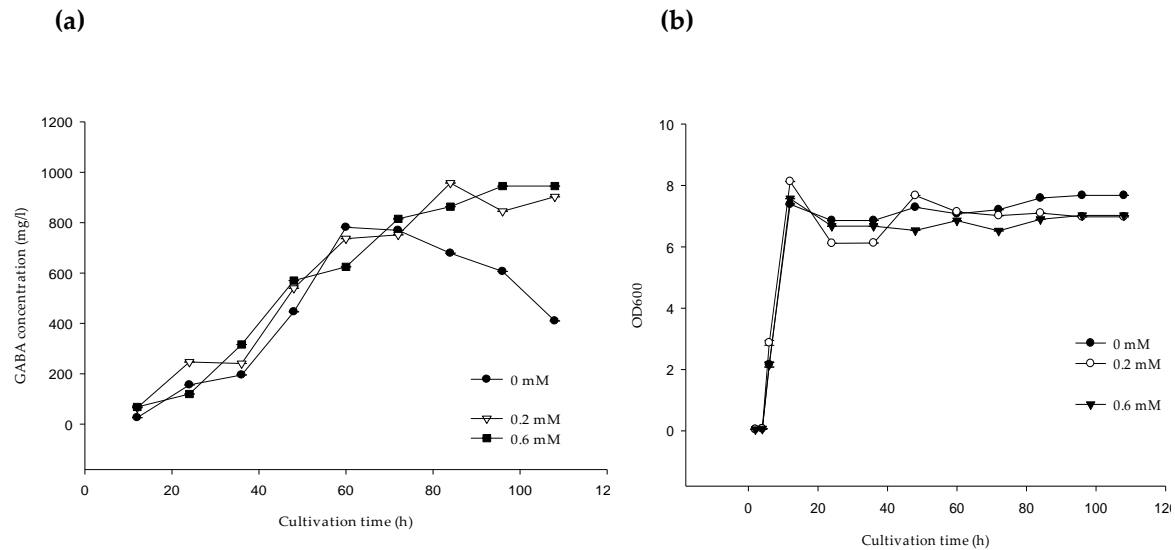


Figure 3. Effect of pyridoxal 5'-phosphate (PLP) on (a) GABA production and (b) cell growth during the cultivation of *L. plantarum* FNCC 260. MRS medium were supplemented with 118 mM MSG and PLP at various concentrations (0, 0.2 mM, 0.6 mM). The strain was incubated at 37°C for 108 h. Data expressed as means \pm SD with SD less than 5%. The experiments were conducted at least in duplicate.

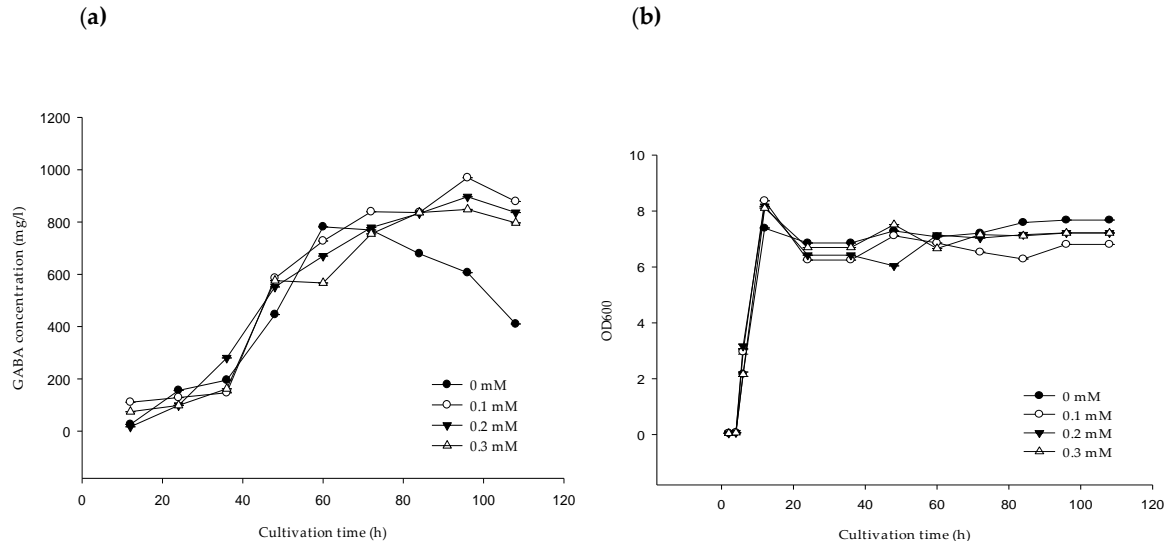


Figure 4. Effect of pyridoxine on (a) GABA production and (b) cell growth during the cultivation of *L. plantarum* FNCC 260. The medium was supplemented with 118 mM MSG and various concentrations of pyridoxine 0, 0.1mM, 0.2 mM, 0.3 mM. The strain was incubated at 37°C for 108 h. Data expressed as mean \pm SD with SD less than 5%. The experiments were conducted at least in duplicate.

