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## Enhancement of $\gamma$ -Aminobutyric Acid Production by Co-Culturing of Two Lactobacilli Strains

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### ABSTRACT

The  $\gamma$ -Amino Butyric Acid (GABA) is a major inhibitory neurotransmitter in central nervous system and its application in drugs and functional foods has attracted great attention. Most of the fermented food products are known and proven for its high content GABA producer, which makes the food product as potential functional foods. Lactic acid bacteria possess special physiological activities and are generally regarded as safe. This study was aimed at evaluating GABA production ability of some lactic acid bacteria strains isolated from Egyptian dairy products and further to increase the production by combining the best GABA producers. The impacts of pH, temperature, incubation time, initial monosodium glutamate (MSG) concentration and pyridoxal-5'-phosphate (PLP) addition on growth and  $\gamma$ -aminobutyric acid production were investigated. By optimizing these factors at pH5, cultivation temperature of 35°C, cultivation time of 72 h in medium supplemented with 750 M of MSG and 200  $\mu$ M of PLP, *Lactobacillus brevis* NM101-1 and *Lactobacillus plantarum* DSM749 accumulated 168.58 and 140.69 mM of GABA, respectively. Co-culturing of both strains was not only able to enhance GABA production but also producing it in a short time (48 h), reached 224.69 mM. It could be concluded that the use of LAB strains in combination with a high GABA producer strain is a successful approach for reaching a physiologically active concentration of GABA.

**Key words:**  $\gamma$ -aminobutyric acid, fermentation, lactic acid bacteria, mixed cultured

### INTRODUCTION

Gamma-aminobutyric acid (GABA), which is also known as 4-amino-butanoic acid, is a ubiquitous non-protein amino acid that is widely distributed in nature from microorganisms to plants and animals (Li *et al.*, 2010). It is synthesized by glutamate decarboxylase (GAD), pyridoxal-5-phosphate-dependent enzyme that catalyzes irreversible  $\alpha$ -de-carboxylation of L-glutamate to GABA. It acts as the major inhibitory neurotransmitter in the mammalian central nervous system (Huang *et al.*, 2007). In addition, GABA has hypotensive, tranquilizing and diuretic effects and can prevent diabetes (Hayakawa *et al.*, 2005). Also, GABA involved in cardiovascular function regulation such as lowering blood pressure and heart rate (Ueda *et al.*, 2007). Furthermore, GABA would also have an inhibitory effect on cancer cell proliferation, stimulate cancer cell apoptosis. Therefore, GABA has potential as a bioactive component in foods and pharmaceuticals (Kim *et al.*, 2009). However, the direction addition of chemical GABA to food is considered unnatural and unsafe as it causes some diseases such as cancer, heart disease and diabetes (Seok *et al.*, 2008). So it is necessary to find a natural method to produce and increase GABA in food.

In the biological method, microbial fermentation is regarded as the effective one because of its convenience and a high ratio of transformation. Several safe microorganisms including Lactic Acid

Bacteria (LAB) have been widely applied in GABA production (Park and Oh, 2007). The LAB possess special physiological activities and are generally regarded as safe and have been extensively utilized in food industries for a long time (Karahana *et al.*, 2010). In addition, the biosynthetic production of natural GABA produced by LAB for the manufacturing of food can make full use of the health-promoting properties of GABA and LAB themselves. In recent years, the GABA production by using LAB as bacterial cell factories has therefore been a focus of research (Li and Cao, 2010). Some fermented products enriched in GABA using GABA producing LAB as starters such as dairy products and cheese have been developed (Li *et al.*, 2008).

