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WORAHARN, Sasimar; LAILERD, Narissara; SIVAMARUTHI, Bhagavathi Sundaram;
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Screening and kinetics of glutaminase and glutamate decarboxylase producing lactic acid bacteria from fermented Thai foods

Sasimar WORAHARN¹, Narissara LAILERD², Bhagavathi Sundaram SIVAMARUTHI¹,
Wiwat WANGCHAROEN³, Sophon SIRISATTHA⁴, Chaiyavat CHAIYASUT^{1*}

Abstract

L-glutaminase and glutamic acid decarboxylase (GAD) catalyzes the hydrolysis of L-glutamine and glutamate, respectively. L-glutaminase widely used in cancer therapy along with a combination of other enzymes and most importantly these enzymes were used in food industries, as a major catalyst of bioconversion. The current investigation was aimed to screen and select L-glutaminase, and GAD producing lactic acid bacteria (LAB). A total of 338 LAB were isolated from fermented meat, fermented fish, fermented soya bean, fermented vegetables and fruits. Among 338 isolates, 22 and 237 LAB has been found to be positive for L-glutaminase and GAD, respectively. We found that 30 days of incubation at 35 °C and pH 6.0 was the optimum condition for glutaminase activity by G507/1. G254/2 was found to be the best for GAD activity with the optimum condition of pH 6.5, temperature 40 °C and ten days of incubation. These LAB strains, G507/1 and G254/2, were identified as close relative of *Lactobacillus brevis* ATCC 14869 and *Lactobacillus fermentum* NBRC 3956, respectively by 16S rRNA sequencing. Further, improvements in up-stream of the fermentation process with these LAB strains are currently under development.

Keywords: L-glutaminase; glutamic acid decarboxylase; lactic acid bacteria and 16S rRNA sequencing.

Practical Application: LABs based safe starter culture for the fermentation of mushroom to produce high GABA.

1 Introduction

Glutaminase distributed in all the microbes like bacteria, yeast and fungi, and they were reported to produce notable amount of glutaminase (Nandakumar et al., 2003). This enzyme widely used in food industries for the enzymatic degradation of proteins, which present in the raw materials. More specifically, flavor of the fermented soy sauce is due to glutamic acid produced by glutaminase. There are many numbers of glutaminase reported from different microbial sources with a wide range of fermentation conditions as summarized by Nandakumar et al. (2003). More specifically, they differ in molecular weight, optimum pH and substrate specificity. Several glutaminases were reported to have both an optimal and stable temperature of ~ 40-50 °C. Some previous studies have reported about the temperature stability and activity of several glutaminases (Prusiner et al., 1976; Moriguchi et al., 1994; Koibuchi et al., 2000). Glutaminases from *Aspergillus oryzae*, *Micrococcus luteus* K-3 and *Bacillus subtilis* are known for its high-salt tolerance capacity (Yano et al., 1988; Moriguchi et al., 1994; Madern et al., 2000; Kennedy et al., 2001).

The transfer of L-glutamic acid to γ -aminobutyric acid (GABA) and carbon dioxide can be mediated by the enzyme called glutamic acid decarboxylase (GAD) with pyridoxal as cofactor (Krnjević, 1974). GABA is a well-known inhibitory neurotransmitter, and its deficiency leads to Parkinson's disease,

Huntington's chorea and cognitive impairment. GABA also plays a role in controlling blood pressure, cholesterol level and has anti-diabetic property (Shimada et al., 2009; Soltani et al., 2011). As like glutaminase, GAD also presents diversely, and they mostly derived from microbial sources ranging from bacterias, yeasts and molds (Kato et al., 2002; Yang et al., 2008a, b). FDA and WHO have approved the use of these enzymes for food fermentation (Food and Agricultural Organization & World Health Organization, 1987).

LAB genus is known as starter cultures in fermented food and beverage industries. LAB is usually known as safe (generally recognized as safe; GRAS) (Bovo et al., 2014). Fermented foods are a good source for LAB isolation. LAB isolated from fermented food was considerate as safe and suitable for food and beverage applications (Bovo et al., 2014; Fraga Cotel et al., 2013; Pérez-Chabela et al., 2008). LABs are recognized probiotic for human usage particularly in fermented food production (Leroy et al., 2006; Yerlikaya, 2014). *Lactobacillus casei*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus sanfranciscensis*, *Lactobacillus reuteri*, *Lactobacillus lactis* subsp. *cremoris* are reported to produce glutaminase (Kieronczyk et al., 2001; Weingand-Ziadé et al., 2003). Thongsanit et al. (2009) isolated *Leuconostoc citreum* from Thai fermented pork sausage with glutaminase activity. The previous reports have suggested

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¹Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand

²Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

³Faculty of Engineering and agro-industry, Maejo University, Sansai, Chiang Mai, Thailand

⁴Department of Bioscience, Thailand Institute of Scientific and Technological Research, Pathumthani, Thailand

*Corresponding author: chaiyavat@gmail.com

that *Listeria monocytogenes*, *Escherichia coli* and *Lactococcus lactis* can produce GAD (Lin et al., 1995; Sanders et al., 1998; Cotter et al., 2001). GAD of *Lactobacillus brevis* and *L. paracasei* are biochemically well characterized (Hiraga et al., 2008; Komatsuzaki et al., 2008). *L. brevis* OPK-3 was isolated from traditional fermented food of Korea with the ability of producing 84.292 mg/L/h of γ -aminobutyric acid (Park & Oh, 2006). Komatsuzaki et al. (2005) have reported the ability of *L. paracasei* to produce GAD isolated from traditional fermented foods. A study by Yokoyama et al. (2002) explains the use of *L. brevis* IFO-12005 for the production of γ -aminobutyric acid from the residue of alcohol distillery. Recently, a study has reported that *L. reuteri* 100-23 play a role in acid resistance during fermentation owing to the production of GAD (Su et al., 2011).

