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Characterization of Gamma Aminobutyric Acid-producing Lactic Acid Bacteria Isolated from Budu Fish in Padang Pariaman West Sumatra Indonesia and their Potentials as Probiotics

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Abstract

Background and Objective: Screening of lactic acid bacteria for the production of gamma-amino-butyric acid is important due to its pharmacological functions. In this study, isolation and characterization of lactic acid bacteria from Budu fish, an endemic fish to West Sumatra, Indonesia, have been investigated and their gamma amino butyric acid-producing potentials were identified

Material and Methods: The research method was as follows: lactic acid bacteria was isolated from Budu fish bought randomly from a traditional market in West Sumatra, Indonesia. Isolates were characterized morphologically and biochemically. Gram+ isolates were investigated for their capability to produce gamma-amino-butyric acid using thin-layer chromatography. Gamma-amino-butyric acid-positive strains were quantitatively analyzed using spectrometry method. Of the five samples, the highest gamma-amino-butyric acid production was reported in one sample of IB2C isolate. The selected isolates of lactic acid bacteria were molecularly identified using 16S rRNA gene sequencing. Effects of incubation time (20, 40 and 60 h) and monosodium glutamate concentrations (2, 4 and 6%) were investigated on gamma-amino-butyric acid-production strains.

Results and Conclusion: Three isolates were selected for the production of gamma-amino-butyric acid in de Mann Rogosa Sharpe broth containing 1% monosodium glutamate. Semiquantitative analysis via pre-staining paper chromatography was carried out to identify the highest gamma amino butyric acid-producing lactic acid bacteria. The highest gamma-amino-butyric acid level of 22.5 $mg.ml^{-1}$ was achieved using 60 h of incubation and 6% of monosodium glutamate concentration in de Mann Rogosa Sharpe broth. The IB2c isolates were Gram+, catalase-, homofermentative and able to utilize various carbon sources. It inhibited growth of *Escherichia coli* O_{157}, *Staphylococcus aureus* and *Salmonella enteritidis*. The IB2C was further characterized molecularly using 16S rRNA sequencing gene. Results were identified as *Lentilactobacillus parabuchneri* strain M1-40. A 97.69% similarity of lactic acid bacteria isolated from Budu fish with *Lentilactobacillus. parabuchneri* indicates probiotic use of the gamma-amino butyric acid-producing lactic acid bacteria.

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1. Introduction

Lactic acid bacteria (LAB) have widely been studied because of their commercial potentials as starters in fermented products, their abilities to preserve foods and their benefits in health sectors such as preserving stability of the intestinal microflora as well as immunostatic and antitumor abilities [1]. The LAB are microbes that can be isolated from fermented foods in West Sumatra, Indonesia. West Sumatra is an area that includes several fermented food products, one

of which is a fermented fish product called Budu. Fish fermentation is carried out traditionally by coastal communities such as in Padang Pariaman Regency. This Budu product includes distinctive taste and aroma produced during fermentation. It is not clear that which microbes play roles in producing the aroma and taste and no studies have reported what nutrients these microbes metabolize. Budu fish is a filtered fish product with the addition of salt with no definite standards, depending on the location of manufactures and storage times.

Budu is produced by the people of the coastal areas of West Sumatra, Indonesia. The WHO has recommended LAB as Generally Recognized as Safe as a type of microbes that are safe and non-pathogenic. The LAB strains in Budu fish are generally *Lactococcus, Streptococcus, Lactobacillus* and *Leuconostoc* [2]. The isolated LAB were only used as starter cultures, probiotics, antimicrobial agents, bacteriocin producers and enzyme producers, whereas LAB include another potential of amino acid production of γ-amino butyric acid (GABA) [3]. The LAB as facultative anaerobes have historically been used in food preservation and use carbohydrates as the major carbon source to produce lactic acid [4]. The LAB are essential for a variety of fermented foods and are often used as starter cultures in traditional and commercial food fermentation [5]. The LAB as the most essential GABA producers include the potential to produce high GABA contents due to high levels of glutamate decarboxylase (GAD) activity in cells [6]. The GABA is a non-protein amino acid resulting from the decarboxylation of glutamate catalyzed by the enzyme GAD [4]. It is widely reported that GABA is a bioactive substance with a variety of physiological uses [5]. Moreover, GABA is the major inhibitory neurotransmitter in central nervous system [6]. Additionally, GABA is an essential substance that affects behavior, cognition and the body's reaction to stress [7]. Inadequacy of GABA may lead to several diseases including Parkinson's, Schizophrenia, Alzheimer's, Huntington's and depression diseases [8]. Despite the fact that GABA is present in plants and animals, its concentration is typically modest. A study has shown that use of LAB-producing GABA isolated from Budu can decrease effects of stress on broilers in high-density cages [3].

Research on GABA produced by LAB have been carried out by several researchers worldwide. There have been numerous studies to synthesize GABA chemically or biologically due to high commercial demands. Since biological techniques involving microorganisms are more promising, fermentation is used to generate numerous GABA compounds [9]. The GABA-producing LAB is generally isolated from fermented products such as kimchi from Korea [10], cheese from Italy [11], fermented fish [12] and cow milk from Australia [13]. Although numerous studies have reported isolation and identification of GABA-producing LAB strains, advanced studies on the isolation and characterization of LAB are still needed. In food industries, when LAB are used as starters to produce GABA-containing fermented foods, LAB with various physiological characterization may include various acid and flavor profiles when they are used as starters in food industries. Thus, screening for novel strains of LAB-producing GABA is popular [14].

Several factors affect production of GABA by LAB such as nutritional factors in form of carbon and nitrogen, environmental factors such as temperature and pH of the media, fermentation time, use of inducers and use of the microbes. The GABA production can be carried out using solid media and liquid fermentation. However, GABA production using LAB is easy to carry out, compared to that GABA production using solid fermentation is [15]. In additional to the high production, the process in liquid fermentation is simpler, the period is relatively short and hence costs are lower, compared to solid fermentation. Therefore, isolation of GABA-producing LAB from Indonesian biodiversity, especially fish cooked native to West Sumatra, has never been reported or characterized and studied for the potential of probiotic LAB (GABA-producing and non-GABA-producing). It is important to investigate LAB species that can produce GABA using fish native to West Sumatra. Therefore, the first step is to correctly identify isolates within that species. Currently, molecular methods play important roles in assisting and classifying microbes. Technically, 16S rRNA gene is the most frequently used target for this regard [16]. Polymerase chain reaction (PCR) and phylogenetic analysis based on the 16S rRNA gene sequence have widely been used to successfully identify isolates from various processed foods [13]. Thin layer chromatography (TLC) was used to screen GABA synthesis in well-characterized strains and the quantity of GABA produced was assessed using spectrophotometer [17]. The successful discovery of LAB from budu fish in Padang Pariaman Regency, West Sumatra, with the ability of producing GABA can be an alternative environmental friendly method.

