Molecular gene cloning and sequencing of *glutamate decarboxylase* gene from *Lactobacillus delbrueckii* and *Lactobacillus reuteri*

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ABSTRACT

Glutamate decarboxylase enzyme produces γ-aminobutyric acid (GABA) in a non-reversible decarboxylation reaction of glutamate. GABA is a major inhibitory neurotransmitter of the brain and it is also present at high concentration in other organs such as pancreatic islets. GABA has effects on blood pressure, diabetes, inflammation, sleeplessness and depression. Some bacteria such as *Lactobacillus* strains are capable of GABA production. Identification of these bacteria is important both for researchers and industry. The aim of this study was molecular gene cloning and sequencing of *glutamate decarboxylase* (*gad*) from *Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842* and *Lactobacillus reuteri ATCC 23272*. These strains were cultured in MRS medium at 37°C for 24 hours. For cloning *gad* gene from these strains, polymerase chain reaction (PCR) was performed using specific primers designed by Oligo7 software. PCR production was extracted from agarose gel and was inserted into PGEM-T vector using T4 DNA ligase enzyme and then it was transformed to *E. coli XL1Blue*. In the final step, white colonies were selected and after plasmid extraction, the existence of *gad* gene in recombinants was confirmed by PCR. *Gad* gene was cloned from *Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842* and *Lactobacillus reuteri ATCC 23272*. It is for the first time that the *gad* gene sequences from these bacteria were registered on NCBI with accession numbers KF751355 and KF751352 respectively. The result of this research indicates that the two aforementioned bacteria contain *glutamate decarboxylase* gene and therefore they possibly can be used for industrial γ-aminobutyric acid production.

Key words: Glutamate decarboxylase; γ-aminobutyric acid; *Lactobacillus delbrueckii subsp. Bulgaricus*; *Lactobacillus reuteri*

INTRODUCTION

Glutamate decarboxylase or glutamic acid decarboxylase (GAD) is a vitamin B6 dependent pyridoxal enzyme, thus it uses pyridoxal 5′-phosphate (PLP) as a cofactor. This enzyme catalyzed carboxylation of glutamic acid and results in γ-aminobutyric acid (GABA) and carbon dioxide production [1]. GAD can be found in eukaryotes and prokaryotes including bacteria, fungi and yeasts. It exists in gram-negative bacteria such as *E. coli* as well as gram-positive bacteria like *Listeria monocytogenes, Lactobacillus delbreuckii, Lactobacillus paracasei, Lactobacillus brevis, and Lactobacillus reuteri*. GAD in these bacteria has beneficial roles, including maintenance of the physiological pH under acidic conditions and ATP production during glutamate metabolism [2]. In vertebrates and mammals like human being, the GAD enzyme exists in brain in two isoforms with molecular weight of 65 and 67 kilo Dalton and has prominent physiological roles in neural network system of the body by production of gamma amino butyric acid (GABA) [3-6].

GABA is a four-carbon amino acid with molecular formula of C4H9NO2 which cannot be found in the structure of protein. It exists in extensive amounts in microorganisms, plants and animals. GABA is one of the most important inhibitory neurotransmitter in central neural system of vertebrates [3, 6]. It has many important functions in human body such as: improving mental health [7], tranquilizing effects,

curing insomnia and depression [8], treatment of autoimmune disease [9], reducing blood pressure in human and laboratory animals [10-13], immune cells stimulator [14], controlling diabetes [15, 16], anti-tumor properties [17] and treatment of neurological abnormalities such as epilepsy, schizophrenia, spasm, Parkinson's and Alzheimer's diseases [5].

As mentioned before, GABA is produced by glutamate decarboxylase enzyme, and because of this, identification of bacteria that have this gene as well as cloning and sequencing of *gad* gene in order to produce GABA with low cost is of great importance. A number of microorganisms such as *E .coli* were widely used in biological systems for the production of GABA. Not all bacteria are safe for GABA production. For example *E. coli* could produce Shiga toxin and it may have pathogenicity, therefore, in recent years, GABA production by lactic acid bacteria is further considered [18, 19].