There have been no detailed reports regarding the production and characterization of glutaminase and GAD from lactic acid bacteria isolated from Thai fermented food. Thus, this study has focused on the isolation and identification of glutaminase and GAD-producing LAB from various Thai fermented foods and subsequently characterization of glutaminase and GAD activity.

2 Materials and methods

2.1 Isolation of lactic acid bacteria (LAB)

Total LABs were isolated from fermented foods such as fermented meat, fish, soya bean, vegetables and fruits, which were aseptically collected from different areas of Thailand. Bacterial isolation was carried out by serial dilution and pour plate method. Briefly, 5% of samples were prepared in saline solution (0.90% (w/v) NaCl) and used as a stock. Then, the sample was serially diluted in saline and plated on Man Rogosa Sharpe agar (MRS agar, Labscan, Cat. No. LS 1168) incorporated with 0.005% of bromocresol purple (Fisher scientific, Cat No. B/4330/44) by pour plate method. The plates were incubated at 37 °C for 24-48 h. After incubation, colonies with yellow pigmentation were considered as LAB and selected for further analysis. Pure cultures of the selected LABs were stored at -20 °C with 40% glycerol (Balagurunathan et al., 2010; Aramide et al., 2009; Bertazzoni Minelli et al., 2004).

2.2 Screening for glutaminase activity

LABs were cultured in MRS broth with 1.5% (w/v) of L-glutamine (Sigma, Cat. No. 49419) at 37 °C for 24 h. Then, the cells were harvested by centrifugation at 6000 rpm for 5 min, and the supernatants were subjected to screening of extracellular glutaminase activity. Bacterial pellets were further washed with 0.85% (w/v) of NaCl and dissolved in the same solution. Followed by, the bacterial suspensions were divided into two equal volumes, and one part was subjected to the analysis of cell-wall-associated glutaminase. Rest of the cell suspensions were subjected to ultrasonication process, after the freeze-thaw treatment by an alternative incubation at -20 °C and RT for 30 min, at 120% of ultrasound power for 20 min using ultra-sonicator (Elma, Model no.D-78224 Singen/Htw) for the determination of intracellular glutaminase activity. The activity

of glutaminase was measured by estimating the amount of NH_3 liberated from L-glutamine (Katikala et al., 2009). In brief, 500 μl of 0.04M of L-glutamine prepared in 0.1 M phosphate buffer pH 7.0 was mixed with 500 μl of samples and incubated at 37 °C for 30 min. After incubation, 0.5 ml of 1.5 M of trichloroacetic acid (TCA) was added to stop the enzymatic reaction. Then equal volume of Nessler's reagent (Merck. Cat. No.109028) was added and incubated at RT until the appearance of yellow color (5-15 min). Then the samples were centrifuged at 5000 rpm for 3 min and the absorbance of the supernatant was measured at 450 nm using a multimode detector (Beckman Coulter, Model. No. DTX 880). TCA solution mixed with L-glutamine and Nessler's reagent served as blank. Ammonia content was calculated by using NH_4SO_4 as standard. One international unit of glutaminase was defined as the amount of enzyme that liberates one micromole of ammonia under optimum conditions. The enzyme yield was expressed as unit/ml or as specific activity (U/mg protein).

2.3 GAD activity assay

All strains were tested by rapid colorimetric assay using bromocresol green as pH indicator for GAD activity (Cotter et al., 2001; Olier et al., 2004). Overnight culture of LABs were centrifuged at 8000 rpm for 10 min and the bacterial pellet was dissolved in 500 μl of test solution, consist of 1g of L-glutamic acid (Sigma), 300 μl of Triton X-100(Sigma), 90 g NaCl (Merck), and 0.05 g bromocresol green (Labchem) dissolved in 1 liter of water, after washing with 0.90% (w/v) of NaCl. The development of green or blue color was considered as low or high activity for GAD, respectively. Three independent experiments were carried out for each strain tested.

2.4 Effect of pH and temperature on LABs growth and enzyme kinetics

Selected positive LABs were cultured in modified medium for respective enzymes. Then, these cultures were re-inoculated in the medium containing of 10% (w/v) of cane sugar (CS) and 1.5% (w/v) L-glutamine with range of pH from 6-8, followed by the medium was incubated at different temperatures like 30, 35, 40 °C for 30 days. Glutaminase activity was denoted as U/ml. GAD-producing LABs were inoculated in the medium containing 10% (w/v) CS with 0.1% (w/v) of L-glutamic acid with pH range of 4.5, 5.5 and 6.5 and incubated at different temperatures like 30, 35, 40 °C for 30 days. Level of GABA and L-glutamic acid was measured by HPLC and growth kinetic of the LABs were assessed by OD at 600 nm.