2. Materials and Methods

2.1. Research Equipments and Materials

Tools used in this study included laminar airflow (LabTech, USA), anaerobic jar, incubator (Infors HT Ecotron, USA), refrigerator, autoclave (ALP KT40S), hot plate (Sybron nouveau II), vortex (Labmart 3000, China), analytical balance (Kern ABT 320-4M, Germany), bunsen, Eppendorf tubes, hockey sticks, ose needles, glass slides, pipettes and micropipette tips (DragonLab, BIO-RAD, USA), light microscope (Irmeco, USA), UV Vis spectrophotometer, oven, centrifuge (Eppendorf Centrifuge 5417 R, Germany), TLC chamber and KK, capillary tubes,

96-well microplates, microplate reader (PR 4100, BIO-RAD, USA), pH meter, water bath shaker (IC DK-540B, Japan), microwave (Panasonic NN-GD5795, Japan), polymerase chain reaction (PCR) equipment (Techne TC-312, UK), spinner down (BIO-RAD, USA), electrophoresis equipment (BIO-RAD, USA), gel-doc imager (BIO-RAD, USA) and laboratory standard glassware.

Materials used in this study included 5 Budu fish samples from coastal areas in Padang Pariaman Regency, West Sumatra, Indonesia, de Mann Rogosa Sharpe (MRS) broth and agar (Merck, Germany), distilled water (DW), 70 and 96% ethanol, crystal violet, Lugol solution, safranin solution, hydrogen peroxide, pure (> 99%) monosodium glutamate (MSG), n-butanol, acetic acid, silica gel 60 F254 (Merck, Germany), chromatographic paper with a thickness of ±0.17–2.0 mm, ninhydrin, copper sulfate, ddH_2O, GoTaq Green Master Mix (USA), nuclease-free water, R and F primers, 1.5% agarose gel, Tris/Borate/EDTA (TBE) buffer, GelRed nucleic acid gel stain, loading dye, DNA ladder, 3 M Na-acetate pH 4.5, cold absolute ethanol, 70% ethanol, Tris-EDTA (TE) buffer pH 8, ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, brilliant green and iron(II) sulfate heptahydrate.

2.2. Budu Fish and samples

Budu fish originating from West Sumatra is not the name of a fish but is a form of fermented product produced in the coastal areas of Padang Pariaman, Agam and Pasaman Regencies. Budu fish is usually made from sea fish that are large with white flesh, including mackerel (*Scomberomorus guttatus*) and gutter fish (*Chorinemus tala*) [18]. (Figure 1).

A total of five commercial Budu fish products were collected from 3 sub-districts of the coasts in Padang Pariaman Regency, West Sumatra, Indonesia. The districts are called Sungai Limau District (IB 1 and IB 2 samples), Batang Gasan (IB 3 and IB 4 samples) and Batang Anai (IB 5 sample). Budu fish were then separated by size promptly and transferred to the Microbiology Laboratory, University of West Sumatra, Indonesia, for further analysis using the cold chain. The sampling sites are shown in Table 1.

2.3. Isolation and Purification of the Lactic Acid Bacteria from Five Budu Fish Products

Isolation of LAB was carried out using serial dilution-agar plates. The MRS broth (Merck, Germany) was used to dilute Budu fish samples before incubating them anaerobically at 37 °C for 24 h. Serial dilution was conducted up to 10^{-8} and spread on MRS agar (Merck, Germany) and then incubated anaerobically at 37 °C for 48 h. The convex spherical, shiny and yellowish-white colored single colonies that grew individually with various diameters were reinoculated on MRS agar to purify the isolates. Isolates were incubated at 80 °C in solution of glycerol and MRS broth after purification. Culture was recarried out in MRS broth for 18–24 h before using for further use [19].

2.4 Screening of Gamma Aminobutyric Acid-producing Lactic Acid Bacteria

The LAB isolates were selected based on the formation of clear zones on agar media with 2% $CaCO_3$. Each preferred isolate was grown in media containing MRS broth (Merck, Germany) with the addition of L-glutamate at a concentration of 50 mM. The MRS broth contained 10 g of casein peptone, 8 g of meat extract, 4 g of yeast extract, 20 g of D (+) glucose, 2 g of K_2HPO_4, 1 g of Tween 80, 2 g of di-ammonium hydrogen citrate, 5 g of sodium acetate, 0.2 g of $MgSO_4$ and 0.04 g of $MnSO_4$ per liter. Media were incubated with LAB isolate at 30 °C under anaerobic conditions. Fermentation results were centrifuged at 10,000 g for 20 min at 4 °C [20]

2.4.1 Qualitative Selection

The GABA-producing qualitative assay was qualitatively analyzed using thin-layer chromatography (TLC) based on the procedure of Rayavarapu et al. [21] with slight modifications. The LAB-Budu fish was grown in MRS broth containing 1% of MSG anaerobically at 37 °C for 72 h. The LAB culture was centrifuged at 10,700 g for 10 min at 4 °C. Four solutions of supernatant of LAB culture, standard solution of GABA, MSG and a mixture of MRS broth and 1% MSG were respectively spotted on TLC plates. Separation process was carried out in the mobile phase of n-butanol:acetic acid:equates (5:3:2) using 1.2% ninhydrin. Spots on the chromatogram could be seen after heating at 70 °C for 5 min.

2.4.2 Quantitative Selective

Based on the qualitative analysis of LAB-Budu fish showing GABA spots, semi-quantitative analysis was carried out using KK prestaining method based on a method by Silberbauer et al. [22] with some modifications. In MRS broth media with various concentrations of MSG (2, 4 and 6%) and incubation times (20, 40 and 60 h) at 37 °C anaerobically in Kultul LAB-Budu fish. Then, 2 µl of the LAB culture supernatant were poured on the chromatography paper and separation process was carried out in the mobile phase of n-butanol:acetic acid:aqueous (5:3:2) containing 1.2% ninhydrin for 3 h. Then, chromatographic paper was dried at 70 °C for 80 min for spot color depiction.

The GABA spots formed on the paper were cut and extracted with 5 ml of 75% ethanol (v v^{-1}):0.4% copper sulfate (w v^{-1}) (38:2, v v^{-1}) at 40 °C for 1 h at 50 rpm. Furthermore, measurements were carried out at 512 nm using spectrophotometer. Absorbance measurement was carried out before sample processing using KK pre-staining method to measure absorbance of the GABA standard solution as described previously.