This study was aimed at studying various fermentative parameters such as pH, temperature, cultivation time and media additives to obtain a high production of GABA by *L. brevis* NM101-1 and *L. plantarum* DSM749 isolated from Egyptian dairy products. Effect of co-culturing on growth and GABA production was also evaluated.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions:** The Lactobacilli species were isolated from 24 samples of traditional Egyptian dairy products collected from Cairo markets as described by Rushdy and Gomaa (2013). All strains from the samples were screened for their GABA producing ability. For GABA production, the strains were subsequently inoculated into 250 mL Erlenmeyer flasks containing 50 mL of the production media, which consisted of following compositions ( $\text{g L}^{-1}$ ): glucose, 50; soya peptone, 25;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.01 and Tween 80, 2 mL. Unless otherwise emphasized, the initial monosodium glutamate (MSG) concentration in the medium was 500 mM. The seed medium was composed of ( $\text{g L}^{-1}$ ): glucose, 50; soya peptone, 25;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.01; L-glutamate, 150 mM and Tween 80, 2 mL. Nitrogen sources, glutamate and the other compositions were autoclaved separately at  $121^\circ\text{C}$  for 20 min and mixed together prior inoculation. Lactobacilli strains were cultured in the seed medium at  $30^\circ\text{C}$  for 24 h and then used for seed culture inoculation. The flasks were inoculated with 10% (v/v) seed culture and incubated with shaking at  $30^\circ\text{C}$  for 72 h.

### Strain identification

**16S rRNA sequencing and analysis:** The isolates showing the highest GABA production were identified by 16S rRNA sequencing according to the method of Rochelle *et al.* (1995). The gene sequencing was done by Macrogen (South Korea). The DNA sequences were aligned using Gene Mapper v. 4.1 and data collection v. 3.1 Communication Patch1. To extract the genomic DNA, bacterial colonies were picked up with a sterilized toothpick and suspended in 0.5 mL of sterilized saline then centrifuged at  $8000 \times g$  for 10 min. After removal of the supernatant, the pellet was suspended in 0.5 mL of Insta Gene Matrix (Bio-Rad, USA), incubated at  $56^\circ\text{C}$  for 30 min and then heated to  $100^\circ\text{C}$  for 10 min. After heating, supernatant can be used for Polymerase Chain Reaction (PCR). Bacterial 16S rRNA gene was amplified using the following universal primers for eubacteria: forward primer 27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR was performed using kits with AmpliTaq DNA polymerase (Fenzyme) (Applied Biosystems, USA).

One microlitre of template DNA was added in 20  $\mu\text{L}$  of PCR solution. The 27 F/1492R primers were used for bacteria. Thirty five amplification cycles were performed at  $94^\circ\text{C}$  for 45 sec,  $55^\circ\text{C}$  for 60 sec and  $72^\circ\text{C}$  for 60 sec. The PCR amplicon were purified by using Montage PCR clean-up kit (Millipore). The purified PCR products of approximately 1400 bp were sequenced by using

2 primers 518 F (5'-CCA GCA GCC GCG GTA ATA Cg-3') and 800R (5'-TAC CAG GGTATC TAATCC -3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems). Sequence analysis was performed with sequences in the National Center for Biotechnology Information (NCBI), USA database using Basic Local Alignment Search Tool for Nucleotides (BLASTN) (Altschul *et al.*, 1997). All identified isolates were kept at -80°C in De Man-Rogosa-Sharpe broth (MRS) with glycerol (30% v/v). The strains were serially transferred at least three times prior to use.

**GABA quantification:** The contents of extracellular GABA accumulated in the culture medium were extracted according to the method of Yang *et al.* (2008) with minor modifications. First, the culture broth was separated from cells by centrifugation (8000×g for 15 min at 4°C) and the supernatant was diluted 50 fold with 7% (v/v) of glacial acetic acid. The diluted sample was centrifuged at 8000×g for 15 min at 4°C and the supernatant was filtered using 0.22 µm pore size membranes and then collected for further analysis.