2.5 HPLC for GABA and L-glutamic acid

GABA and L-glutamic acid content in the culture were determined by HPLC analysis. Briefly, 1 ml of culture was centrifuged at 6,000 rpm for 10 min and supernatant was collected for derivatization with PITC (phenyl isothiocyanate). Analysis was performed using HPLC equipped with a C18 column (4.6 \times 250 mm, 5 μm). The elution solvent system consists of 70 mM of sodium acetate pH 5.8 (A) and 55% (v/v) of acetonitrile (B).UV detector at 254 nm was used. The flow

rate was adjusted as 1 ml/min and the sample injection volume was 20 µl. L-glutamic acid and GABA were served as standards.

2.6 Molecular identification of LAB isolates

Genomic DNA of the LAB was isolated by standard procedure (Sambrook & Russell, 2001) and 16S rRNA coding gene was amplified using gene specific primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3'; Reverse primer 5'-GGTTACCTGTTCAGACTT-3'). The amplified DNA fragment was subjected to sequencing by Sanger's method. Sequences were analyzed, and taxonomic strain identification was performed by comparing the sequences of each strain with those reported in EZTaxon (<http://www.ezbiocloud.net/eztaxon/>) database. Phylogenetic for LABs were constructed by the neighbor-joining method (Kim et al., 2012). The topologies of trees analyzed by bootstrap analysis of the sequence with MEGA 6 software based on 100 random resembling.

2.7 Statistical analysis

All the experiments were performed in three independent repeats, and the values were plotted as Mean \pm SD.

3 Results and discussion

3.1 LABs with glutaminase activity

Total strains of 338 lactic acid bacteria (LAB) strains were isolated from various fermented foods as detailed (Supplementary Table 1). All the LABs were screened for glutaminase production as per the procedure stated in materials and methods section. Among 338 isolates, 22 LABs were capable of producing as glutaminase producers, and they were further analyzed for localization of the enzyme. About 3, 12, 7 strains were found to produces extracellular glutaminase, intracellular, cell-wall associated enzyme producers, respectively. Among these positives, 12 strains (4, 6 and 2 strains were attributed for the production of extracellular, intracellular and cell-wall associated enzymes, respectively) were selected as high scorer (Table 1) and both extracellular and cell-wall associated enzyme

activity was observed in a LAB isolate denoted as G415/2. Strains G507/1 (2.88 ± 0.19 U/mg protein), G284/2 (6.07 ± 0.28 U/mg protein) and G270/2 (0.85 ± 0.19 U/mg protein) were found as best extra-cellular, intracellular and cell-wall associated enzyme producers, respectively. These results suggested that intracellular glutaminase producing LABs are more harbored in selected food sources than other strains. Moreover, these strains were reported for its highest enzyme production compared to others (Table 1), based on the enzyme producing ability, strains denoted as G507/1 (extracellular glutaminase producer) and G284/2 were selected for further analysis.

3.2 Kinetics of growth and glutaminase production

Selected two glutaminase producing strains were further kinetically analyzed to study the impact of pH and temperature on enzyme production. Glutaminase production was assessed at different temperatures (30, 35, 40° C) and pH (6, 7, 8) as detailed in materials and methods section. Amount of enzyme production was denoted as U/mg of protein. The results indicated that G507/1 produces highest amount of enzyme (7.46 U/ mg of protein) than other strain at 35° C with the pH of 6.0 after 30 days of incubation and also this is the only strain that can produce, at least, trace amount of enzyme at all the tested conditions (Supplementary Table 2, Figure 1a). Growth rate of G507/1 at 35° C, pH 6.0 was also assessed for 30 days, and it was found to be a typical multiplication (Figure 1b). Previous study by Jeon et al. (2009) has reported that *L. reuteri* KCTC3594 produces the glutaminase with optimum activity at 40 °C and pH 7.5; moreover, it shows 50% of activity at high salt concentration. Marine actinomycetes produce L-glutaminase with optimum activity at 27 °C and pH 9.5 after 96 h of incubation (Sivakumar et al., 2006).

These results suggested that optimum L-glutaminase activity is varied from pH 6.0-9.0 and temperature at 27-40 °C depends on the isolation source. The earlier study related to glutaminase producing bacteria from Thai food by Thongsanit et al. (2009) suggested that food isolate of *Weissella cibaria* can able to produce 0.0085 U/mg of enzyme whereas the present study have reported that LAB isolate can produce 7.46 U/ mg of enzyme.

Table 1. Production of glutaminase producing by LAB strains and classification based on the nature of enzyme in habitation.