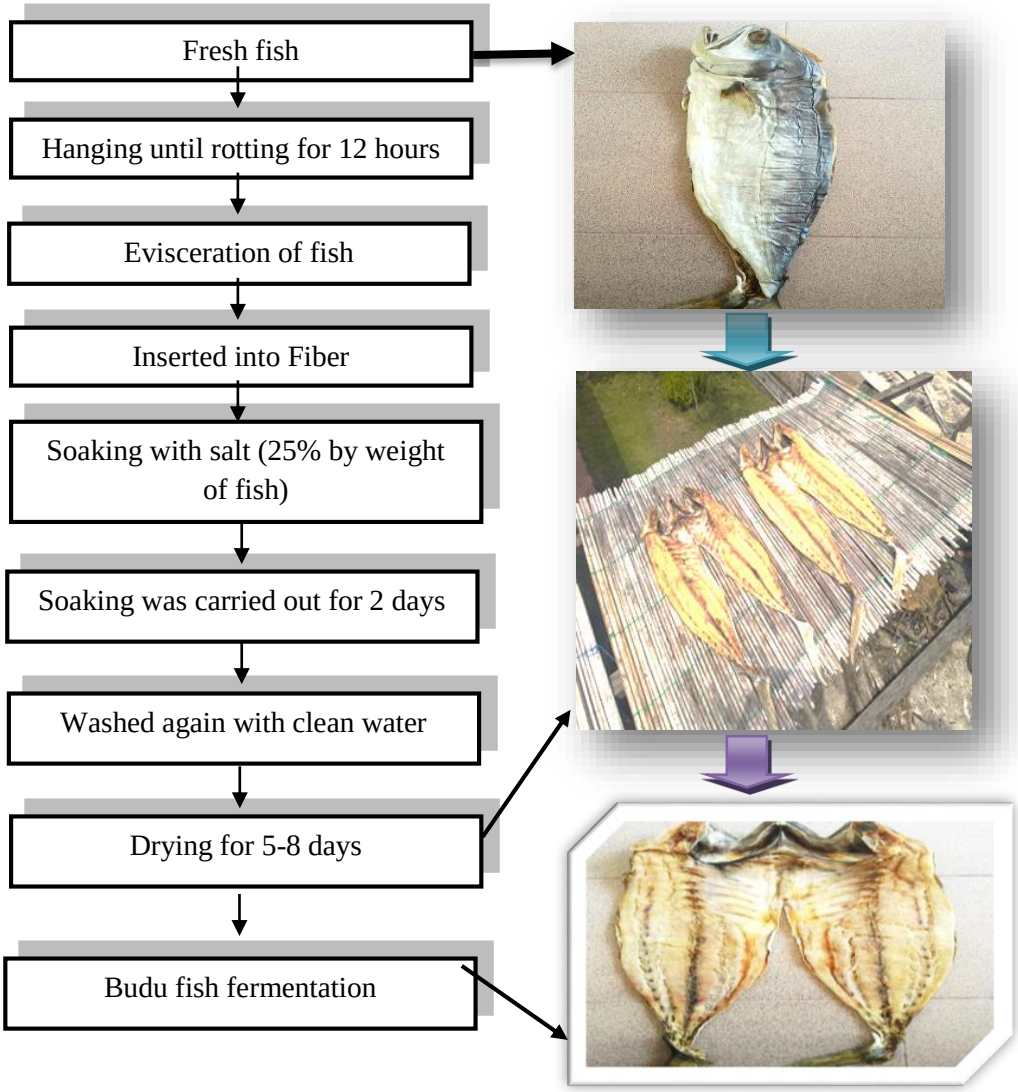

Figure 1. The process of fermenting Budu fish, Padang Pariaman Regency, West Sumatra, Indonesia

Table 1. Sampling locations of Budu Fish, Padang Pariaman Regency, West Sumatra, Indonesia

Sample Code	Location	Fish Type	Drying Time (days)
IB 1	Sungai Limau	Mackerel fish (*S. guttatus*)	8
IB 2	Sungai Limau	Mackerel fish (*S. guttatus*)	6
IB 3	Batang Gasan	Gutter fish (*C. tala*)	7
IB 4	Batang Gasan	Gutter fish (*C. tala*)	5
IB 5	Batang Anai	Mackerel fish (*S. guttatus*)	6

2.4.3. Characterization of Lactic Acid Bacteria Isolates from West Sumatra Budu fish

Morphological characterization was carried out macroscopically by inoculating LAB cultures on MRS agar to study shape, color and diameter of the LAB isolates. Then, morphological characterization was carried out microscopically using Gram staining to investigate shape and color of the cells. Furthermore, catalase and fermentation tests were used to characterize biochemical characteristics and the test for the inhibition of pathogenic bacteria was used to investigate potency of the probiotics [23].

2.5 Isolation and Characterization of the 16S rRNA of Lactic Acid Bacteria

Genome isolation was carried out using Genomic DNA Mini Kit (Invitrogen, Pure-Link, USA) following the manufacturer's instructions. Lysozyme (PureLink, USA)

was used at 20 mg.ml^{-1} to lyse bacterial cell walls and hence the efficiency of protein or nucleic acid extraction could increase. Selected bacterial genomic DNA was used for 16S rRNA gene amplification. Amplification was carried out using forward primer of 63F (5'-CAG GCC TAA CAC ATG CAA G TC-3') and reverse primer of 1387R (5'-GGG CGG GGT GTA CAA GGC-3'). The PCR mixture included 25 µl of DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, USA), 22 µl of MQ, 1 µl of each forward or reverse primer (10 µM each, IDT synthesis) and 1 µl of the template. The conditions of amplification were set as follows: preheating at 95 °C for 5 min, denaturation step at 95 °C for 30 s, primer annealing at 58 °C for 30 s, extension step at 72 °C for 1 min and 35 cycles of post-cycling extension step at 72 °C for 5 min according to Dowarah et al. [24] using thermal cycler (Biometra T-Personal Thermal Cycler, USA).

2.6 Basic Local Alignment Search Tool and Phylogenetic Analysis

The analysis of phylogenetics was carried out based on formerly reported techniques [25]. BioEdit software was used to collect the sequencing data and data were converted to FASTA format and analyzed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nl-m.nih.gov-/blast/cgi). Analysis of phylogeny was carried out by exporting DNA sequences into Clustal W2 http://ww-w.ebi.ac.uk/Tools/clustalw2/). The BLAST MEGA v6.0 (http://www.megasoftware.net) was used to create a phylogenetic tree using neighbor-joining (NJ) method.

2.7 Data analysis

Data were achieved from three analysis times and shown as mean ±SD (standard deviation). Statistical significance was calculated using one-way analysis of Variance (ANOVA) and SPSS software v.26. Turkey method was used to calculate significant differences between the mean results and $p<0.05$ was expressed as the significant level.

3. Results and Discussion

3.1 Isolation of Lactic Acid Bacteria from Budu Fish, Padang Pariaman Regency, West Sumatra, Indonesia

A total of five commercial products of Budu fish originating from 3 sub-districts on the coasts of Padang Pariaman Regency, namely Sungai Limau, Batang Gasan and Batang Anai sub-districts, were selected by simple random sampling. Budu fish products were symbolized by IB 1, IB 2, IB 3, IB 4 and IB 5. The LAB isolation was carried out through serial dilution-agar plates using selective LAB media (MRS broth and agar) with nutrient source and pH appropriate for LAB growth [1]; thus, LAB could grow and reproduce appropriately. Serial dilutions are carried out to decrease density of inoculated LAB; hence, LAB could grow in colonies separately or not overlapping. Budu fish samples that had been grown in MRS Broth and carried out serial dilution were inoculated on MRS Agar. Single colonies that were convex round in shape, yellowish white and shiny growing individually with various diameters were reinoculated on MRS agar to achieve pure LAB isolates of Budu fish using scratch method. A total of 15 LAB isolates were isolated and purified in detail, including IB 1, three isolates (IB1a, IB1b and IB1c); IB 2, four isolates (IB 2a, IB2b, IB2c and IB2d); IB3, three isolates (IB3a, IB3b and IB3c); IB4, one isolate (IB4) and IB5, four isolates (IB5a, IB5b, IB5c and IB5d).