Pyridoxine (vitamin B6) is a water-soluble vitamin that is widely found in nature. Pyridoxine can be taken up by the cells at the plasma membrane and is subsequently phosphorylated to form PLP within the cytoplasm [51]. The utilization of pyridoxine could be an alternative to replace PLP since PLP is significantly more expensive with low availability. Therefore, we hypothesized that the addition of pyridoxine could improve GABA production. It is shown that the addition of 0.1 mM pyridoxine had a better enhancement on GABA production compared to higher concentrations of

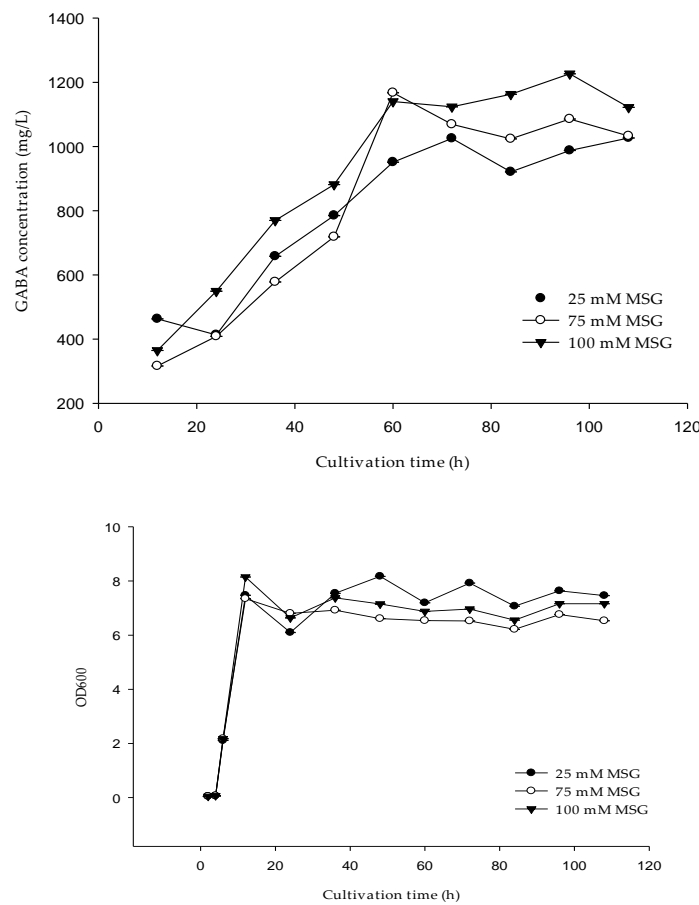
pyridoxine tested. The addition of 0.1 mM pyridoxine enhanced GABA production reaching 727.5 mg/L and 969.5 mg/L after 60 h and 96 h of cultivation, respectively (Figure 4a), which are significantly higher compared to the cultivation without pyridoxine. This observation suggests that pyridoxine can be taken up by the cells and is phosphorylated to form PLP, which is essential for GAD activity. However, GABA production decreased to 878.8 mg/L after 108 h of cultivation (Figure 4a). This could be due to degradation of pyridoxine during cultivation and hence it lost its activity as cofactor [52]. The addition of pyridoxine did not have notable effects on cell growth during the cultivation of *L. plantarum* FNCC 260 (Figure 4b). In both cases of PLP and pyridoxine additions, we observed that the growth reached $OD_{600} \sim 8$ within 12 h and then maintained at $OD \sim 6-7$ during the entire cultivation time up to 108 h. Li et al. reported that the addition of cofactor did not improve or inhibit the cell growth of *L. brevis* NCL912 [28].