S. No.	Strains	Specific activity of glutaminase (U/mg protein)		
		Extracellular enzyme	Intracellular enzyme	Cell-wall-associated enzyme
1	G314/2	ND	1.37 \pm 0.30	ND
2	G256/1	ND	2.78 \pm 0.20	ND
3	G256/2	ND	4.35 \pm 0.25	ND
4	G284/1	ND	3.67 \pm 0.17	ND
5	G284/2	ND	6.07 \pm 0.28	ND
6	G479/1	0.70 \pm 0.24	ND	ND
7	G339/1	1.43 \pm 0.19	ND	ND
8	G415/2	0.18 \pm 0.15	ND	0.52 \pm 0.26
9	G30/2	ND	ND	0.34 \pm 0.23
10	G26/1	ND	3.02 \pm 0.18	ND
11	G507/1	2.88 \pm 0.19	ND	ND
12	G270/2	ND	ND	0.85 \pm 0.19

Values are represented as mean \pm SD of three experiments. (ND: Not detected).

3.3 Screening and kinetics of GAD activity

All the isolated LABs (338) were screened for GAD activity as detailed in materials and methods section. A total of 17, 36 and 215 isolates were categorized as high, medium and low amount of GAD producers based on the color development during the screening test, respectively (Figure 2).

Previous studies have reported that bacterial pathogens could synthesize GAD with pH optima in the acid range, so they can function even if the internal pH is varied (Foster, 2001). Two isolates (G30/1 and G254/2) were selected for kinetic analysis, and the efficiency of the GAD was assessed by measuring the GABA production and residual glutamic acid in the medium (Supplementary Table 3 and 4). The isolate G254/2 produces a maximum amount of GABA (12.73 ± 1.0 mg/ml) at 40 °C, pH of 6.5 after ten days of incubation (Figure 3a). The process was continued up to 30 days to study the consistency or enzyme activity, and the results indicated that increased incubation decreases the activity. When temperature and pH level is decreased GABA production also reduced. Therefore, GAD production by G254/2 depends on these two physical parameters (Figure 3a). Bacterial growth were assessed during optimum test condition and found to be normal (Figure 3b).

Another strain, G30/1, produced the maximum amount of GABA (8.96 ± 0.3 mg/ml) at 35 °C, pH of 4.5 after 15 days of incubation. In this case, increase in pH and temperature affects the productivity (Supplementary Table 3).

Previous studies reported that *L. paracasei*, isolated from Japanese traditional fermented fish, can produce GABA at a concentration of 302 mM (Komatsuzaki et al., 2005), *L. plantarum* DW12 produce about 4000 mg/l in a fermented red seaweed beverage system after 60 days of incubation (Ratanaburee et al., 2011). *L. lactis* (Lu et al., 2009; Siragusa et al., 2007), *Lb. buchneri* (Cho et al., 2007; Park & Oh, 2006), *Lb. delbrueckii* subsp. *bulgaricus* (Siragusa et al., 2007), *Bifidobacterium dentium* (Barrett et al., 2012), and *Streptococcus salivarius* subsp. *thermophilus* (Yang et al., 2008b) are reported to produce GAD with different efficiency and optimum conditions.

The current study has identified two GAD producing strains that are varied in their ability to produce GABA and optimum physical parameters like temperature and pH. The residual glutamic acid analysis also supported the above said statements (Supplementary Table 4).

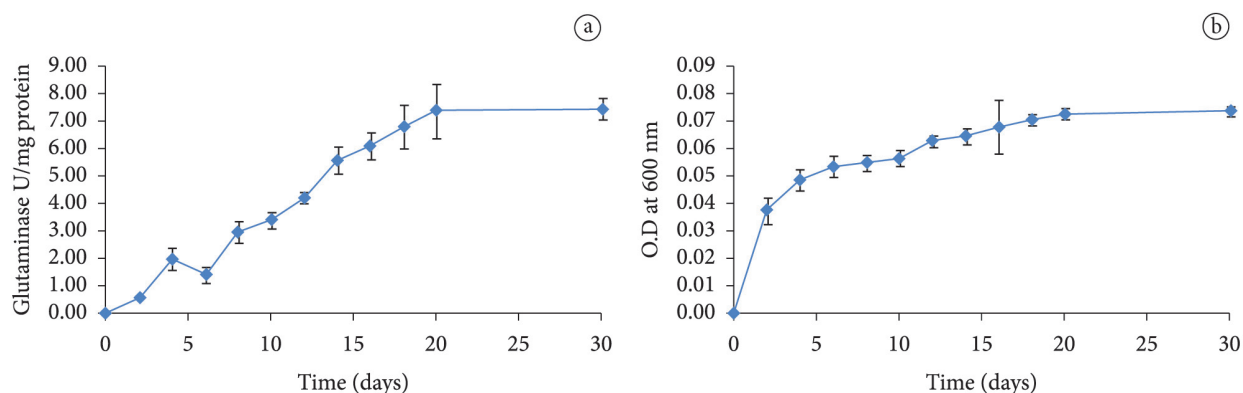


Figure 1. Kinetics of glutaminase production (a) and growth pattern (b) of G507/1 in optimum condition (Temperature at 35 °C, pH 6.0, 30 days of incubation).