3.2 Qualitative Gamma Aminobutyric Acid-producing Lactic Acid Bacteria Screening

Screening was carried out by detecting isolates capable of producing extracellular GABA. The GABA-producing LAB screening media consisted of MRS broth enriched with 50 mM L-glutamate. The qualitative test used TLC (Figure 2). From a total of 15 LAB isolates achieved, only three isolates were detected producing extracellular GABA (Table 2). This could occur because differences in types of the bacterial isolates are estimated; therefore, there were differences in their ability to produce GABA caused by variations in genetic materials. Santos-Espinosa et al. [26] reported that types of LAB could produce GABA while others could not. For example, *Lactococcus lactis* subsp. *lactic* was a LAB strain that could produce GABA while *L. lactis* subsp. *cemoris* could not produce GABA. Based on the results of a study by Li et al. [10], the GAD CB gene, one of the GABA-producing coding genes, was present in *L. lactis* subsp. *cemoris* but did not experience rearrangements by insertion or deletion in its large fragment. However, deletions of adenine and one thymine insert were detected in the coding region, resulting in a frameshift mutation. Frame shifts resulting from the insertion or deletion of one base within the coding region render the translated protein nonfunctional.

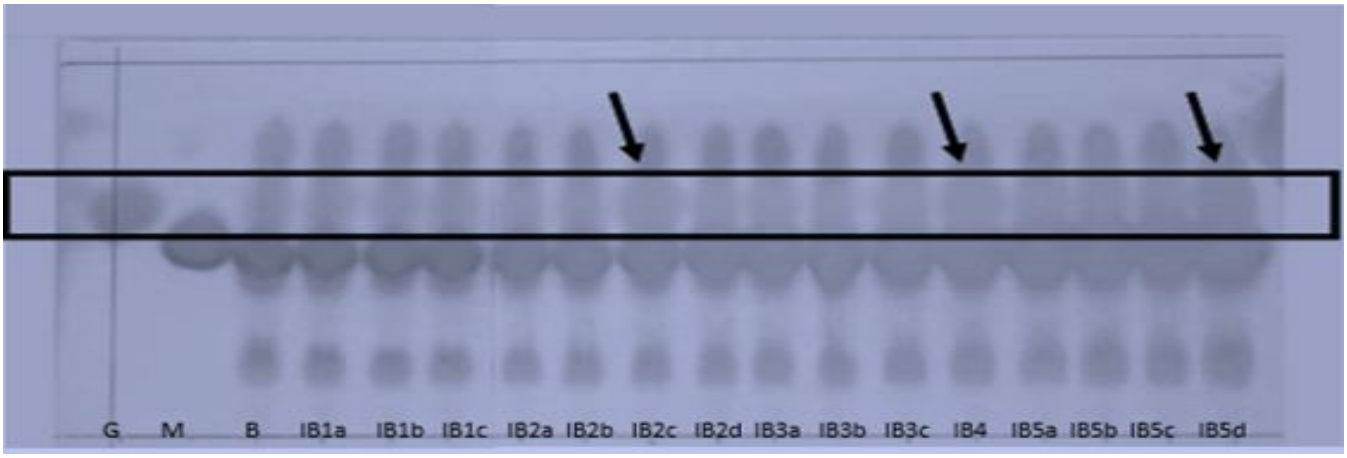

Figure 2. Thin layer chromatography image for the qualitative analysis of gamma aminobutyric acid-producing lactic acid bacteria-Budu fish. Lactic acid bacteria-Budu fish was cultivated in de Mann

Table 2. Gamma aminobutyric acid-producing lactic acid bacteria-Budu fish at various incubation times and monosodium glutamate concentrations

LAB isolate	GABA concentration (mg.ml^{-1})*		
	20 H	40 H	60 H
IB2c + 2% MSG	1.46 ± 0,77^{ab}	2.50 ± 2.59^{ab}	3.46 ± 1.24^{a}
IB2c + 4% MSG	2.89 ± 0.24^{a}	3.01 ± 1.39^{bc}	4.42 ± 2.05 ^{ab}
IB2c + 6% MSG	4.96 ± 1.86^{b}	9.44 ± 1.70^{d}	22.5 ± 2.60^{d}
IB4 + 2% MSG	0,6 ± 0.86^{a}	1.14 ± 0.11^{a}	0.59 ± 0.75^{a}
IB4 + 4% MSG	1.24 ± 0.13^{a}	2.56 ± 0.32^{a}	2.33 ± 0.27^{a}
IB4 + 6% MSG	3.98± 1.85^{ab}	3.40 ± 1.24^{ab}	4.74 ± 0.89^{ab}
IB5d + 2% MSG	2.16 ± 2.06^{ab}	4.72 ± 3.61^{ab}	1454 ± 2.65^{a}
IB5d + 4% MSG	3.10 ± 0.80^{a}	5.29 ± 2.14^{ab}	10.48 ± 1.70^{a}
IB5d + 6% MSG	3.89 ± 0.43^{ab}	7.44 ± 3.82^{ab}	12.26 ± 2.82^{a}

*Data are presented as the mean and standard deviation of three repeats of the paper chromatography pre-staining method measurement of GABA level. Superscripts with distinct letters pointing in the column's direction denote substantially different results (P<0.05).

3.3 Qualitative Analysis of Lactic Acid Bacteria-Budu Fish Producing Gamma Aminobutyric Acid

The ability of LAB of Budu fish to generate GABA was qualitatively assessed using thin layer chromatography (TLC), stationary phase of silica gel 60 F254 and mobile phase of a mixture of aqueous:acetic acid:n-butanol (2:3:5). This was an established method since it did not need expensive chemicals and special pre-treatment of the sample. Various qualitative methods such as pH indicator method and enzyme-based microtiter plate assay (EBMPA) include a longer processing time and further expensive method such as GABase enzyme [27]. Naturally, LAB can produce GABA, a non-protein amino acid produced by glutamate decarboxylation and catalyzed by GAD enzyme, because LAB include GAD enzyme activity in their cells [28]. The GAD can convert GABA from its glutamate substrate or its salts. Sometimes, GABA-producing LAB fermentation media are glutamate-rich food sources such as kimchi, cheese, place and sorrel and hence no additional glutamate or salt is needed in the fermentation process [29]. However, researchers have explained that addition of glutamate or its salts can increase concentration of GABA in various culture media [5]. In this study, MSG was used as a substrate for the GAD enzyme in GABA production.

Generally, LAB produces the highest level of GABA at the end of the stationary phase since acidity and lack of nutrition damage the metabolic activity of LAB [12]. Acidic condition activates GAD enzyme [30], which can catalyze GABA formation in the cytoplasm and secrete it into the culture media (extracellular GABA) [13]. Moreover, GABA can be produced intracellularly; however, its content is low and it is difficult to extract GABA directly from the cells [11]. Thus, GABA level of LAB-fish Budu in this study was calculated in supernatant LAB-Budu fish culture through its extracellular GABA content (LAB-Budu fish culture supernatant). Fifteen isolates were cultivated in MRS broth containing 1% MSG and GABA level was calculated in the culture supernatant using TLC. The three selected LAB isolates (IB2c, IB4 and IB5d) and TLC chromatograms are shown in Figure 2. Chromatograms showed that those three selected LAB isolates included clearer GABA spots than that the other isolates did. These results are similar to the previous

results by Sanchart et al. [31] that six out of 22 selected LAB isolates included the GABA-producing ability based on the clearness of the spots on the TLC plates. Furthermore, GABA-producing ability of the isolates (IB2c, IB4 and IB5d) was analyzed semi-quantitatively regarding the incubation time (20, 40 and 60 h) and MSG concentration (2, 4 and 6%) variations in MRS broth with the paper chromatography staining.