In this research, identification and sequencing of *glutamate decarboxylase* gene from *Lactobacillus delbrueckii subsp bulgaricus ATCC 11842* and *Lactobacillus reuteri ATCC 23272* was performed.

MATERIALS AND METHODS

Bacterial strains and plasmids:

In the current research, *Lactobacillus delbrueckii* subsp *bulgaricus ATCC 11842* and *Lactobacillus reuteri ATCC 23272* were bought from Pasteur Institute of Iran (Tehran, Iran). *E.coli XL1 Blue* (Novagen, Inc., San Diego, CA, USA) was used for plasmid manipulations. PGEM-T (Promega, Madison, WI) plasmid which is a T/A vector and has a sticky end was used as the clone vector.

Culture condition:

Lactobacillus delbrueckii subsp bulgaricus ATCC 11842 and *Lactobacillus reuteri ATCC 23272* bacteria were cultivated in MRS medium at 37°C for 24 hours. *E.coli XL1 Blue* was cultivated in liquid LB medium at 37°C with shaking. Ampicillin was added, when necessary, at a final concentration of 100 mg/ml.

Genomic DNA extraction and PCR amplification:

Genomic DNA extraction of *Lactobacillus delbrueckii subsp bulgaricus* and *Lactobacillus reuteri* was performed using a DNA isolation kit (CinnaGen Co., Tehran, Iran) according to manufacturer's instructions. The concentrations of extracted DNA samples were determined by using spectrometry method.

The genomic DNA which was extracted from two aforementioned bacteria was used as a pattern for *gad* gene manipulation. The primers were designed using Oligo7 software according to the nucleotide sequence of *gad* gene of Lactobacilli in the National Center for Biotechnology Information (NCBI) [20]. The forward and reverse primers were as follows: F (5' - ATGGCAAAACACACGCATGAAA -3'), R (5'- TCAGTGCGTGAACCCGTATTC -3').

PCR reaction was performed in 0.5 ml microtube with a total volume of 25 µl mixture containing: 2 µl template DNA, 1 µl MgCl2, 1 µl of forward primer, 1 µl reverse primer, 1µl dNTP, 2.5 µl 10X PCR buffer, 0.5 µl Taq DNA polymerase and 16 µl distilled water.

PCR amplification was performed using T3 Thermo cycler device as follows: a first cycle (denaturation) was done for 5 min at 95°C followed by 30 cycles containing: 1 min at 95°C, 45 s at 58°C, 2 min at 72°C and finally a final extension was continued for 10 minutes at 72°C.

PRC production was loaded on 1% agarose gel and then it was run in TAE (Tris acetate EDTA) buffer under the voltage of 110 V for 45 minutes. The agarose gel was colored with ethidium bromide and then it was observed using UV transillumination. Amplification products were stored at -20 ^{0}C.

PCR product was purified from gel using a DNA Extraction Kit (Fermentas) from agarose gel according to manufacturer's instructions.

Cloning of *gad* gene and sequence analysis:

The purified PCR products were ligated to pGEM-T vector by T4 DNA ligase. In this process, 5 µl of 10X buffer with 1 µl of pGEM-T vector, 1 µl T4 DNA ligase and 3 µl of purified PCR products (*gad* gene) were added to a 0.5 ml microtube. Next, the microtube was incubated at room temperature for 1 hour and then at 4°C for a day. pGEM-T vector includes lacZ gene in multiple cloning site (MCS). This gene encodes the β-galactosidase enzyme that could hydrolyze X-gal and produce colored material. Therefore colonies containing external pieces (*gad* gene) could be identified through blue-white screening considering that white colonies contain external pieces inside MCS of pGEM-T plasmid while there is no external piece in blue colonies.