3.4. The Effect of MSG on GABA Production

The presence of MSG is a key factor in producing GABA. The optimal culture conditions for GABA production were determined by measuring the GABA content in the cultivation medium of *L. plantarum* FNCC 260 with different initial MSG concentrations in the MRS medium. As shown in Figure 5a, increasing MSG concentrations increased GABA production and maintained cell viability. The maximum GABA production was achieved at 1226 mg/L at 96 h in an MRS medium containing 100 mM MSG. It appears that prolonged incubation time did not increase GABA productivity of the strain. A possible reason led to such circumstances was due to GABA catabolism, resulting from GABA transaminase activity. The activity of GABA transaminase could decrease GABA production by converting GABA to succinic semi-aldehyde (SSA).

Similarly, high concentrations of MSG resulted in decreased GABA production of the strains *L. brevis* CRL 1942, *S. thermophilus* Y2 and *L. paracasei* NFRI 7415 [2,53,54]. It was suggested that high glutamate concentrations become more toxic to some strains of LAB and suppressed the expression of *gadB* genes [55]. In this study, we observed that MSG concentration up to 100 mM did not have effects on bacterial growth (Figure 5b). Indicating that GABA is consumed by the cells to maintained its viability during the cultivation period. However, as it was shown in Figure 2, when the initial concentration of MSG in cultivation medium was 118 mM, the production of GABA was significantly lower compared to the production obtained with 100 mM MSG. It suggests that the observations from previous studies [2,28,52] about the negative effects of high glutamate concentrations on bacterial growth of some LAB strains and the expression of *gadB* genes could be an explanation for a similar observation in our study.

(a)



(b)

Figure 5. Effect of various MSG concentrations on (a) GABA production and (b) the growth of *L. plantarum* FNCC 260 cultivated in MRS medium at 37°C. Data expressed as mean \pm SD with SD less than 5%. The experiments were conducted at least in duplicate.

3.5. Cloning, Expression of Glutamate Decarboxylase from *L. plantarum* FNCC 260 in *E. coli* and purification of the enzyme

The *gadB* gene from *L. plantarum* FNCC 260 was cloned and its complete open reading frame consists of 1410 bp, encoding 469 amino acids. The predicted molecular mass of GAD is 53.57 kDa and the theoretical isoelectric point (pI) is 5.62 as calculated using ExPASy program (www.expasy.org). The GadB sequence from *L. plantarum* FNCC 260 shared 98% homology with the GadB from *L. futsaii* CS3 (accession number AB839950), *L. plantarum* Taj-Apis362 (accession number AHG59384) and *L. plantarum* WCFS1 (accession number CCC80401.1).

The *gadB* gene was cloned into the expression vector pET-21a(+) (Novagen, Merck KGaA, Darmstadt, Germany). The resulting vector pET21Gad was subsequently transformed into *E. coli* T7 Express carrying the plasmid pGRO 7 for the enhanced expression of the chaperones GroEL/GroES (*E. coli* T7 Express GRO). *E. coli* cells were cultivated in LB medium and induced with 0.5 mM IPTG as described in Materials and Methods. The obtained expression yield was 1.38 kU/L fermentation medium with a specific activity 0.24 U/mg. The recombinant GAD was purified with a single-step purification using the His-trap HP column and the specific activity of the purified enzyme was 1.12 U/mg with a purification factor of 4.5. The apparent molecular mass as judged by SDS-PAGE and native PAGE was estimated to be ~51 kDa and ~140 kDa, respectively (Figure 6a and b). The size of the protein was also confirmed by LC-ESI-MS and it was determined to be 51.79 kDa (data not shown). Several bands were found in native PAGE with the largest band was ~140 kDa. The LC-ESI-MS analysis revealed that these bands on native PAGE contain components of the subunit. The first

band represent the intact dimeric enzyme and the other bands with lower molecular masses result from degradation of the intact protein. It also suggested that GadB from *L. plantarum* FNCC 260 is a homodimeric enzyme. GadB from *L. plantarum* ATCC 14917 was also reported as a homodimer [55].

The deduced amino acid sequence of *L. plantarum* FNCC 260 GAD contains a highly conserved catalytic domain that belongs to the PLP-dependent decarboxylase superfamily (Figure 7). A lysine residue (K280) is known as the PLP-binding site for most bacterial GADs [50,55]. Lysine residue is also found in plant GADs since high homology between bacterial and plant GADs has been revealed [56,57]. In addition, the two residues T215 and D247 are crucial to promote decarboxylation [50]. Furthermore, the consensus sequence HVDAASGG is highly conserved in many bacterial GADs (Figure 7), and is also found in several GADs from *Lactobacillus* spp including *L. futsaii* CS3, *L. brevis* HYE1, *L. zymae*, and *L. paracasei* NFRI 7415 [19,34,36,50].