One hundred microlitre aliquot of the extracellular GABA were derivatives to Phenylthiocarbamyl-GABA (PTC-GABA) and analyzed by HPLC according to Rossetti and Lombard (1996) with minor modifications. The dried residue containing PTC-GABA was dissolved in 100 µL of mobile phase, consisting of a mixture of 80% solution A (aqueous solution of 13.608 g sodium acetate-3-hydrate, 0.5 mL triethylamine, 0.7 mL acetic acid and 5.0 mL acetonitrile in 1000 mL deionized water) and 20% solution B (acetonitrile-water (60:40)) adjusted to pH 5.8. Isocratic HPLC separation was performed using HPLC (Shimadzu Asia PTe.) with UV set of 254 nm and PDA detector. The used column was puerospher STAR RP-18 LiChroCART (250-4.6 Merck, Darmstadt, Germany) and the column temperature was set at 35°C. The flow rate for the mobile phase of isocratic elution was 1.0 mL min<sup>-1</sup>. GABA were detected by comparison of their retention time (R<sub>t</sub>) values and spectra with known standards and determined by peak areas from the HPLC chromatograms.

**Cell growth:** Cell growth was monitored by measuring the optical density at 600 nm using spectrophotometer (UV-VIS Double Beam PC, Labomed INC). Ten milliliter culture medium was centrifuged at 10,000 rpm, 4°C for 15 min and the cell pellet was washed with 10 mL distilled water. The cell pellet was harvested by centrifugation and dried at 105°C for 48 h or till constant weight was obtained. Cell mass concentration was determined by the standard calibration curve between OD<sub>600</sub> and cell dry weight.

**Effect of initial pH, temperature and cultivation time on GABA production:** The effect of pH, temperature and cultivation time on GABA production were determined by cultivating the strains in a culture medium using the following conditions pH (4, 5, 6, 7 and 8), temperatures (25, 30, 35, 37 and 40°C) and cultivation time (24, 48, 72, 96 and 120 h)

**Effect of monosodium glutamate (MSG) concentrations and addition of pyridoxal-5-phosphate (PLP) concentration on GABA production:** The effect of different concentrations of MSG (250, 500, 750 and 1000 mM) and PLP addition (50, 100, 150 and 200 µM) on the GABA production were investigated at optimum pH, temperature and cultivation time.

**Statistical analysis:** All experiments were done in triplicate and the results were presented as Mean±Standard Deviation. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of  $p < 0.05$ .

## RESULTS AND DISCUSSION

The consumer attention towards the selection of health beneficial foods makes the significant growth of GABA-enriched foods. GABA is present in small quantities in many plant sources, vegetables, for example spinach, potatoes, cabbage asparagus (broccoli), tomatoes, fruits, for example apples, grapes, in cereals, for example barley, maize. The increased amount is found mainly in fermented products, especially fermented dairy products, soy sauce (Yamakoshi *et al.*, 2007), cheeses (Siragusa *et al.*, 2007) and so on. Natural addition of GABA is in high demand over the addition of chemical nutrient GABA since consumers prefer naturally occurring substances and the fermentation helps to reduce the cost of the foods due to the omission of chemical addition of GABA and also provides attractive foods with better taste and at the same time replaces the chemical GABA by natural GABA (Li and Cao, 2010). Therefore, GABA production by naturally occurring microorganisms during fermentation is getting higher request.

The most interesting and practical group of bacteria for GABA production is LAB, which produce high levels of GABA (Watanabe *et al.*, 2007). Lactic acid bacteria possess special physiological activities and are generally regarded as safe. Therefore, using lactic acid bacteria as cell factories for gamma-aminobutyric acid production is a fascinating project and opens up a vast range of prospects for making use of GABA and LAB. In the present study, twenty three lactobacilli species were isolated from 24 dairy samples of traditional Egyptian dairy products. Out of 23 bacterial isolates, eight strains showed GABA productivity. The strains exhibited the highest GABA production were identified by 16S rRNA gene sequence analysis. The sequence alignment using BLASTN software for the comparison up to 1500 bp of the analysis gave a high homology of 99% homology to *L. brevis* NM101-1 and *L. plantarum* DSM749, respectively.

**Factors affecting GABA synthesis:** Generally, bacterial glutamate decarboxylases are located in cytoplasm, whereas glutamate and GABA can be transported through membrane and exchanged, which results in the extracellular accumulation of GABA. Different fermentation factors affect the rate of extracellular GABA production by microorganisms. Among them, the most common and essential factors are pH, temperature, cultivation time and media additives of culture.