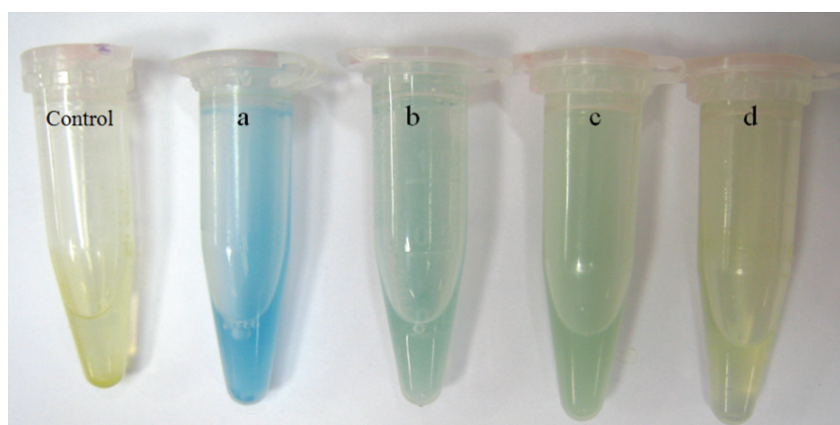


Figure 2. The representative picture shows high (a), medium (b) and low (c) level of GAD production during rapid colorimetric assay using bromocresol green as pH indicator for GAD activity. Medium control and negative results were denoted as control and (d), respectively.

3.4 Bacterial identification

16s rRNA based molecular identification of bacterial strains was employed to identify the glutaminase producer-G507/1 and GAD producer-G254/2. Sequencing and phylogenetic tree were construed as explained in materials and methods. G507/1, which is a glutaminase-producing LAB was identified as *Lactobacillus brevis* ATCC 14869 (Accession number KI271266) with 99.86%

of 16s rRNA sequence similarity (Figure 4a). The strain G254/2, which is a GAD-producing LAB was identified as *Lactobacillus fermentum* NBRC 3956 (Accession number AP008937) with 99.86% of 16s rRNA sequence similarity (Figure 4b). There is no detailed report about *L. fermentum* mediated GABA production. A previous report by Yokoyama et al. (2002) has enlightened the use of *L. brevis* for the production of γ -aminobutyric acid from the residue of alcohol distillery.

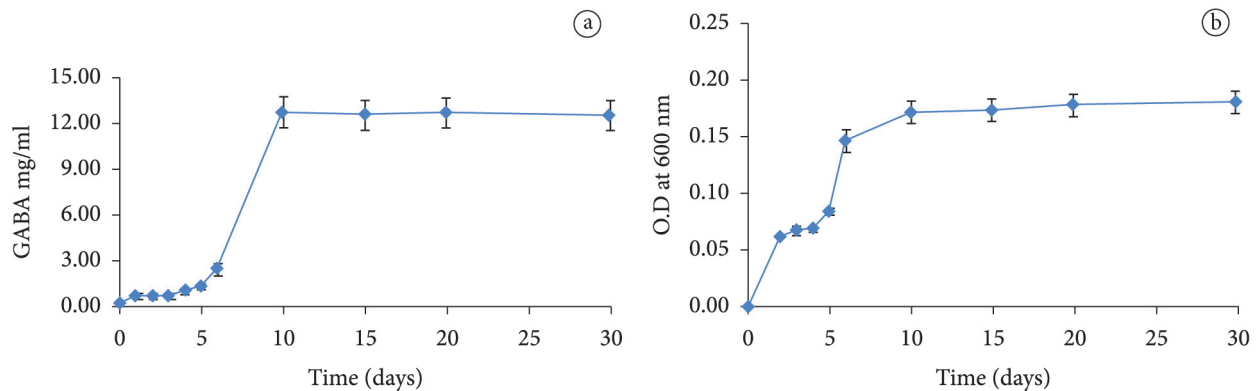


Figure 3. Kinetics of GABA production (a) and growth pattern (b) of G254/2 in optimum condition (Temperature at 40 °C, pH 6.5, 10 days of incubation).