Rogosa Sharpe broth containing 1% monosodium glutamate for 60 h, then drizzled on the spot 1 (G) thin layer chromatography plate: gamma aminobutyric acid standard (1 $mg.ml^{-1}$); spot 2 (M): standard monosodium glutamate (1 $mg.ml^{-1}$); spot 3 (B): de Mann Rogosa Sharpe broth and monosodium glutamate; spot to spot 18:15 isolates of lactic acid bacteria-Budu Fish

3.4 Semi-Quantitative Analysis of Gamma Aminobutyric Acid-producing Lactic Acid Bacteria-Budu Fish

The GABA-producing LAB-Budu fish was analyzed quantitatively by pre-staining paper chromatography (KK) according to Sanchart et al. [31] with variations in MSG concentrations (2, 4 and 6%) and incubation times (20, 40 and 60 h). Concentration of GABA in LAB-Budu fish culture was calculated by inserting the absorbance value derived from the regression equation of the GABA standard solution [$y = 0.0464x + 0.0292$ ($R^2 = 0.9974$)]. The GABA generated by the LAB IB2c, IB4 and IB5d isolates with various incubation times and MSG concentrations are shown in Table 2. Concentration of GABA has continuously increased up to 60 h of incubation and 6% MSG and the highest GABA concentration was achieved at 60 h of incubation. It has been stated previously that the maximum GABA is produced at the end of the stationary phase. This is linked to the highest GABA concentration achieved at 60 h. In addition to the incubation time, MSG concentration affects GABA production by LAB. This is caused by increases in substrates around the cytosolic enzyme GAD [13].

Based on Table 2, almost all LAB-Budu fish isolates showed similar potencies to produce GABA with the highest concentration achieved after 60 h of incubation. However, decreases were reported in GABA concentration of the IB5d isolate after 60 h of incubation. This could be because the IB5.d bacterial cells began to enter the death phase, a phase where cells quickly lose their ability to divide and die within hours [27]. This could occur because differences were seen in the types of bacteria from these isolates and therefore differences were reported in their ability to produce GABA caused by variations in genetic materials. Several types of LAB can produce GABA while others cannot [32]. For example, *Lactococcus (L.). lactis* subsp. *lactis* is a LAB strain that can produce GABA while *L. Lactis* subsp. *femoris* cannot. Naturally, GABA is formed via decarboxylation of L-glutamate, a reaction catalyzed by enzymes that depend on pyridoxal phosphate [33] This glutamate decarboxylase enzyme is produced by several microorganisms such as LAB. Studies by Moe et al. [34] reported that LAB produced glutamate from curd, which was a precursor for the GABA formation. The GABA biosynthesis by microorganisms is regulated by several factors that affect the fermentation process. The affecting factors include pH of the media, temperature, and duration of the fermentation process, nutritional content such as carbon and nitrogen sources and addition of inducers, which affect yields of the fermented products. Biochemical processes in each type of LAB for producing GABA include various optimum conditions.

The maximum concentration of GABA was generated by isolating IB2c at 60 h of incubation and 6% concentration of MSG in MRS broth (22.5 $mg.ml^{-1}$), which was significantly distinguished from other concentrations of GABA generated by other isolates. These results were better than results from previous studies, showing that GABA levels of 18,825 and 16,023 $mg.ml^{-1}$ were achieved from *Lactobacillus* (*L.*) *parabuchneri* OPY-1 and *L. brevis* OPK-3 isolated from kimchi [35]. The GABA level of *L. parabuchneri* BJ20 from fermented seaweed was 19,465 $mg.ml^{-}$ [27], the GABA level of *L. brevis* IFO was 10.047 $mg.ml^{-1}$[6] and the GABA level of *L. parabuchneri* CECT8183 isolated from cheese in Spain was 20.1 $mg.ml^{-1}$. The higher the activity of the GAD enzyme in its cells, the higher the level of GABA produced by IB2 [36]. However, GABA concentration in IB2 was not higher than that by *L. fermentum* GABA 100 from black raspberry juice, which produced 30.6 $mg.ml^{-}$ of GABA [10]. The current results were higher, compared to those from [37], who isolated LAB from traditional foods in form of fermented fish using *L. paracasei* NFRI 7415 with a GABA production of 20.145 $mg.ml^{-1}$. The optimum fermentation conditions for each microorganism varied. This was due to differences in the characteristics of the GAD enzyme for catalyzing glutamic acid to GABA. The IB2c isolates incubated for 60 h in MRS broth and 6% MSG were molecularly identified using phylogenetic analysis.

3.5 Characterization of Gamma Aminobutyric Acid-producing Lactic Acid Bacteria

Further investigations needed to be carried out on the IB2c isolate due to its highest production of GABA (Table 2). The IB2c isolate was subjected to a catalase test to differentiate microorganisms producing the catalase enzyme for the detection of hydrogen peroxide toxicity. The catalase-positive reaction is verified when oxygen is formed by air bubbles. The IB2c was unable to synthesize the catalase enzyme, converting H_2O_2 to water and oxygen. Therefore, IB2c is a catalase-negative bacteria. Similarly, You et al. [38] reported catalase-negative bacteria in fermented goat milk producing nine LAB. Chen et al. [39] showed that LAB demonstrated catalase-negative results. Results of this study

were similar to those by Sokovic et al. [40], who reported negative catalase in LAB isolates from mangoes. Results of this study were also similar to the results of a study by Costa et al. [41], reporting catalase negative in LAB isolated from bekasam. The IB2c isolate did not show formation of O_2 bubbles after dropping H_2O_2, indicating negative catalase. The catalase test aims to show ability of a microorganism to produce the catalase enzyme. This test is carried out by dripping a 3% H_2O_2 solution on the test microorganisms. however, LAB are catalase negative, which means that they cannot produce the catalase enzyme and hence cannot convert H_2O_2 into water and O_2 as shown by Prima et al. [23]. Based on the results achieved, no O_2 bubbles were formed by the IB2c isolate and hence the LAB isolate was catalase negative.

Another biochemical characteristic of LAB is through testing the type of fermentation. LAB can be homofermentative or heterofermentative based on the major fermented product. If lactic acid is produced, then LAB is homofermentative. In contrast, heterofermentative LAB can produce ethanol, CO_2 and acetic acid in addition to producing lactic acid. This fermentation test was carried out by observing if gas bubbles formed in Durham tubes set upside down in LAB liquid culture after 48 h of incubation. If gas is present in the Durham tube, LAB is declared to be heterofermentative. In contrast, if there is no gas in Durham tube, then LAB is categorized as homofermentative [41]. This corresponds to the statement by Bungenstock et al. [42]. In this study, LAB-Budu fish IB2c isolate was homofermentative. Results were similar to those by Song et al. [20], who stated that homofermentative LAB involved Embden-Meyernof-Parnas pathway of glycolysis, producing lactic acid, 2 mol ATP from 1 glucose/hexose molecule under normal conditions, no CO and a cell biomass twice as much as that of heterofermentative LAB.