**Effect of initial pH:** The biosynthesis of GABA in microorganisms is mainly regulated by pH, which usually has the most pronounced effect for a fermentation process (Yang *et al.*, 2008). To investigate the effect of different pH levels on biomass and the production of GABA by *L. brevis* and *L. plantarum*, the initial pHs of the media were adjusted to 4, 5, 6, 7 and 8. As shown in Fig. 1a, b, pH has a significant effect on the production of biomass and GABA production. The optimum GABA production by *L. brevis* and *L. plantarum* reached 80.47 and 75.82 mM was recorded at pH 5. Further increase in the pH led to a decrease of GABA production. These results accorded with the previous reports about the optimal pH values for maintaining the activity of LAB GAD were in the range of 4.0-5.0 (Huang *et al.*, 2007; Komatsuzaki *et al.*, 2008). The higher or lower pH may lead to the partial loss of the GAD activity. Co-culturing of *L. brevis* and *L. plantarum* in a ratio of 1:1 showed more drastic difference on the production of GABA, where the extensively large amount of 96.14 mM was recorded at pH5 (Fig. 1c). The GABA production appeared to correlate with the acidification of the medium, hence, it is probable that two strains collaborated in the production of GABA as a result of acid-resistant response.

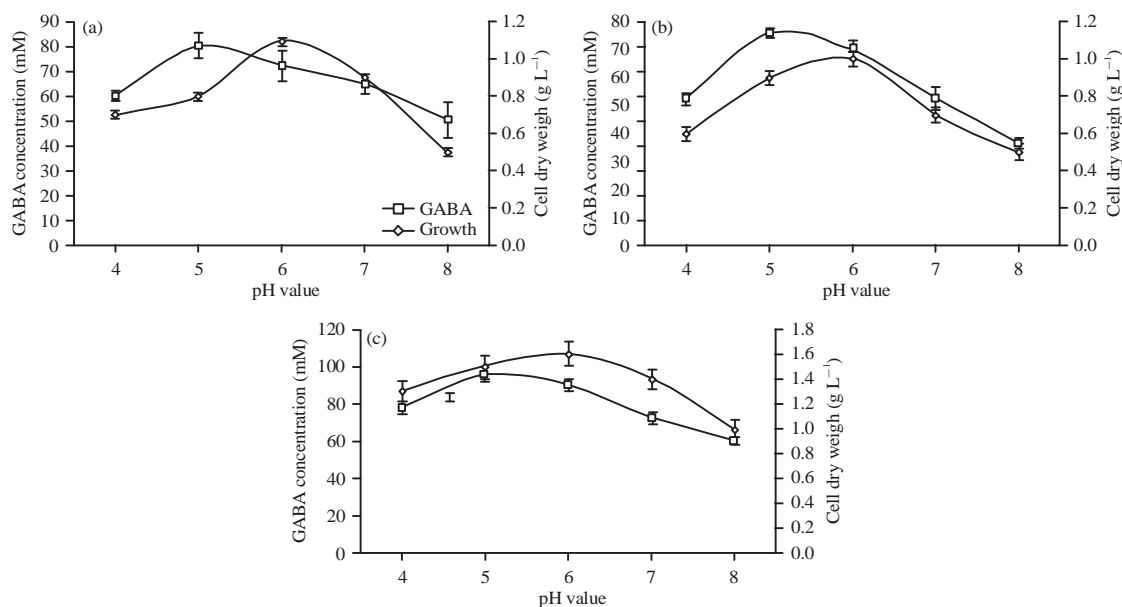


Fig. 1(a-c): Effect of the initial pH on growth and extracellular production of GABA of (a) *L. brevis*, (b) *L. plantarum* and (c) Co-cultured of both *L. brevis* and *L. plantarum*, results are means of three independent determinations and error bars represent the standard deviation