Preparation of competent cells from *E. coli XL1 Blue* was performed according to the calcium chloride method [21] and then the transformation of vector containing the desired DNA to the competent cells was done by means of heat shock as follows.

In a sterile micro-tube, 20 µl of ligation reaction product was mixed with 100 µl competent cells and was put on ice for 30 min. Then the microtube was placed in a hot water bath at 42°C for 2 min and after heat shock, the sample was placed in a mixture of water and ice (4°C) for 5 min. One milliliter of liquid LB medium was added to each microtube and it was incubated at 37°C for 1 hour. After initial incubation and centrifugation at 5000 g, the recovered cells were plated onto LB agar plates containing ampicillin, IPTG (Fermentas) and X-Gal (Fermentas) and incubated at 37°C for 18 hour for screening of blue and white colonies. Then some of the white colonies were randomly selected as containing the recombinant vector colonies and were inoculated in liquid LB medium containing ampicillin for 24 hours at 37°C.Then the recombinant plasmids (pGEMT-GAD) were extracted by a plasmid extraction kit (Fermentas) according to its instruction.

For verifying the existence of *gad* gene in recombinant plasmid, plasmid DNA extracted from white colonies bacteria was used as a template to amplify the *gad* gene by PCR performed in 25 µl of solution under condition as previously described. The PCR products were analyzed by electrophoresis. The final validated positive clone of pGEMT-GAD was sent to Faza Biotech Company (Tehran, Iran) using T7 forward and SP6 reverse primers for sequence determination.

RESULTS

Cloning of *Lactobacillus delbrueckii* subsp *bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272 gad* gene:

In order to clone the *gad* gene from *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC 23272*, the PCR method was used for cloning experiment as mentioned in materials and methods. The PCR product for the entire open reading frame (ORF) of the *gad* gene, obtained by the PCR amplification, was cloned into pGEM-T vector and sequenced (FigURE. 1).

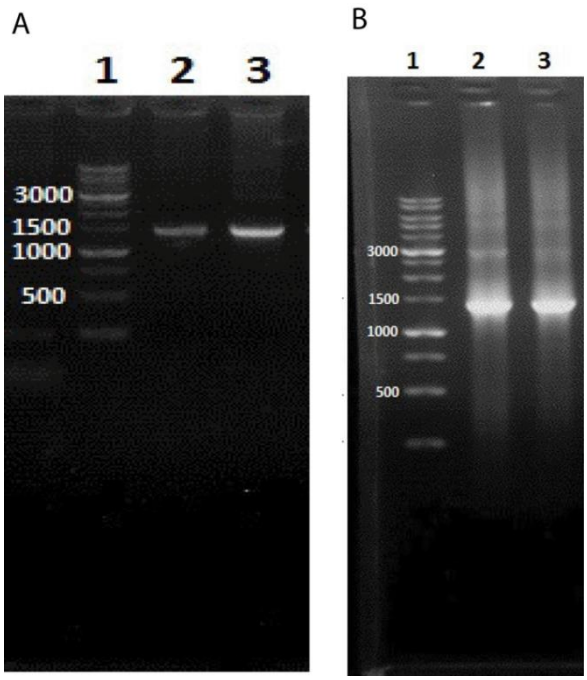

Figure 1. (A) PCR amplification and gel electrophoresis of PCR product. Lane 1: 1kb DNA ladder, lanes 2: PRC product for *Lactobacillus delbrueckii subsp bulgaricus*, lanes 3: PRC product for *Lactobacillus reuteri*. (B) Agarose gel electrophoresis of PCR amplification of *gad* gene products with pGEMT-GAD. Lane 1: 1Kbp DNA ladder. Lane 2: PCR amplification of *gad* gene from *Lactobacillus delbrueckii subsp bulgaricus* with pGEMT-GAD. Lane 3: PCR amplification of *gad* gene from *Lactobacillus reuteri* with pGEMT-GAD.