Furthermore, Gram staining was carried out to study the morphological characteristics of the isolates microscopically to figure out color and shape of the cells. Microscopic identification is an identification based on the physiological characteristics of LAB isolates. Gram staining is used to identify the type of bacteria in form of color (Gram-positive or Gram-negative) and shape of the bacteria [43,44]. Results showed that the LAB IB2c isolate belonged to Gram-positive bacilli. Results of this study were not much different from the results of a study by Dowarah et al.[24] on tilapia which reported all isolates as Gram-positive bacteria. This was associated to statements by Song et al. [20].

The selected IB2c isolate Budu fish can inhibit the growth and development of pathogenic bacteria of *Escherichia coli* O_{157}, *Staphylococcus* (*S.*) *aureus* and *Salmonella enteritidis*. Results from the study showed a clear/inhibitory zone area of IB2c isolate in *E. coli* O_{157} with a diameter of 14.46 mm. The largest inhibitory zone diameter for *S. aureus* ATCC 25923 in IB2c isolate was 14.40 mm and the largest diameter of the inhibitory zone against *Salmonella Enteritidis* ATCC 13076 by the IB2c isolate was16.60 mm. Results showed that the inhibition zone of the IB2c isolate against pathogenic bacteria included a high antimicrobial activity. These results were similar to those by Prima et al. [23], stating that inhibition zone activity was grouped into four groups of weak activity (< 5 mm), moderate (5–10 mm), strong (> 10–20 mm) and very strong (> 20–30 mm). Therefore, it could be stated that LAB IB2c isolate from fermentation of Budu Fish included a clear zone of the strong category against the three bacteria. An important criterion used in selecting LAB isolates as probiotic agents included their ability to inhibit growth of pathogenically enteric bacteria that inhabit normal function of the digestive tract. Another requirement of the probiotic bacteria includes ability to produce antimicrobial substances [12].

3.6 Identification of the Selected IB2c Isolates from Budu Fish Using 16S rRNA

3.6.1 Results of 16S rRNA Gene Amplification Using Polymerase Chain Reaction

Electrophoresis results exposed that the bacterial 16S rRNA gene from Budu fish LAB isolates was successfully amplified. The PCR primers of 27F AGAGTTTGATCC-TGGCTGAG for the forward direction and primer 1429 R GTTTACCTTACGACTT for the backward direction yielded fragments with sizes of 1,500 bp. The aim of LAB 16S rRNA gene amplification was to verifying results of the genomic DNA isolation process and identifying LAB. Additionally, nucleotide sequencing was carried out after 16S rRNA gene amplification. Electrophoresis results of the PCR products from LAB isolates are present in Figure 3.

The aim of the molecular study was to analyze genetic relationships of LAB based on ribosomal RNA sequence comparisons. Due to its petite size, 16S rRNA can be directly sequenced without cloning PCR amplicons. Results of the 1,500-bp DNA fragment amplification on agarose gels are present in Figure 3. This finding shows that the specific primers used in this study can identify bacteria down to the strain levels. Chromatograms of the sequencing results from the forward and reverse primer reading directions of the 16S rRNA gene from IB2c isolate were aligned using SeqMan program. Amplicon length of the 16S rRNA gene amplified with the primers 16S rRNA _27F and 16S rRNA _1525R was 1,529 bp. Moreover, chromatogram of the forward 16S rRNA _27F primer reading was 726 bp in size. Then, the 16S rRNA _1525R reverse primer reading direction length was 1,131 bp. As a weakness of the Sanger Sequencing method, several bases at the ends of the chromatogram had peaks that overlaped with low qualities. Therefore, these bases were deleted [45]. The forward 16S rRNA _27F primer bases were removed at the 5' end by 20 bp and at the 3' end by 48 bp. Bases of the reverse primer 16S rRNA _1525R were

removed at the 3' end by 105 bp and at the 5' end by 23 bp. After the editing process, length of the sequence of the 16S rRNA gene fragment from the IB2c isolate included 1,444 bp. The edited chromatogram was as follows.

>Bacterial 16S rRNA Gene Sequence of the IB2c Isolate

GGATGGACCCGGGCGTATTAGCTGTTGGTAGGT
ACGGCCTACCAAGGCAATGATCGTAGCCGACCTGA
AGGGTAATCGGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTAGGGATCTTC
CACAATGGACGAAAGTCTGATGGAGCAACGCCGC
GTGAGTGATGAAGGGTTTCGGCTCGTAAACTCTGT
TGTTGGAGAAGAACAGGTGTGAGAGTAACTGTTCA
CATCTTGACGGTATCCAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAG
CGCAGGCGGTTTCTTAGGTCTGATGTGAAAGCCTT
CGGCTTAACCGGAGAAGTGCATCGGAAACCAGGA
GACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAAGAA
CACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACT
GACGCTGAGGCTCGAAAGCATGGGTAGCGAACAG
GATTAGATACCCTGGTAGTCCATGCCGTAAACGAT
GAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGA
GTACGACCGCAAGGTTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGATGCTACGCGAAGAACCTTACCAGGTCTT
GACATCTTCTGCCAACCTAAGAGATAGCGTTCCCT
TCGGGGACGAATGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTGGGTAAGTCCCGCAA
CACGCACCCTTATGTAGTTGCCAGCATTCAGTTGG
GCACTCTAGCAGATGCCGGTGACAACCGGAGGAA
GGTGGGGATGACGTCAATCATCATGCCCTTATGAC
CTGGG

Base sequence of the bacterial Isolate IB2c 16S rRNA gene was then analyzed using BLAST. The BLAST results showed information based on 15 sequence data of *L. parabuchneri* bacteria in the GenBank selected for further use in genetic relationship analysis.

Based on Table 3, IB2c isolate included a similarity of 97.69% to the genome sequence and partial strains of *Bacillus* spp. Based on the statement of Matti et al. [46] that a sequence is homologous if it includes more than 70% similarity, analysis was carried out using BLAST and then visualized using MEGA v7.0. The sequences were previously aligned using BioEdit. According to the phylogenetic visualization, it was stated that the fermented Budu fish isolates in the study included apomorphic relationships with *L. parabuchneri* as Liang et al [47] stated that the characters were apomorphic and plesiomorphic. Apomorphic characteristics are usually inherited and seen in ingroups, whereas synapomorphic characteristics are inherited and seen in monophyletic groups. Results of the image were created by selecting 15 species from the BLAST results based on the query coverage values. Then, top 15 species were downloaded in FASTA format for use in phylogenetic studies.