It was reported that GABA production can be effectively improved by maintaining cellular pH of the medium to acidic conditions because GAD must consume an H<sup>+</sup> ion for GABA production (Yang *et al.*, 2008). In addition, pH-regulated genes encoding a GABA transporter was shown to have an acidic pH optimum and preferentially expressed at acidic ambient pH (Espeso and Arst, 2000). In microorganisms, GABA can be decomposed through this pathway: GABA transaminase catalyses the reversible conversion of GABA to succinic semi aldehyde using either pyruvate or  $\alpha$ -ketoglutarate as amino acceptor and succinic semi aldehyde dehydrogenase catalyses the reversible conversion of succinic semialdehyde to succinate. Since the above mentioned enzymes have the alkaline pH optimum (Kumar *et al.*, 2000), therefore, acidic condition can block the enzymes that cause GABA to decompose resulted in enhanced accumulation of GABA in the medium.

Initial pH of the culture medium can influence the growth rate of Lactobacilli. The initial pH value of 6.0 caused the highest growth rate of *L. brevis* and *L. plantarum*. In the same regard, Zacharof and Lovitt (2010) reported that the maximum growth rate of *L. plantarum* occurs at moderately acidic initial pH of 6.5. High cell density, in general, is also required for efficient GABA biosynthesis (Li and Cao, 2010). The GAD is the enzyme to make GABA from L-glutamate and utilizes a single proton during the catalysis. This consumption of the intracellular proton is responsible for shifting the intracellular pH toward neutral, hence, it helps microbes to survive under the acidic environment. Since growth and subsequently expression of the GAD gene requires this type of pH changing therefore, stabilization of pH e.g., using buffer system would suppress the gene expression and subsequently GABA production.

**Effect of temperature:** The incubation temperature is also a major factor affecting maximum GABA yield by fermentation. In addition to an effect on biocatalyst activity and stability,

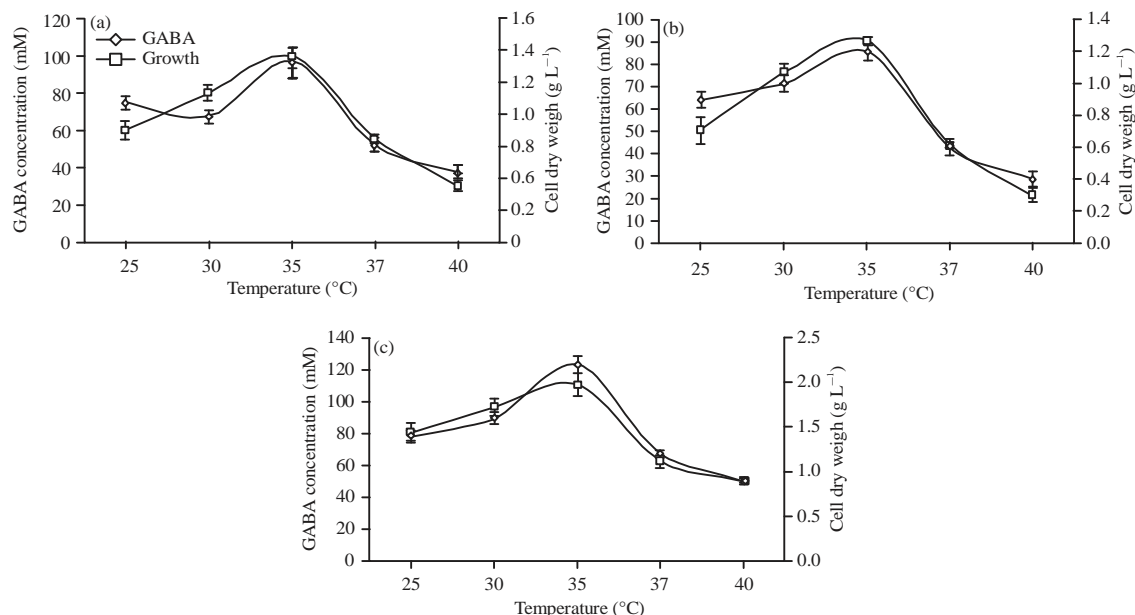


Fig. 2(a-c): Effect of cultivation temperature on growth and extracellular production of GABA of (a) *L. brevis*, (b) *L. plantarum* and (c) Co-cultured of both *L. brevis* and *L. plantarum*, results are means of three independent determinations and error bars represent the standard deviation