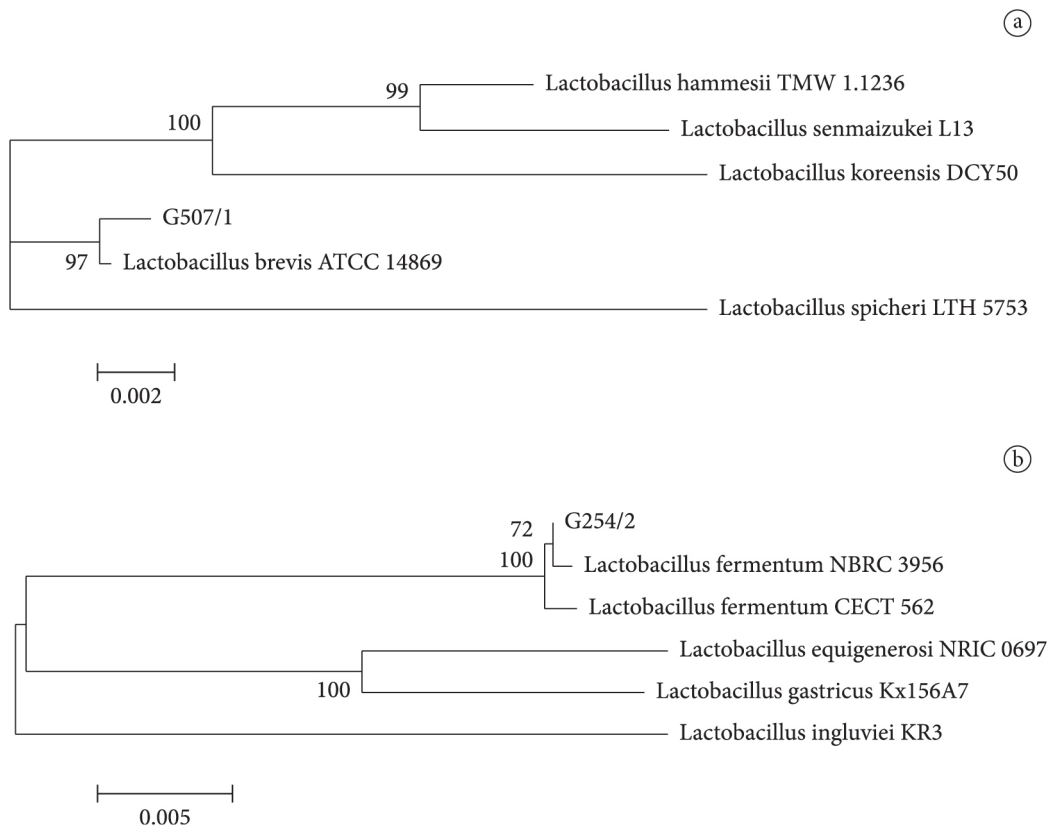


Figure 4. Phylogenetic representation of identified LABs (a) G507/1 displayed 99.86% of similarity with *Lactobacillus brevis* ATCC 14869 (Accession number KI271266) and (b) G254/2 was identified as *Lactobacillus fermentum* NBRC 3956 (Accession number AP008937) with 99.86% of similarity.

A study has reported the cloning and expression of GAD from *L. brevis* OPK-3 in *E. coli* (Park & Oh, 2007), but the enzyme was unstable. The GAD produced by *L. brevis* was biochemically well characterized previously (Hiraga et al., 2008). *L. brevis* PM17 was also reported for the GABA-producing ability (Siragusa et al., 2007).

4 Conclusions

The current investigation revealed that tested Thai fermented foods are rich in LABs with glutaminase and GAD producing capacity. Isolated *L. brevis* ATCC 14869 and *L. fermentum* NBRC 3956 produces relatively higher amount of glutaminase and GAD, respectively which can further used for fermentation processes as a safe starter. Further molecular characterization of these strains and other isolates will provide the comprehensive inspiration about use of these strains for the fermentation process in the future.

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Supplementary Table 1. Details of LAB and positives for glutaminase and GAD production isolated from fermented foods.

Samples	No. of Samples	No. of LAB isolates	Positive for Glutaminase	Positive for GAD
Fermented fruit	16	51	1	38
Fermented vegetable	24	119	10	88
Soy paste	9	40	2	31
Shrimp paste	5	21	3	9
Fermented fish	11	55	5	31
Fermented crab	3	21	-	14
Fermented pork and meat	4	31	1	26
Total	72	338	22	237

Supplementary Table 2. Impact of temperature and pH on glutaminase production. Values are representative of mean \pm SD of three independent experiments (ND: Not detected).