The nucleotide sequence of Lactobacillus *gad* gene consisted of approximately 1500bp (Fig. 1).

Colonies have two white and blue colors. Colonies with white color are those bacteria that contain recombinant vector. If the gene does not enter plasmid, colonies would not be white. Colony PCR was performed to be sure that T/A vector (pGEMT-GAD) extracted from white colonies contains *gad* gene.

Sequencing analysis of Lactobacillus delbrueckii subsp bulgaricus ATCC11842 and Lactobacillus reuteri ATCC23272 gad gene:

Recombinant vectors were sent for sequencing of amplified *gad* genes (Faza Pajouh, Iran). The nucleotide sequence of the *gad* gene for *Lactobacillus delbrueckii subsp. bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272* was determined and submitted to the GenBank nucleotide sequence database under the accession numbers KF751355 and KF751352 respectively.

Sequence analysis showed that the *gad* gene in *Lactobacillus delbrueckii subsp. bulgaricus ATCC11842* contained a complete ORF of 1413 bp that encoded a protein of 470 amino acids with predicted a calculated molecular weight of 53.57 kDa and a pI of 5.9 and the *gad* gene in *Lactobacillus reuteri ATCC23272* contained a complete ORF of 1410 bp that encoded a protein of 469 amino acids with a calculated molecular weight of 53.59 kDa and a pI of 5.84.

The deduced amino acid sequence was multiply aligned with other GAD sequences obtained from GenBank. This was done using MultAlin software that its results are summarized in Fig. 2. As shown in Fig. 2 the amino acid sequence of GAD deduced from the *Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842* (NCBI blastp) showed 92% identity with *Lactobacillus delbrueckii subsp. bulgaricus*, partial cds (Accession: ABP37998) GAD sequences (Fig. 2A).

The amino acid sequence homology of *Lactobacillus reuteri ATCC 23272* with *Lactobacillus reuteri strain LTH5448* (GenBank KGE72363) and *Lactobacillus reuteri* strain 100-23 (GenBank EDX42043) was 64% and 63 % respectively (Fig 2B).

DISCUSSION

Glutamate decarboxylase has been extracted from lactic acid bacteria and its biochemical properties has been defined clearly [1, 10, 22, 23]. In lactic acid bacteria, this enzyme is an intracellular enzyme [12, 24, 25] that is induced in this type of bacteria in response to acidic stress and the activity of glutamate decarboxylase is increased by adding sulfate ions [10]. Although decarboxylation reaction is the same for glutamate decarboxylase of lactic acid bacteria, the primary structure in N- terminal and C- terminal is considerably different. The variations in this primary structure of enzyme can impact on its capability in GABA production [23].

Given that GABA is a proficient neurotransmitter of inhibition in the central nervous system moreover, it influences on human health, so identifying *gad* gene nucleotide sequence and also its protein sequence will be of great value for the capability of this enzyme in producing GABA. Therefore in the current research the sequence of *gad* gene in *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272* was determined. Such a result is in consistence with the study reported by Siragusa et.al. [26].

In 1953, Najjar and Fisher completed the process of GAD enzyme purification [27, 28]. In 1961 the existence of *gad* gene in *E. coli* was verified by Halpern and Umbarger [29], but as mentioned earlier in this paper, due to the existence of Shiga toxin and its pathogeneicity, *E. coli* is not used for GABA production [18] and recently scientists have focused on producing GABA from those acid lactic bacteria that contain *gad* gene [12].

The full length of *glutamate decarboxylase* gene from *Lactobacillus reuteri 100-23*[30], *Lactobacillus brevis 877G* [31], *Lactobacillus brevis IFO12005* [32], and the main pieces of *glutamate decarboxylase B* gene from *Lactobacillus delbrueckii subsp bulgaricus* have been cloned and the sequences have been determined [26]. In 1991 the human *glutamate decarboxylase* gene was separated from chromosome 10 [33]. In this research, the sequences of *glutamate decarboxylase* gene from *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272* was determined and registered in NCBI website.