temperature has an effect on the thermodynamic equilibrium of a reaction (Kim *et al.*, 2009; Li *et al.*, 2010). Figure 2a, b shows that the considerable variation in the growth of *L. brevis* and *L. plantarum* and GABA production under different fermentation temperatures. The bacterial growth increased with the increase of temperature and peaked at 35°C, then decreased with the increase of temperature. For GABA production, a trend similar to the bacterial growth was observed. The effective synthesis of GABA reached 100 mM and 90.6 mM by *L. brevis* and *L. plantarum*, respectively was recorded at 35°C. Results illustrated in Fig. 2c show that co-culturing of both *L. brevis* and *L. plantarum* resulted in enhancement of GABA production reached 110.66 mM. These data indicated that appropriate temperature was beneficial to produce GABA and excessively high temperature was unfavorable to the GABA production (Tung *et al.*, 2011). Results indicated that high efficient conversion glutamate to GABA needed not only high cell density but also appropriate temperature.

**Time course study of extracellular GABA content:** The time factor plays an important role in the fermentation and the production of GABA as temperature and pH do. *L. brevis* and *L. plantarum* required 72 h of fermentation to reach the highest production of GABA at 100 and 90.68 mM, respectively (Fig. 3a, b). In the same trend, Di Cagno *et al.* (2010) reported that *L. plantarum* DSM19463 required 72 h of fermentation to reach the highest production of GABA at 4.83 mM.

From the results depicts in Fig. 3c, it is worth mentioning that co-culturing of both *L. brevis* and *L. plantarum* was not only able to enhance GABA production reached 125.56 mM but also it produced it in a short time (48 h).

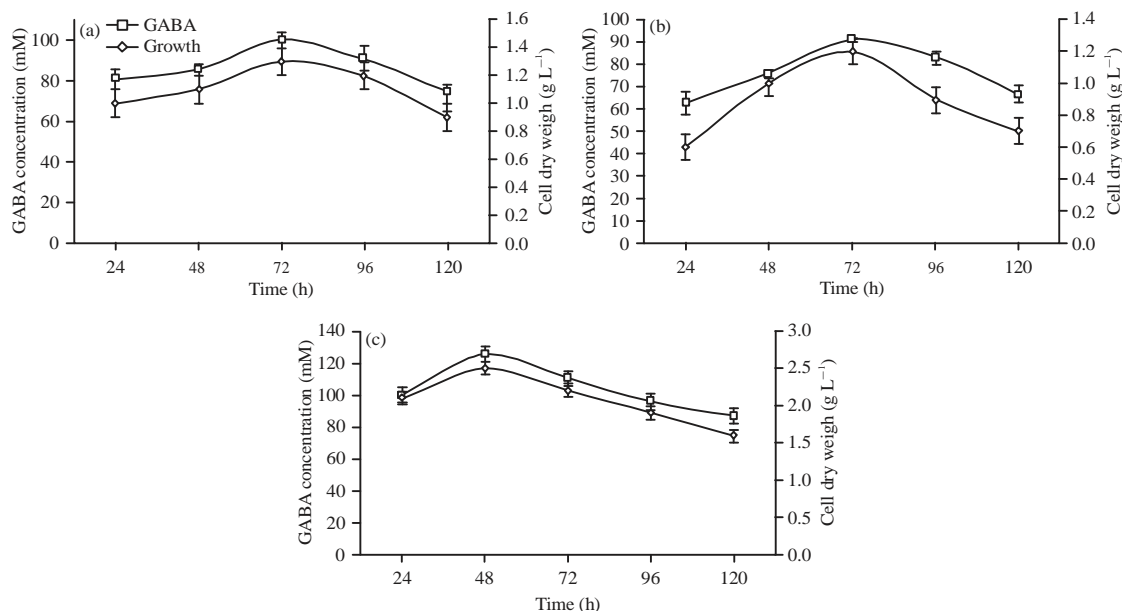


Fig. 3(a-c): Time profiles of growth and extracellular production of GABA of (a) *L. brevis*, (b) *L. plantarum* and (c) Co-cultured of both *L. brevis* and *L. plantarum*, results are means of three independent determinations and error bars represent the standard deviation