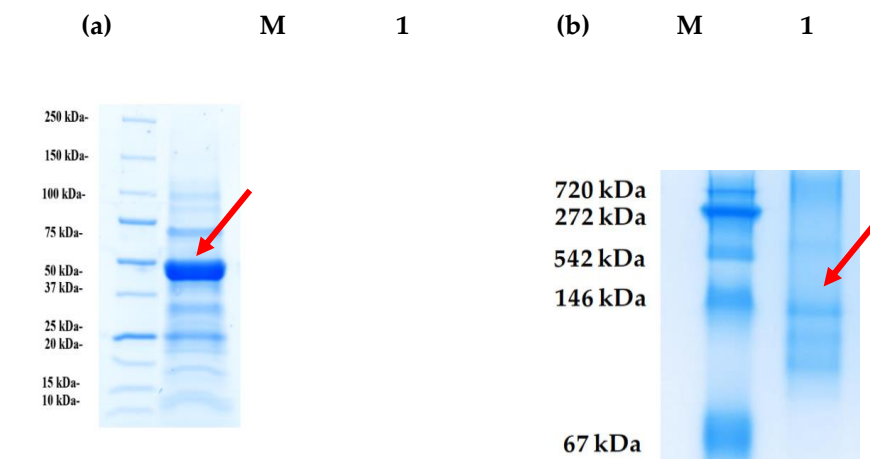


Figure 6. (a) SDS-PAGE analysis of purified recombinant GAD from *L. plantarum* FNCC 260 expressed in *E. coli*. Lane M; protein marker, 1; purified GAD. The arrow indicated GAD with molecular masses of approximately 53 kDa. **(b)** Native PAGE analysis of purified recombinant GAD. The molecular masses of GAD was estimated to be 140 kDa, Lane M; protein marker, 1; purified GAD.

Ldelbrueckii	1	MAMLYGKHNHEAEEYLEPVGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL
Lclactis	1	MAMLYGKHNHEAEEYLEPVGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL
St.thermophilus	1	MAMLYGKHNHEAEEYLEPVGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL
Lp260	1	MAMLYGKHNHEAEEYLEPVGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL
Lfutsaii	1	MAMLYGKHNHEAEEYLEPVGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL
Lherbarum	1	--MLYGKHDHETEEYLTPTFGNPAEQHDLPKYRLAQHATAPREADRLVRDELLDEGNSRL
Lmudanjiangensi	1	MAMLYGKHDHETNKFTPTFGDPAEQHDLPKYRLAQHATAPREADRLVRDELLDEGNSRL
consensus	1	maMLYGKHnHEaeeyLePvFGaPsEQHDLPKYRLpKsLsPREADRLVRDELLDEGNSRL

Ldelbrueckii	61	NLATFCQTYMEPEAVELMKDTRANKKPIDKSEYPRTAEIEKRGVNNIANLWHAPDDEHFT
Lclactis	61	NLATFCQTYMEPEAVELMKDTLA-KKTIIDKSEYPRTAEIENRGVNI IANLGHPPDDEHFT
St.thermophilus	61	NLATFCQTYMEPEAVELMKDTLA-KKPIDKSEYPTAEIENRGVNI IANLWHPPDDEHFT
Lp260	61	NLATFCQTYMEPEAVELMKDTLA-KNAIDKSEYPRTAEIENRCVNI IANLWHAPDDEHFT
Lfutsaii	61	NLATFCQTYMEPEAVELMKDTLA-KNAIDKSEYPRTAEIENRCVNI IANLWHAPDDEHFT
Lherbarum	59	NLATFCQTYMEPEAVELMKDTLA-KNAIDKSEYPRTAEIENRCVNI IANLWHAPENEKFT
Lmudanjiangensi	61	NLATFCQTYMEPEAVELMKDTLA-KNAIDKSEYPRTAEIENRCVNI IANLWHAPSEKFT
consensus	61	NLATFCQTYMEPEaveLMKDTLAKnaIDKSEYPrTAEIEnRcvNi IANLwHaPddEhfT