Figure2 shows the alignment of *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272* GAD with its homologues. The deduced amino acid sequence of GAD from *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272* was shown to be the most homologous to that of *Lactobacillus delbrueckii subsp. Bulgaricus*, partial cds (Accession: ABP37998) 92 % identity. There was also 99 % nucleic acid identity with *Lactobacillus plantarum JDM1*. Both GAD proteins from *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272* contained highly conserved catalytic domains.

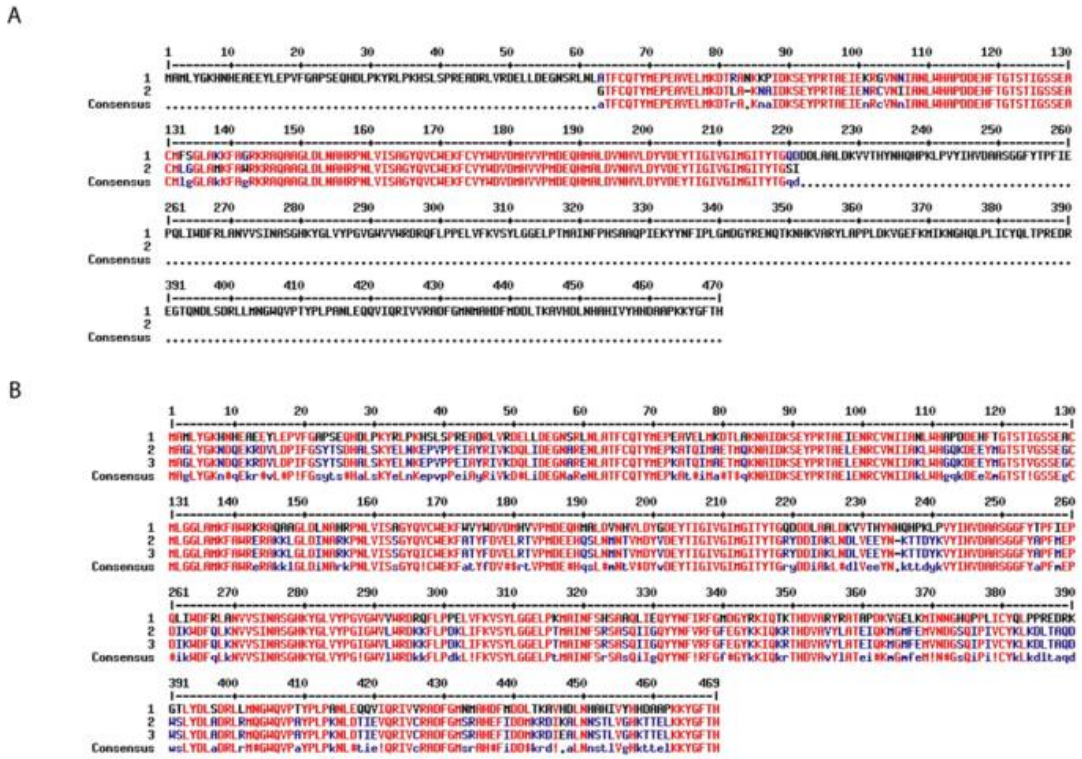

Figure 2. Alignment of GAD sequences. (A) 1 is cloned gad (*Lactobacillus delbrueckii subsp. bulgaricus* ATCC11842) and 2 is *Lactobacillus delbrueckii* subsp. Bulgaricus, partial cds (Accession: ABP37998). (B) 1 is cloned gad (*Lactobacillus reuteri* ATCC23272). 2 and 3 are *Lactobacillus reuteri* strain LTH5448 (Accession: KGE72363.1) and *Lactobacillus reuteri* 100-23 (Accession: EDX42043.1) respectively.