**Effect of initial monosodium glutamate concentration:** Sodium glutamate is known flavour enhancer in foods but its higher intake is associated with human body disorders like stroke, autism and Alzheimer's disease because of its excitatory neurotransmitter effect in mammalian nervous system. Hence, it is very crucial to minimize the usage of glutamate in food industry for human consumption (Ali *et al.*, 2009).

The content of produced GABA depends on various conditions such as the amount of precursor, an addition of salt, enzyme and the effect of pH (Mazur *et al.*, 2011). As shown in Fig. 4a and b, the highest production of GABA by *L. brevis* and *L. plantarum* reached 110.89 and 99.12 mM, respectively were recorded in the presence of 750 M of monosodium glutamate (the precursor), however, when the concentration of monosodium glutamate exceeded 750 M, GABA production decreased. Co-culturing of both *L. brevis* and *L. plantarum* resulted in enhancement of GABA production reached 160.57 mM (Fig. 4c). So it can be deduced that the GABA production may be improved with increased concentration of MSG added to the culture medium and the MSG concentration above a certain level would inhibit cell growth.

In the same regard, GABA concentration reached 161 mM after cultivation in the medium containing 500 mM of glutamate by *L. paracasei* NFRI 7415 (Komatsuzaki *et al.*, 2005). *Lactobacillus brevis* also increased GABA production by the addition of 6% glutamic acid (Binh *et al.*, 2014). However, *S. salivarius* subsp. *thermophilus* Y2 did not increase GABA production significantly when glutamate was added 10-20 g L<sup>-1</sup> of media, suggesting that these concentrations of glutamate are not appropriate for the synthesis of GABA in this species (Yang *et al.*, 2008).

The addition time for the GABA substrate also affects the final GABA yield as well as the concentration of the substrate in the medium. A significant difference in GABA yield among various times of MSG addition was shown in Fig. 4a-c. It is clear that individual strains produced the