Ldelbrueckii	121	GTSTIGSSEACMFSGLAKKFAGRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVD
Lclactis	120	GTSTIGSSEACMLGGLAMKFAWRKRAQAAGLNLNAHRPNLVISAGYQVCWEKFCVYWDVD
St.thermophilus	120	GTSTIGSSEACMLGGLAMKFAWRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVD
Lp260	120	GTSTIGSSEACMLGGLAMKFAWRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVD
Lfutsaii	120	GTSTIGSSEACMLGGLAMKFAWRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVD
Lherbarum	118	GTSTIGSSEACMLGGLAMKFAWRKRATKAGLDLNAORPNLVISAGYQVCWEKFCVYWDID
Lmudanjiangensi	120	GTSTIGSSEACMLGGLAMKFAWRKRACKAGLDLNAORPNLVISAGYQVCWEKFCVYWDID
consensus	121	GTSTIGSSEACmlgGLAmKFawRKRAqaAGLdLNahRPNLVISAGYQVCWEKFCVYWDvD

Ldelbrueckii	181	MHVVPMDQHMALDVNHVLDYVDEYTGIVGIMGITYTGQDDDLAALDKVVTHYNHQHHPK
Lclactis	180	MHVVPMDQHMALDVNHVLDYVDEYTGIVGIMGITYTGQDDDLAALDKVVTHYNHQHHPK
St.thermophilus	180	MHVVPMDQHMALDVNHVLDYVDEYTGIVGIMGITYTGQDDDLAALDKVVTHYNHQHHPK
Lp260	180	MHVVPMDQHMALDVNHVLDYVDEYTGIVGIMGITYTGQYDDLALDKVVTHYNHQHHPK
Lfutsaii	180	MHVVPMDQHMALDVNHVLDYVDEYTGIVGIMDITYTGQYDDLALDKVVTHYNHQHHPK
Lherbarum	178	MHVVPMDHHMSLDVHDVLDYVDYTGITIGILGITYTGQYDNLKLDTVVTKYNQHHSK
Lmudanjiangensi	180	MHVVPMDHQMALDTHVLDYVDYTGITIGIMGITYTGQYDDLSTLDAIVDKYNHHHTE
consensus	181	MHVVPMDEqhMaLDvnHVLdYVDeYTIGIvGImGITYTGQyDdLaaLDkvVthYnHqHpk

Ldelbrueckii	241	LPVYIHVDAASGGFYTPFIEPQLIWDFRLANVSINASGHKYGLVYPGVGVVWVRQRQFL
Lclactis	240	LPVYIHVDAASGGFYTPFIEPQLIWDFRLANVSINASGHKYGLVYPGVGVVWVRQRQFL
St.thermophilus	240	LPVYIHVDAASGGFYTPFIEPQLIWDFRLANVSINASGHKYGLVYPGVGVVWVRQRQFL
Lp260	240	LPVYIHVDAASGGFYTPFIEPQLIWDFRLANVSINASGHKYGLVYPGVGVVWVRQRQFL
Lfutsaii	240	LPVYIHVDAASGGFYTPFIEPQLIWDFRLANVSINASGHKYGLVYPGVGVVWVRQRQFL
Lherbarum	238	LPVYIHVDAASGGFYTPFIEPQLIWDFRLKNVSINASGHKYGLVYPGVGVVWVRQRQFL
Lmudanjiangensi	240	LPVYIHVDAASGGFYAPFVDSQLIWDFRLKNVSINASGHKYGLVYPGVGVVWVRQRQFL
consensus	241	LPVYIHVDAASGGFYTPFIEpQLiWDFrLaNVsINASGHKYGLVYPGVGVvvWRDrqfL

Ldelbrueckii	301	PELVFKVSYLG-GELPTMAINFPHSAAQPIEKYYNFIPLGMDGYRENQTKNHKVARYLA
Lclactis	300	PELVFKVSYLG-GELPTMAINFSSHAAQLIEQYYNFIRFGMDGYREIQTKTHEVARYLA
St.thermophilus	300	PELVFKVSYLRWGNCRPTMAINFSSHAAQLIGQYYNFIRFGMDGYREIQTKTHDVARYLA
Lp260	299	PELVFKVSYLG-GELPTMAINFSSHAAQLIGQYYNFIRFGMDGYREIQTKTHDVARYLA
Lfutsaii	300	PELVFKVSYLG-GELPTMAINFSSHAAQLIGQYYNFIRFGMDGYREIQTKTHDVARYLA
Lherbarum	298	PELVFKVSYLG-GELPTMAINFSSHAAQLIGQYYNFIRFGMDGYKEIQTKTHNVARYLA
Lmudanjiangensi	300	PELVFKVSYLG-GELPTMAINFSSHAAQLIGQYYNFIRFGMDGYREIQTKTHNVARYLA
consensus	301	PpeLVFKVSYLgGelpTMAINFsHSAaQlIgqYYNFirfGmDGYrEiQTKtHeVArYLA