CONCLUSION

In this study *gad* gene was cloned and sequenced from *Lactobacillus delbrueckii subsp bulgaricus ATCC11842* and *Lactobacillus reuteri ATCC23272*. To our knowledge, just a few reports have been concerning the cloning and sequencing *gad* gene from these bacteria. The capacity of these bacteria for synthesizing GABA may open new perspectives on production of GABA-enriched functional food products. Therefore, these bacteria could be largely used in a variety of fermented functional foods.

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REFERENCES

1. Kim S-H, Shin B-H, Kim Y-H, Nam S-W, Jeon S-J. Cloning and expression of a full-length glutamate decarboxylase gene fromLactobacillus brevis BH2. Biotechnology and Bioprocess Engineering. 2007;12(6):707-12.

2. Capitani G, Biase DD, Aurizi C, Gut H, Bossa F, Grütter MG. Crystal structure and functional analysis of Escherichia coli glutamate decarboxylase. The EMBO journal. 2003;22(16):4027-37.

3. Elliott K, Jasper HH. Gamma-aminobutyric acid. Physiol Rev. 1959;39:383-406.

4. Esmaeili A, Dehghan M. Partial cloning and nucleotide sequencing of glutamate decarboxylase gene isoform 65 from human brain. Research in Molecular Medicine. 2015;3(2):0-.

5. Esmaeili A, Ghaedi K. GABAA receptors as novel drug targets for treatment of mental disorders. Journal of Paramedical Sciences. 2010;1(3).

6. Dhakal R, Bajpai VK, Baek K-H. Production of gaba (γ-aminobutyric acid) by microorganisms: A review. Brazilian Journal of Microbiology. 2012;43(4):1230-41.