Strains	Days	Glutaminase production (U/mg protein)								
		Temperature								
		30 °C			35 °C			40 °C		
		pH6.0	pH7.0	pH8.0	pH6.0	pH7.0	pH8.0	pH6.0	pH7.0	pH8.0
G284/2	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
G507/1	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
G284/2	2	0.09 \pm 0	0.21 \pm 0.10	ND	1.14 \pm 0.10	ND	ND	ND	0.07 \pm 0.00	ND
G507/1	2	ND	ND	ND	0.54 \pm 0.10	ND	ND	ND	ND	ND
G284/2	4	0.20 \pm 0.02	0.22 \pm 0.10	ND	1.08 \pm 0.20	0.23 \pm 0.10	ND	ND	0.09 \pm 0.01	ND
G507/1	4	0.02 \pm 0	ND	ND	1.95 \pm 0.40	0.00 \pm 0.00	ND	ND	0.09 \pm 0.00	ND
G284/2	6	0.24 \pm 0.04	0.24 \pm 0.10	ND	1.37 \pm 0.30	0.37 \pm 0.10	ND	ND	0.17 \pm 0.01	ND
G507/1	6	0.07 \pm 0	ND	ND	1.37 \pm 0.30	0.00 \pm 0.00	ND	0.10 \pm 0.00	0.13 \pm 0.01	ND
G284/2	8	0.30 \pm 0.05	0.29 \pm 0.10	ND	2.31 \pm 0.30	0.64 \pm 0.10	ND	ND	0.24 \pm 0.02	ND
G507/1	8	0.26 \pm 0.10	0.02 \pm 0.00	ND	2.95 \pm 0.40	0.96 \pm 0.30	1.68 \pm 0.30	0.40 \pm 0.02	0.21 \pm 0.03	ND
G284/2	10	0.32 \pm 0.03	0.31 \pm 0.10	ND	2.51 \pm 0.30	0.59 \pm 0.10	0.35 \pm 0.10	ND	0.25 \pm 0.04	ND
G507/1	10	0.34 \pm 0.10	0.10 \pm 0.02	ND	3.38 \pm 0.30	1.29 \pm 0.20	2.75 \pm 0.20	0.47 \pm 0.10	0.31 \pm 0.01	ND
G284/2	12	0.43 \pm 0.20	0.50 \pm 0.10	ND	2.48 \pm 0.40	2.21 \pm 0.20	0.53 \pm 0.20	0.09 \pm 0.00	0.23 \pm 0.10	ND
G507/1	12	0.45 \pm 0.10	0.15 \pm 0.05	0.02 \pm 0	4.21 \pm 0.20	1.73 \pm 0.30	2.28 \pm 0.20	0.63 \pm 0.07	0.30 \pm 0.01	ND
G284/2	14	0.56 \pm 0.10	0.59 \pm 0.10	0.04 \pm 0	3.04 \pm 0.30	2.07 \pm 0.20	0.93 \pm 0.20	0.19 \pm 0.01	0.30 \pm 0.10	ND
G507/1	14	0.45 \pm 0.20	0.20 \pm 0.10	0.19 \pm 0.04	5.59 \pm 0.50	2.37 \pm 0.30	3.84 \pm 0.80	0.60 \pm 0.03	0.42 \pm 0.10	ND
G284/2	16	0.57 \pm 0.20	0.75 \pm 0.20	0.18 \pm 0.10	3.64 \pm 0.30	2.61 \pm 0.30	1.59 \pm 0.30	0.26 \pm 0.02	0.43 \pm 0.10	ND
G507/1	16	0.73 \pm 0.10	0.25 \pm 0.10	0.30 \pm 0.10	6.09 \pm 0.50	2.88 \pm 0.40	3.95 \pm 0.40	0.82 \pm 0.10	0.50 \pm 0.10	ND
G284/2	18	0.60 \pm 0.10	0.75 \pm 0.20	0.41 \pm 0.20	4.40 \pm 0.40	4.05 \pm 0.50	3.40 \pm 0.40	0.36 \pm 0.06	0.39 \pm 0.10	ND
G507/1	18	0.96 \pm 0.10	0.38 \pm 0.10	0.51 \pm 0.20	6.81 \pm 0.80	3.62 \pm 0.30	4.26 \pm 0.20	1.11 \pm 0.10	0.71 \pm 0.10	0.04 \pm 0.00
G284/2	20	0.99 \pm 0.10	1.11 \pm 0.10	0.87 \pm 0.10	4.70 \pm 0.70	5.06 \pm 0.50	3.56 \pm 0.50	0.50 \pm 0.10	0.81 \pm 0.10	ND
G507/1	20	1.52 \pm 0.10	0.41 \pm 0.10	0.96 \pm 0.10	7.39 \pm 1.00	4.92 \pm 0.40	5.29 \pm 0.50	1.28 \pm 0.10	0.86 \pm 0.10	0.16 \pm 0.03
G284/2	30	1.11 \pm 0.10	1.24 \pm 0.10	0.61 \pm 0.10	4.55 \pm 0.50	4.92 \pm 0.40	3.53 \pm 0.50	0.73 \pm 0.10	0.87 \pm 0.10	ND
G507/1	30	1.35 \pm 0.10	0.61 \pm 0.10	1.16 \pm 0.10	7.46 \pm 0.40	4.63 \pm 0.30	4.91 \pm 0.50	1.27 \pm 0.10	0.96 \pm 0.10	0.48 \pm 0.10

Supplementary Table 3. GABA production by LABs at different temperature and pH (ND: Not detected).