Ldelbrueckii	360	PELDKVGEFKMIKNGHQLPLICYQLTPREDREGTQNDLSDRLLMNGWQVPTYPLPANLEQ
Llactis	359	PAEKVGEYKMINNGHQLPLICYQKTPRENREWTPTYDLSRLIMNGWKVPTYPLPANLEQ
St.thermophilus	360	AALDKVGEFKMINNGHQLPLICYQLAPREDREWTLTYDLSDRLLMNGWQVPTYPLPANLEQ
Lp260	358	AALDKVGEFKMINNGHQLPLICYQLAPREDREWTLTYDLSDRLLMNGWQVPTYPLPANLEQ
Lfutsaii	359	AALDKVGEFKMINNGHQLPLICYQLAPREDREWTLTYDLSDRLLMNGWQVPTYPLPANLEQ
Lherbarum	357	TALKQLGEFTIINDGHQLPLICYQLTPREDREWTLTYDLSDRLLMSGWQVPYPLPANLEQ
Lmudanjiangensi	359	KATEQLGEFSLINNGQLPLICYQLVPRSDREWTLTYDLSDRLLMNGWQVPTYPLPANLEQ
consensus	361	aldkvGEfkmInnGhQLPLICYQLPRedREwTlydLSdRLlMnGWqVPtYPLPANLeq
Ldelbrueckii	420	QVIQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH-
Llactis	419	QVIQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH-
St.thermophilus	420	QVIQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH-
Lp260	418	QVIQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYLFTHC
Lfutsaii	419	QVIQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH-
Lherbarum	417	QVIQRIVVRADFGMNMAHDFMDDLTTAVNDLNHAHIVYHAEAPKKYGFTH-
Lmudanjiangensi	419	QVIQRIVVRADFGMNMAHDFMDDLTAATKALNCAHIVYHAEAPKKYGFTH-
consensus	421	QVIQRIVVRADFGMNMAHDFmDDLTKAvhdLNhAHIVYHhDaaPKKYgFTH

Figure 7. Alignment of amino acid sequences of GAD from *L. plantarum* FNCC 260 and six other GADs from LAB. The consensus sequence HVDAASGGF indicated by a smaller red box is highly conserved in GAD sequences. The sequence SINASGHKYGLVYPGVGVVWR in the bigger red box is the PLP-binding domain [49]. GadB sequences shown are from *L. delbrueckii* (Ldelbrueckii), *Lactococcus lactis* (Llactis), *S. thermophilus* (St. thermophilus), *L. plantarum* FNCC 260 (Lp260), *L. futsaii* (Lfutsaii), *L. herbarum* (Lherbarum), and *L. mudanjiangensi* (Lmudanjiangensi).

3.6 GABA Synthesis by Recombinant Glutamate Decarboxylase from *L. plantarum* FNCC 260.

For enzymatic GABA synthesis, we performed the conversion of MSG using 0.64 U/mL purified recombinant GAD in a 2-mL scale of reaction mixtures. Most GADs from *Lactobacillus* spp. have optimum activities at acidic pH values [33,36,56,57], and the recombinant GAD from *L. plantarum* FNCC 260 showed an optimum pH at pH 4.5 as expected (data not shown). MSG (100 mM) in 50 mM citrate-phosphate buffer (pH 4.5) containing 0.2 mM PLP was used as substrate and the reaction was performed at 30°C. GABA synthesis reached its highest yield at 6450 mg/L (63 mM) within 6 h of reaction (Figure 8), and the enzyme GAD retained 73% of its initial activity after 6 h of reaction. It was clear that the use of purified GAD was more efficient in terms of both GABA production and conversion time. Enzymatic synthesis of GABA using purified recombinant GAD from *L. plantarum* FNCC 260 showed 5 to 7-fold higher product concentrations than microbial fermentations in a significantly shorter time. Furthermore, UPLC analysis was performed to confirm GABA production in both microbial fermentation and enzymatic conversion with a retention time of GABA at 8.5 min (Figure 9).