7. Parvez S, Malik K, Ah Kang S, Kim HY. Probiotics and their fermented food products are

beneficial for health. Journal of applied microbiology. 2006;100(6):1171-85.
8. Esmaeili A, Lynch JW, Sah P. GABAA receptors containing gamma1 subunits contribute to inhibitory transmission in the central amygdala. Journal of neurophysiology. 2009;101(1):341-9.
9. Okada T, Sugishita T, Murakami T, Murai H, Saikusa T, Horino T, et al. Effect of the defatted rice germ enriched with GABA for sleeplessness, depression, autonomic disorder by oral administration. Nippon Shokuhin Kagaku Kogaku Kaishi= Journal of the Japanese Society for Food Science and Technology. 2000;47(8):596-603.
10. Hiraga K, Ueno Y, Oda K. Glutamate decarboxylase from Lactobacillus brevis: activation by ammonium sulfate. Bioscience, biotechnology, and biochemistry. 2008;72(5):1299-306.
11. Lin Q, Yang S, Lü F, Lu Z, Bie X, Jiao Y, et al. Cloning and expression of glutamate decarboxylase gene from Streptococcus thermophilus Y2. The Journal of general and applied microbiology. 2009;55(4):305-10.
12. Li H, Cao Y. Lactic acid bacterial cell factories for gamma-aminobutyric acid. Amino acids. 2010;39(5):1107-16.
13. Mazur R, Kovalovská K, Hudec J. Changes in selectivity of gamma-aminobutyric acid formation effected by fermentation conditions and microorganisms resources. Journal of Microbiology, Biotechnology and Food Sciences. 2011;1(2):164-71.
14. Oh S-H, Oh C-H. Brown rice extracts with enhanced levels of GABA stimulate immune cells. Food Science and Biotechnology. 2003;12(3):248-52.
15. Yan P-M, Xue W-T, Tan S-S, Zhang H, Chang X-H. Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. Food Control. 2008;19(1):50-5.
16. Taneera J, Jin Z, Jin Y, Muhammed S, Zhang E, Lang S, et al. γ-Aminobutyric acid (GABA) signalling in human pancreatic islets is altered in type 2 diabetes. Diabetologia. 2012;55(7):1985-94.
17. Wu TY, Tsai CC, Hwang YT, Chiu TH. Effect of antioxidant activity and functional properties of chingshey purple sweet potato fermented milk by Lactobacillus acidophilus, L. delbrueckii subsp. lactis, and L. gasseri Strains. Journal of food science. 2012;77(1):M2-M8.
18. Lu X, Chen Z, Gu Z, Han Y. Isolation of γ-aminobutyric acid-producing bacteria and optimization of fermentative medium. Biochemical Engineering Journal. 2008;41(1):48-52.
19. Binh TTT, Ju W-T, Jung W-J, Park R-D. Optimization of γ-amino butyric acid production in a newly isolated Lactobacillus brevis. Biotechnology letters. 2014;36(1):93-8.
20. Galperin MY, Rigden DJ, Fernández-Suárez XM. The 2015 nucleic acids research database issue and molecular biology database collection. Nucleic acids research. 2015;43(D1):D1-D5.
21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: Cold spring harbor laboratory press New York; 1989.
22. Park K-B, Oh S-H. Cloning and expression of a full-length glutamate decarboxylase gene from Lactobacillus plantarum. J Food Sci Nutr. 2004;9(4):324-9.
23. Komatsuzaki N, Nakamura T, Kimura T, Shima J. Characterization of glutamate decarboxylase from a high γ-aminobutyric acid (GABA)-producer, Lactobacillus paracasei. Bioscience, biotechnology, and biochemistry. 2008;72(2):278-85.
24. Ueno Y, Hayakawa K, Takahashi S, Oda K. Purification and characterization of glutamate decarboxylase from Lactobacillus brevis IFO 12005. Bioscience, biotechnology, and biochemistry. 1997;61(7):1168-71.
25. Huang J, Mei L, Sheng Q, Yao S, Lin D. Purification and Characterization of Glutamate Decarboxylase of Lactobacillus brevis CGMCC 1306 Isolated from Fresh Milk. Chinese Journal of Chemical Engineering. 2007;15(2):157-61.
26. Siragusa S, De Angelis M, Di Cagno R, Rizzello C, Coda R, Gobbetti M. Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Applied and environmental microbiology. 2007;73(22):7283-90.
27. Rodwell A. The histidine decarboxylase of a species of Lactobacillus; apparent dispensability of pyridoxal phosphate as coenzyme. Journal of general microbiology. 1953;8(2):233-7.
28. Shukuya R, Schwert GW. Glutamic acid decarboxylase I. Isolation procedures and

properties of the enzyme. Journal of Biological Chemistry. 1960;235(6):1649-52.
29. Marcus M, Halpern YS. Genetic and physiological analysis of glutamate decarboxylase in Escherichia coli K-12. Journal of bacteriology. 1969;97(3):1509-10.
30. Su MS, Schlicht S, Gänzle MG. Contribution of glutamate decarboxylase in Lactobacillus reuteri to acid resistance and persistence in sourdough fermentation. Microb Cell Fact. 2011;10(Suppl 1):S8.
31. Seo M-J, Nam Y-D, Lee S-Y, Park S-L, YI S-H, Lim S-I. Expression and characterization of a Glutamate decarboxylase from Lactobacillus brevis 877G producing γ-aminobutyric acid. Bioscience, biotechnology, and biochemistry. 2013;77(4):853-6.
32. Yokoyama S, Hiramatsu J-i, Hayakawa K. Production of γ-aminobutyric acid from alcohol distillery lees by Lactobacillus brevis IFO-12005. Journal of bioscience and bioengineering. 2002;93(1):95-7.
33. Karlsen AE, Hagopian WA, Grubin CE, Dube S, Disteche CM, Adler DA, et al. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proceedings of the National Academy of Sciences. 1991;88(19):8337-41.