3.6.2 Phylogenetic Tree

Based on the BLAST results, a phylogenetic tree was plotted to see degrees of relationships between the isolates and other species in NCBI. Phylogenetic tree was created using Mega X with aligned sequences. Genetic distance was analyzed using 2-parameter Kimura method. Phylogenetic tree was constructed using neighbor-joining method with a bootstrap value of 1,000. Evolutionary distance was analyzed using Kimura 2-parameter method. The phylogenetic tree showed relationships between the IB2c isolate and ten bacteria from the GenBank based on the 16S rRNA gene fragment sequence. In the BLAST results, IB2c included close relationships with *Bacillus parabuchneri* with 100% similarity. Based on the table of BLAST results from IB2c isolate, it is clear that the samples belonged to *Bacillus* spp. Then, creation of a phylogenetic tree for the two isolates was carried out simultaneously. Results showed that the two isolates were 100% linked to each other (Figure 4). This was carried out because it was suggested that these isolates belonged to the same genus and could produce GABA. The phylogenetic tree showing relationships between the IB2c isolate and 15 other bacteria from the GenBank based on the 16S rRNA gene fragment sequence is demonstrated in Fig 4.

This is similar to the statement of [48], reporting that the neighbor-joining approach can be used to reveal an organism's evolutionary history. In phylogenetic trees, organisms with identical taxon usually were grouped in clusters and have higher bootstrap values [49]. In this study, a phylogenetic tree was created to investigate the evolutionary distances using p-distance method. [50] detected that a total of 15 nucleotide sequences and codon regions, using MEGA 7.0 [23]. The phylogenetic tree (Fig. 4) was reconstructed. The constructed phylogenetic tree resulted in two major branches consisting of the 15 bacteria. This was because the 16S rRNA gene fragment sequences included 100% similarities, as verified by the results of BLAST, alignment and genetic distance calculations. Thus, identification of the IB2c isolate could be carried out up to the genus level. Based on this fact, it was concluded that the IB2c isolate was *Lentilactobacillus* (*Len.*) *parabuchneri* strain M1-40. This microorganism is homofermentative (only produces lactic acid) and cannot utilize pentoses (carbohydrates with C5 atoms).

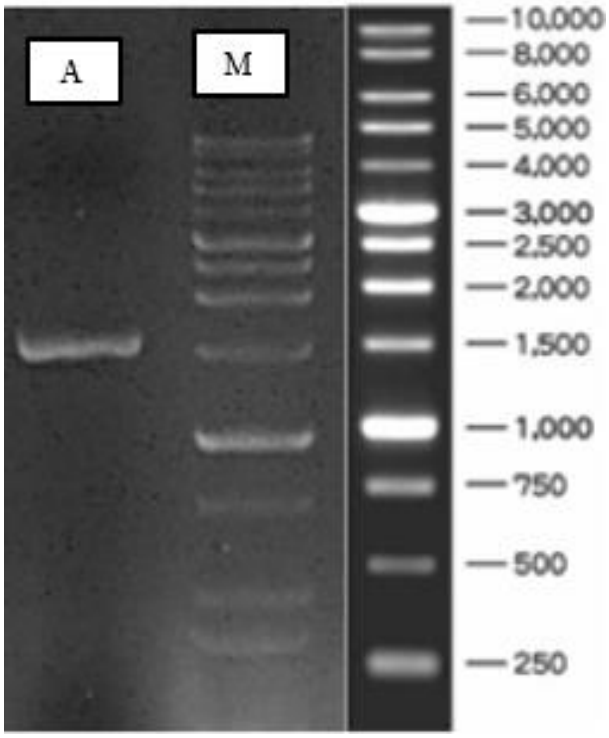

Figure 3. Polymerase chain reaction electrophoresis results of the isolate IB2c from Budu fish (M, Marker; A, IB2c isolate from Budu fish)

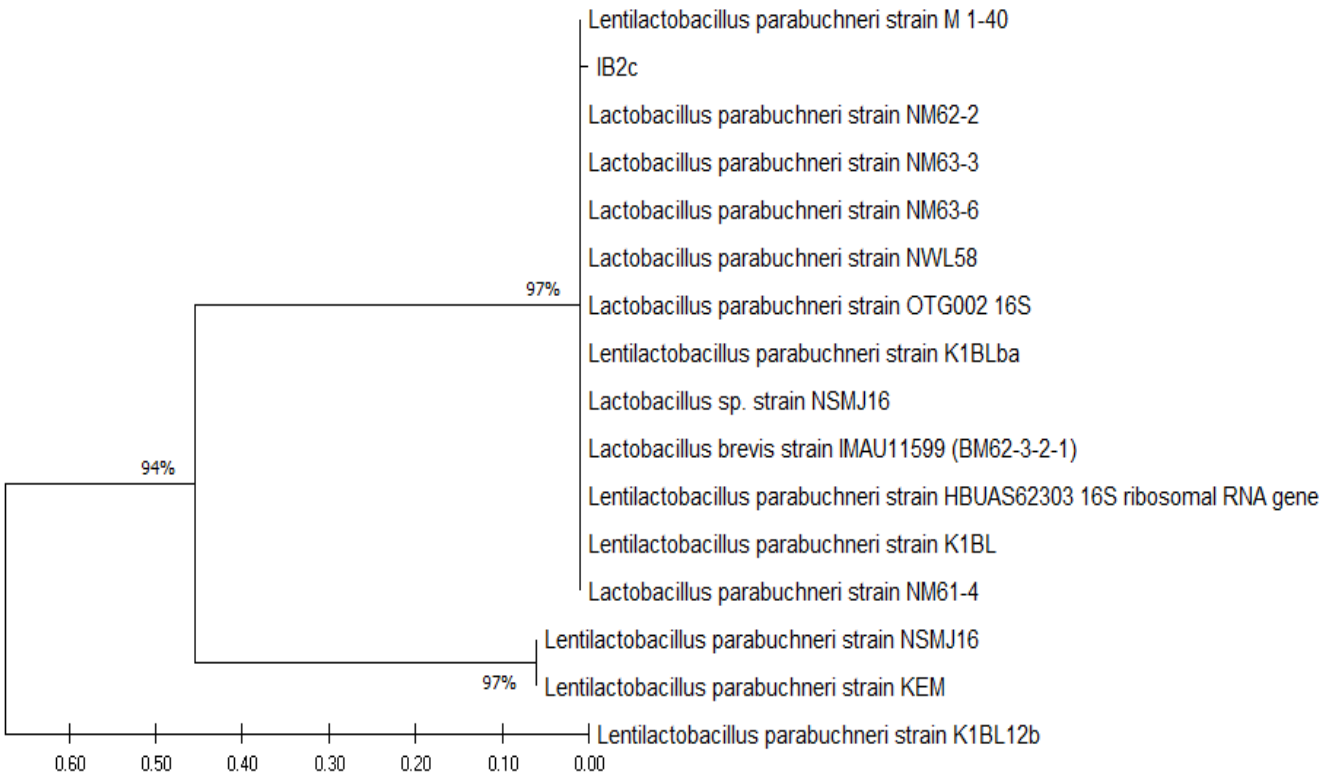

Figure 4. Phylogenetic tree of the IB2c isolate

Table 3. Basic Local Alignment Search Tool results of lactic acid bacteria (IB2c) of Bilih Budu fish