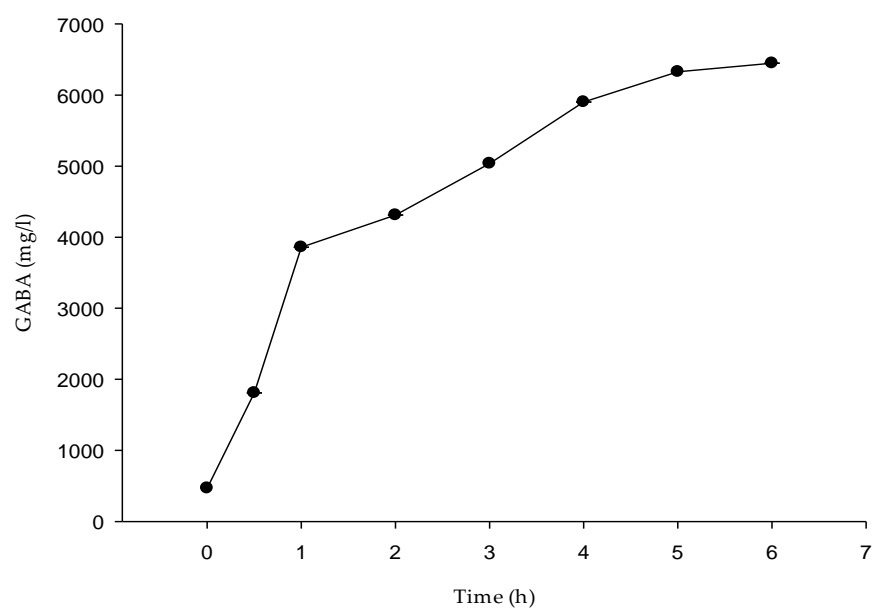
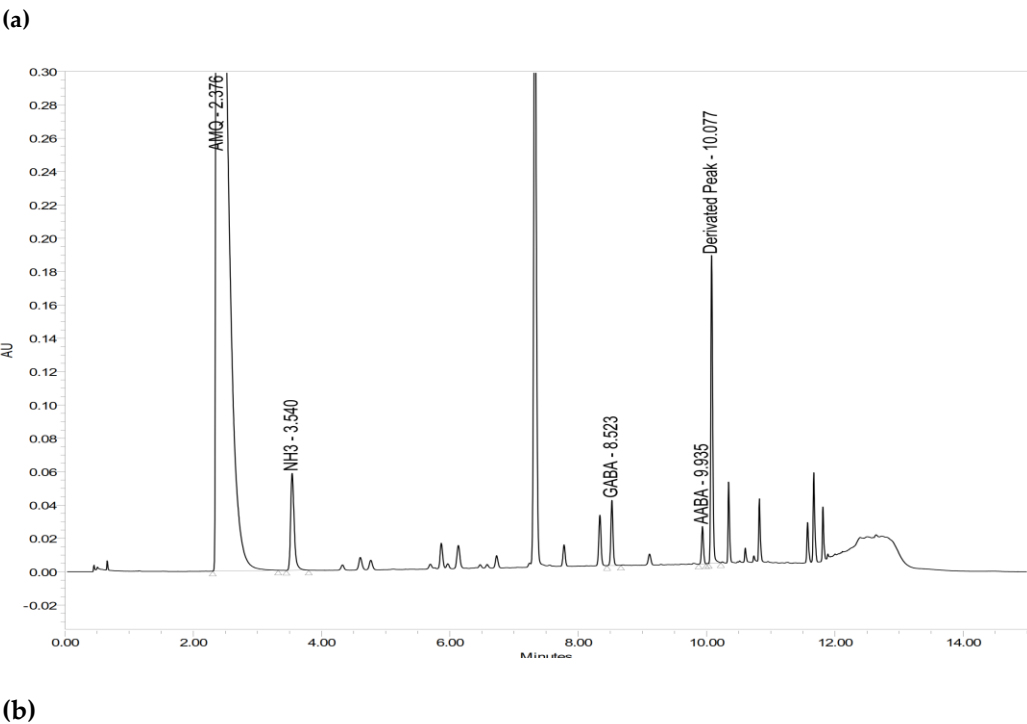


Figure 8. Enzymatic conversion of MSG to GABA using purified recombinant GAD from *L. plantarum* FNCC 260. The conversion was performed using 0.64 U/ml of GAD in 2 ml of 50 mM citrate-phosphate buffer (pH 4.5) containing 100 mM MSG and 0.2 mM PLP at 30°C. Data expressed as mean \pm SD with SD less than 5%. The experiments were conducted at least in duplicate.