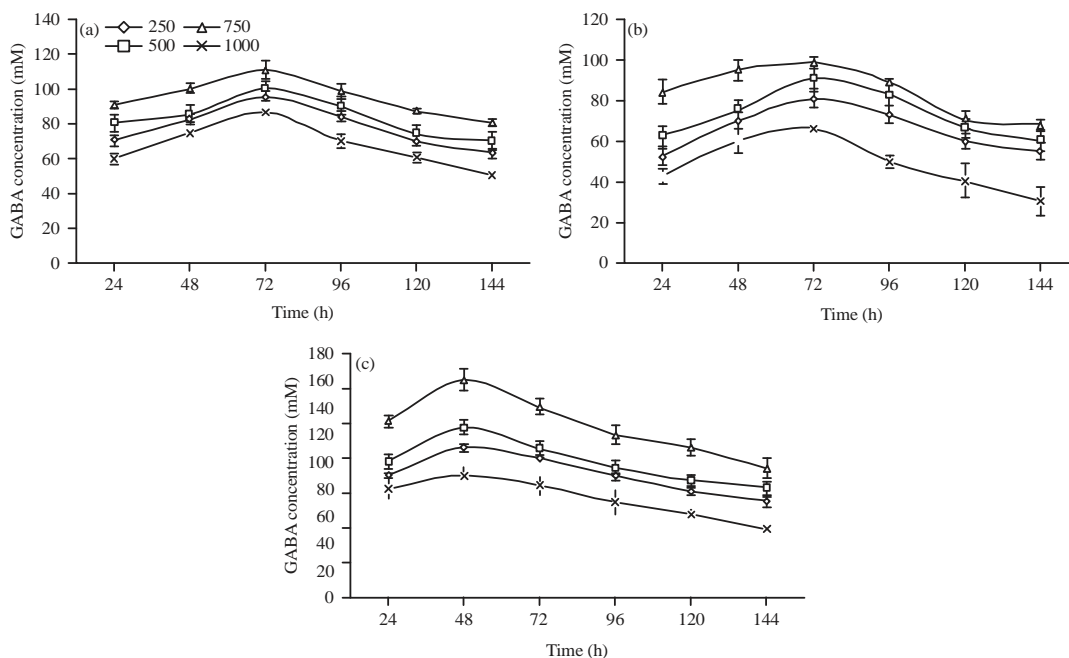


Fig. 4(a-c): Effect of initial monosodium glutamate (MSG) concentration on extracellular production of GABA by (a) *L. brevis*, (b) *L. plantarum* and (c) Co-cultured of both *L. brevis* and *L. plantarum*. Results are means of three independent determinations and error bars represent the standard deviation

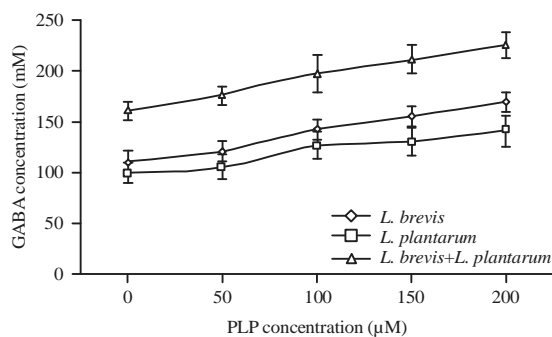


Fig. 5: Effect of PLP addition on extracellular production of GABA by *L. brevis*, *L. plantarum* and co-cultured of both *L. brevis* and *L. plantarum*, Results are means of three independent determinations and error bars represent the standard deviation

highest GABA at 72 h while co-culturing of both strains was at 48 h. These results indicate that the highest GABA production by microorganisms can depend on the addition of appropriate medium additives and optimum additional time for the additives.

**Effect of PLP addition on extracellular GABA production:** Glutamic acid decarboxylase (GAD, EC 4.1.1.15) catalyzes the irreversible  $\alpha$ -decarboxylation of glutamate to produce GABA. The GAD uses PLP as coenzyme. So, it might be hypothesized that addition of PLP to the culture medium would improve GABA production. The effect of PLP addition was examined by analysis

of extracellular GABA content in culture broth of *L. brevis* and *L. plantarum* with PLP at different concentrations. Figure 5 shows that the higher the concentration of PLP was the higher level of extracellular GABA the strain would produce. The highest concentration of GABA reached 168.58 and 140.69 mM produced by *L. brevis* and *L. plantarum*, respectively were recorded at 200  $\mu$ M PLP. In the same line, Lin (2013) reported that *L. rhamnosus* YS9 in medium supplemented with 200 mM of monosodium glutamate and 200  $\mu$ M of pyridoxal phosphate (PLP), produced 187 mM of GABA. These results differed from which reported by Li *et al.* (2010) that PLP did not affect the cell growth and gamma-aminobutyric acid production of *L. brevis* NCL912.

Co-culturing of both *L. brevis* and *L. plantarum* resulted in enhancement of GABA production reached 224.69 mM at 200  $\mu$ M PLP. The results suggest that co-culturing of the two strains raises the each other's properties of the acidification of the medium and production of GABA or acid resistance (Watanabe *et al.*, 2011).

## CONCLUSION

*Lactobacillus brevis* and *L. plantarum* can be potential starter cultures for GABA-enriched functional foods. Co-culturing of microbes is beneficial not only for food production but also for human health since a large number of microbes live together in the animal intestines, in other word they are co-culturing. The microbes may collaborate to carry out important biological functions; however, very little is understood. The mechanism of the co-culturing of different microbes needs to be explored and the findings can be extended to the industrial application as well as well as the improvement of human health.

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