No	Description of Lactobacillus Bacterial strains	Query cover (%)	Accession number	Percent identification (%)
1	*L. brevis* strain IMAU11599		KP763877.1	
2	*Len. parabuchneri* strain HBUAS62303		OM978660.1	
3	*L. sp*. strain NSMJ16		MT397049.1	
4	*Len. parabuchneri* strain K1BL		MW698734.1	
5	*Len. parabuchneri* strain K1BLba		MW697452.1	
6	*Len. parabuchneri* strain K1BL12b		MW697447.1	
7	*L. parabuchneri* strain OTG002 16S		MW412495.1	
8	*Len. parabuchneri* strain NSMJ16	100%	CP050493.1	97,69%
9	*Len. parabuchneri* strain KEM		CP062473.1	
10	*L. parabuchneri* strain NWL58		HQ293081.1	
11	*L. parabuchneri* strain NM63-6		HM218286.1	
12	*L. parabuchneri* strain NM63-3		HM218284.1	
13	*L. parabuchneri* strain NM62-2		HM218278.1	
14	*L. parabuchneri* strain NM61-4		HM218276.1	
15	*L. parabuchneri* strain M 1-40		OP962314.1	

Studies state that *Len. parabuchneri* is a GABA-producing LAB species, similar to *L. fermentum* and several other species. This is due to the presence of the GAD enzyme in *Len. parabuchneri*. [51] reported that *Len. parabuchneri* was one of the species; in which, the gene encoding GAD enzyme was detected. A similar finding was reported by [52], who stated that *Len. parabuchneri* was a LAB species that produced GAD enzyme and hence could catalyze formation of GABA from glutamate or its salts. The GAD enzyme in *Len. parabuchneri* has been cloned, purified, immobilized and used to convert glutamate to GABA [53]. Results of this study are different from those achieved by [2] on isolation of LAB from tuna fish. Several LAB strains were isolated such as *Enterococcus faecalis, Tetragenococcus muriaticus, L. delbrueckii* subp. *delbrueckii* and *Carnobacterium divergens*. These species of bacteria varied because types of the fermented fish varied from the fresh fish. Moreover, ingredients for the fermentation and fermentation methods varied as well. Results of this study were different from those by [11], who identified LAB from cheese, including *L. brevis, L. pentosus* and *Pediococcus pentosaceus*.

4. Conclusion

A total of 15 isolates of LAB have been isolated from Budu fish and identified with bacilli-shaped cells. Of the 15 LAB-Fish Budu isolates, three isolates (IB2c, IB4 and IB5d) were capable of producing GABA that IB2c isolate produced the highest level of GABA (22.5 $mg.ml^{-1}$) after 60 h of incubation with addition of 6% MSG. Using biochemical characterization, the highest GABA-producing IB2c isolates were Gram-positive, catalase-negative and homofermentative and could utilize various carbon sources for each isolate. Based on the analysis of 16S rRNA gene sequence, IB2c isolate from Budu fish was identified as *Len. parabuchneri* strain M1-40 (accession no. OP962314.1), which IB2c isolate included the best potential to produce GABA and was classified as a probiotic bacterium. It is recommended to quantitatively analyze concentration of GABA in LAB-Budu fish and use of GABA-producing LAB-Budu fish as a starter to produce functional products that are safe and halal.

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6. Conflict of Interest

The authors report no conflict of interest.

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تعیین خصوصیات باکتری‌های اسید لاکتیک تولید کننده گاما آمینوبوتیریک اسید جدا شده از ماهی بودو در پادانگ پاریمان غرب سوماترا اندونزی و قابلیت‌های کاربرد آنها به‌عنوان زیست‌یار

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چکیده

سابقه و هدف: غربالگری باکتری‌های لاکتیک اسید برای تولید گاما آمینو بوتیریک اسید، به‌دلیل عملکردهای دارویی آن، مهم است .در این مطالعه، جداسازی و شناسایی باکتری‌های لاکتیک اسید از ماهی بودو، ماهی بومی غرب سوماترا، اندونزی، مورد بررسی قرار گرفته و توانایی تولید گاما آمینو بوتیریک اسید آنها شناسایی شد.

مواد و روش ها: روش تحقیق به‌شرح زیر بود: باکتری لاکتیک اسید از ماهی بودو که به‌طور تصادفی از بازار سنتی در غرب سوماترا، اندونزی خریداری شده بود، جدا شد. جدایه‌ها از نظر ریخت‌شناسی [۱] و بیوشیمیایی بررسی شدند. جدایه‌های گرم مثبت به‌دلیل قابلیت تولید گاما آمینو بوتیریک اسید با استفاده از کروماتوگرافی لایه نازک مورد بررسی قرار گرفتند. سویه‌های مثبت گاما آمینو بوتیریک اسید با استفاده از طیف سنجی مورد تجزیه و تحلیل کمی قرار گرفتند. از بین پنج نمونه، بیشترین تولید گاما آمینو بوتیریک اسید در نمونه ایزوله IB2C گزارش شد. ساختار مولکولی جدایه های انتخابی باکتری‌های لاکتیک اسید با استفاده از توالی‌یابی ژن [۲] S rRNA ۱۶شناسایی شدند. اثر زمان گرمخانه‌گذاری (۲۰، ۴۰ و ۶۰ ساعت) و غلظت مونو سدیم گلوتامات (۲، ۴ و ۶ درصد) بر سویه‌های تولید اسید گاما آمینو بوتیریک مورد بررسی قرار گرفت.

یافته ها و نتیجه‌گیری: از ۱۵ جدایه، برای تولید گاما آمینو بوتیریک اسید در براث de Mann Rogosa Sharpe حاوی ۱٪ مونوسدیم گلوتامات، سه جدایه انتخاب شدند. تجزیه و تحلیل نیمه کمی با کروماتوگرافی کاغذی پیش رنگ‌آمیزی برای شناسایی باکتری لاکتیک اسید با بالاترین میزان تولید گاما آمینو بوتیریک اسید انجام شد. بالاترین میزان گاما آمینو بوتیریک اسید (۲۲/۵ میلی‌گرم در میلی‌لیتر) با ۶۰ ساعت گرمخانه‌گذاری و غلظت ۶ درصد مونوسدیم گلوتامات در آبگوشت de Mann Rogosa Sharpe به‌دست آمد. جدایه های IB2c، گرم مثبت، کاتالاز منفی، هموفرمانتیو و قادر به استفاده از منابع گوناگون کربن بودند، رشد *اشریشیا کلی* O_{157}، *استافیلوکوکوس اورئوس* و *سالمونلا انتریتیدیس* را مهار کردند. ساختار مولکولی IB2C با استفاده از توالی‌یابی ژن S rRNA ۱۶تعیین شد. نتایج به‌عنوان سویه *لنتیلاکتوباسیلوس پارابوچری* سویه M1-40 شناسایی شد. شباهت ۹۷/۶۹ درصدی باکتری لاکتیک اسید جدا شده از ماهی بودو با *Len. parabuchneri* بر استفاده زیست‌یار از باکتری لاکتیک اسید تولید کننده گاما آمینو بوتیریک اسید دلالت دارد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- تولید GABA
- تخمیر میکروبی
- مشخصات مولکولی
- ماهی بومی
- سویه *Len. parabuchneri* M1-40

***نویسنده مسئول**

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