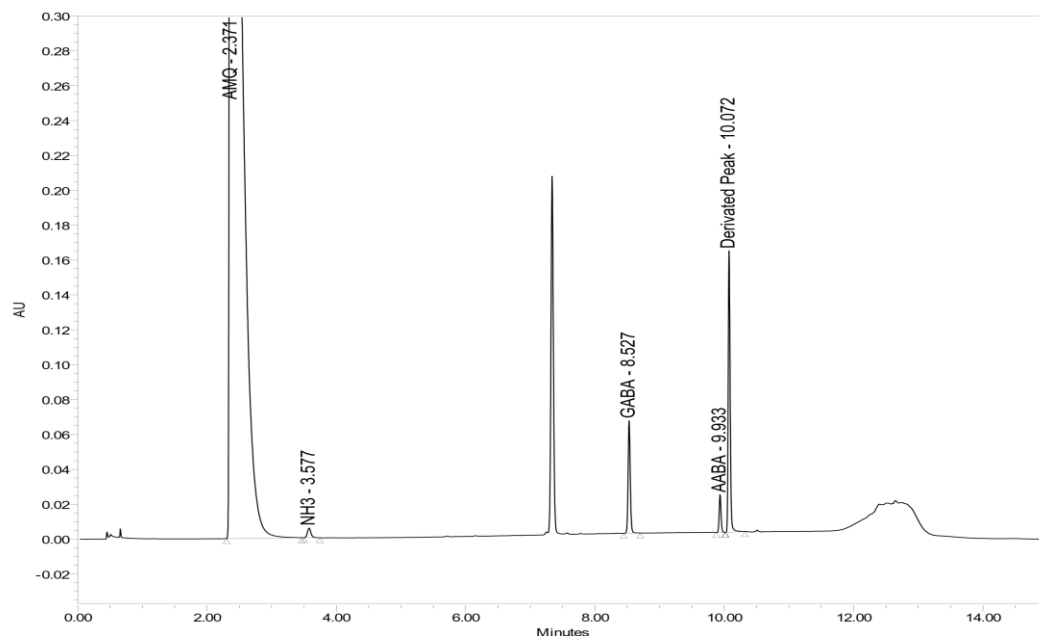


Figure 9. UPLC analysis of GABA in the culture medium of microbial fermentation (a) and in the reaction mixture of enzymatic conversion of MSG (b). The strain was cultivated in MRS broth containing 118 mM MSG and incubated at 37°C for 48 h. The culture broth was centrifuged and the supernatans were collected. Conversion reactions were performed with 0.64 U/ml purified GAD using 200 mM MSG in 50 mM citrate-phosphate buffer (pH 4.5) containing 0.2 mM of PLP. The reactions were carried out at 30°C with 300 rpm agitation. All samples (microbial fermentations and enzymatic conversion) were hydrolyzed and derivatized prior to UPLC analysis.

4. Conclusions

In the present study, we compared GABA synthesis between microbial fermentation of *L. plantarum* FNCC 260, which was isolated from Indonesian fermented cassava, and enzymatic conversion of glutamate using recombinant glutamate decarboxylase (GAD) from *L. plantarum* FNCC 260 expressed in *E. coli*. MSG, PLP and pyridoxine were shown to positively affect GABA production during the cultivations of *L. plantarum* FNCC 260. Enzymatic synthesis of GABA using purified recombinant GAD from *L. plantarum* FNCC 260 showed at least 5-fold higher GABA titres than microbial fermentations in a significantly shorter time. The newly isolated GABA-producing LAB is of great interest to extend the area of applications. *L. plantarum* FNCC 260 should be considered as a potential candidate for GABA production via both fermentation and enzymatic synthesis and can be also developed as functional starter culture.

Author Contributions: Conceptualization, I.B.A.Y, E.S.R, D.H and T.H.N; methodology, I.B.A.Y, S.K, T.H.N and D.H; software, I.B.A.Y.; validation, I.B.A.Y, S.K and T.H.N; formal analysis, I.B.A.Y and S.K ; investigation, I.B.A.Y, S.K and T.H.N.; resources, I.B.A.Y.; data curation, I.B.A.Y and S.K.; writing—original draft preparation, I.B.A.Y.; writing—review and editing, I.B.A.Y and T.H.N, D.H, K.D.; visualization, I.B.A.Y.; supervision, T.H.N, D.H, K.D and E.S.R.; project administration, I.B.A.Y.; funding acquisition, D.H and T.H.N All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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