Strains	Days	GABA production (mg/ml)								
		Temperature								
		30 °C			35 °C			40 °C		
		pH4.5	pH5.5	pH6.5	pH4.5	pH5.5	pH6.5	pH4.5	pH5.5	pH6.5
G30/1	0	0.23 ± 0.10	0.38 ± 0.20	0.36 ± 0.10	ND	0.09 ± 0.01	0.25 ± 0.10	ND	ND	0.25 ± 0.10
G254/2	0	0.33 ± 0.10	0.49 ± 0.20	0.39 ± 0.10	0.20 ± 0.01	0.49 ± 0.20	0.25 ± 0.10	0.34 ± 0.10	0.30 ± 0.10	0.25 ± 0.10
G30/1	5	0.90 ± 0.10	1.89 ± 0.20	1.39 ± 0.10	2.26 ± 0.20	0.52 ± 0.20	0.37 ± 0.10	0.47 ± 0.10	0.43 ± 0.10	0.38 ± 0.10
G254/2	5	1.48 ± 0.20	1.74 ± 0.10	2.62 ± 0.10	0.76 ± 0.10	7.59 ± 1.00	7.51 ± 0.50	0.49 ± 0.10	0.70 ± 0.20	1.29 ± 0.20
G30/1	10	1.03 ± 0.10	1.72 ± 0.20	1.57 ± 0.10	8.93 ± 0.50	0.83 ± 0.10	0.51 ± 0.10	0.53 ± 0.10	0.65 ± 0.10	0.47 ± 0.10
G254/2	10	1.31 ± 0.10	1.43 ± 0.20	2.53 ± 0.10	8.17 ± 0.10	9.24 ± 0.20	8.74 ± 0.50	1.48 ± 0.20	5.03 ± 1.00	12.73 ± 1.0
G30/1	15	1.19 ± 0.10	1.61 ± 0.20	1.66 ± 0.10	8.96 ± 0.30	0.86 ± 0.20	0.50 ± 0.10	0.55 ± 0.10	0.66 ± 0.10	0.42 ± 0.10
G254/2	15	1.22 ± 0.20	1.58 ± 0.20	2.30 ± 0.10	8.17 ± 0.30	9.35 ± 0.30	8.76 ± 0.20	1.48 ± 0.20	5.44 ± 1.00	12.53 ± 1.0
G30/1	20	1.30 ± 0.20	1.49 ± 0.20	2.32 ± 0.20	8.94 ± 0.50	0.90 ± 0.10	0.61 ± 0.10	0.56 ± 0.10	0.68 ± 0.10	0.46 ± 0.10
G254/2	20	1.13 ± 0.10	1.44 ± 0.20	2.40 ± 0.05	8.21 ± 0.10	9.38 ± 0.30	8.82 ± 0.30	1.52 ± 0.30	5.90 ± 1.00	12.69 ± 1.0
G30/1	30	1.36 ± 0.30	1.51 ± 0.10	2.18 ± 0.10	8.96 ± 0.50	0.87 ± 0.20	0.58 ± 0.10	0.55 ± 0.10	0.65 ± 0.10	0.41 ± 0.20
G254/2	30	1.07 ± 0.10	1.45 ± 0.20	2.34 ± 0.04	8.18 ± 0.20	9.34 ± 0.30	8.77 ± 0.30	1.48 ± 0.20	5.65 ± 1.00	12.54 ± 1.0

Supplementary Table 4. Residual glutamic acid content at different temperature and pH. Values are represented as mean \pm SD of three experiments. (ND: Not detected).

Residual glutamic acid (mg/ml)								
Temperature								
30 °C			35 °C			40 °C		
pH4.5	pH5.5	pH6.5	pH4.5	pH5.5	pH6.5	pH4.5	pH5.5	pH6.5
9.06 \pm 1.00	9.67 \pm 1.00	10.53 \pm 1.00	29.98 \pm 1.0	20.23 \pm 2	10.74 \pm 1	29.26 \pm 1.0	16.70 \pm 2	17.06 \pm 2
8.15 \pm 1.00	8.85 \pm 1.00	16.72 \pm 1.00	34.10 \pm 2.0	19.63 \pm 2	8.86 \pm 2	30.27 \pm 2.0	16.13 \pm 2	15.76 \pm 1
0.15 \pm 0.02	0.03 \pm 0.004	0.03 \pm 0.001	16.66 \pm 2.0	12.49 \pm 2	7.83 \pm 0.8	23.93 \pm 1.0	8.37 \pm 1	10.26 \pm 1
0.34 \pm 0.03	0.03 \pm 0.001	0.03 \pm 0.002	23.52 \pm 1.0	0.37 \pm 0.1	ND	24.29 \pm 2.0	8.77 \pm 2	10.07 \pm 2
0.10 \pm 0.01	0.03 \pm 0.002	0.03 \pm 0.002	2.09 \pm 0.15	12.27 \pm 1	6.91 \pm 1	23.14 \pm 2.0	6.29 \pm 1	6.34 \pm 1
0.03 \pm 0.001	0.03 \pm 0.003	0.03 \pm 0.003	6.57 \pm 0.50	0.25 \pm 0.1	ND	21.29 \pm 2.0	5.03 \pm 2	0.05 \pm 0.02
0.03 \pm 0.01	0.03 \pm 0.003	0.03 \pm 0.004	1.98 \pm 0.1	12.18 \pm 1	6.70 \pm 1	22.92 \pm 1.0	6.07 \pm 1	6.26 \pm 1
0.03 \pm 0.001	0.03 \pm 0.002	0.03 \pm 0.004	6.46 \pm 0.04	0.24 \pm 0.1	ND	19.75 \pm 2.0	4.76 \pm 2	0.03 \pm 0.01
0.03 \pm 0.01	0.03 \pm 0.003	0.03 \pm 0.002	1.95 \pm 0.1	11.91 \pm 2	6.38 \pm 1	22.60 \pm 2.0	5.83 \pm 1	6.13 \pm 1
0.29 \pm 0.04	0.01 \pm 0.001	0.03 \pm 0.004	6.35 \pm 0.3	0.23 \pm 0.1	ND	18.66 \pm 2.0	4.46 \pm 2	0.04 \pm 0.01
0.03 \pm 0.01	0.03 \pm 0.002	0.03 \pm 0.004	1.92 \pm 0.45	12.06 \pm 2	6.18 \pm 1	22.71 \pm 2.0	5.71 \pm 1	5.57 \pm 1
0.03 \pm 0.002	0.03 \pm 0.004	0.03 \pm 0.004	6.57 \pm 0.5	0.22 \pm 0.1	ND	17.64 \pm 2.0	4.46 \pm 2	0.02 \pm 0.01