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A high γ-aminobutyric acid-producing *Lactobacillus brevis* isolated from Chinese traditional *paocai*

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Abstract - To find a high γ-aminobutyric acid-producing lactic acid bacterium, more than 1000 strains of lactic acid bacteria isolated from *paocai* samples in various areas of China were screened by the ability in production of γ-aminobutyric acid, analysed with paper chromatography, HPLC and HPLC-MS. Among them, one strain NCL912 exhibited high ability to convert sodium glutamate to γ-aminobutyric acid. The strain accumulated 149.05 mM of γ-aminobutyric acid in a modified MRS medium containing 3% sodium glutamate after 48 h of static cultivation at 30 °C. This strain was identified as *Lactobacillus brevis* according to its phenotypic and phylogenetic characteristics.

Key words: *paocai;* lactic acid bacteria; γ-aminobutyric acid; screening and identification; *Lactobacillus brevis*.

INTRODUCTION

γ-Aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature (Manyam *et al*., 1981). It is the major inhibitory neurotransmitter in the mammalian brain. GABA has several well-known physiological functions, such as neurotransmission, induction of hypotensive, diuretic effects, and tranquilizer effects (Jakobs *et al*., 1993; Wong *et al*., 2003). A recent study showed that GABA is a strong secretagogue of insulin from the pancreas (Adeghate and Ponery, 2002) and effectively prevents diabetic conditions (Hagiwara *et al*., 2004).

GABA is produced by microorganisms including bacteria (Maras *et al*., 1992; Smith *et al*., 1992), fungi (Kono and Himeno, 2000), and yeasts (Hao and Schmit, 1993). Several GABA-producing lactic acid bacteria (LAB) have been reported, including *Lactobacillus brevis* isolated from Kimchi (Ueno *et al*., 1997; Park and Oh, 2007), fresh milk (Huang *et al*., 2007a), alcohol distillery lees (Yokoyama *et al*., 2002) and cheese (Siragusa *et al*., 2007), *Lactococcus lactis* from cheese (Nomura *et al*., 1998; Siragusa *et al*., 2007), *Lactobacillus paracasei* from cheese (Siragusa *et al*., 2007) and Japanese traditional fermented fish (Komatsuzaki *et al*., 2005), *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus plantarum* from cheese (Siragusa *et al*., 2007). Glutamate decarboxylase (GAD; EC 4.1.1.15) is considered responsible for GABA production in GABA-producing strains of LAB. GAD has been isolated from a wide variety of LAB and its biochemical properties have been characterised (Ueno *et al*., 1997; Nomura *et al*., 1998, 1999, 2000; Huang *et al*., 2007a).

GABA-producing ability is widely various among strains of LAB (Yokoyama *et al*., 2002; Komatsuzaki *et al*., 2005; Huang *et al*., 2007b; Park and Oh, 2007). GABA-producing LAB have the potential for the development of functional food containing GABA. In recent years, yogurt and cheese containing GABA (Nomura *et al*., 1998; Park and Oh, 2007), have been developed with GABA-producing LAB as starters.

Paocai is a traditional fermented vegetable in China. It is made with many kinds of vegetables, spices and other ingredients by anaerobic fermentation in a special container. *Paocai* fermentation is initiated by various microorganisms presented in the raw materials, and LAB become the dominate bacteria finally. Many LAB have been isolated from Chinese *paocai* and some of them have been shown to have useful properties. However, GABA-producing LAB isolated from Chinese *paocai* have not yet been reported. A GABA-producing LAB was isolated from Chinese traditional *paocai*, and the fermentation ability and conditions were investigated in the present research.

MATERIALS AND METHODS

Materials. GABA standard was purchased from Aldrich Co. (Milwaukee, WI, USA). Methanol and acetonitrile were of HPLC-grade. All other reagents used were of analytical grade. A *N*-butanol-acetic acid-water (5:3:2) solvent was prepared. Boric acid buffer solution (0.4 M, pH 10.4) and sodium acetate buffer (0.02 M, pH 7.3) containing 200 µl triethylamine were also prepared. Ortho-phthalaldehyde (OPA)-2-mercaptoethanol (MCE) reagent was made by adding 10 mg OPA and 10 µl MCE to 2.5 ml acetonitrile. The GABA standard solution was prepared in the concentration of 5 g l^{-1}. The mobile phase was the mixture of 1.64

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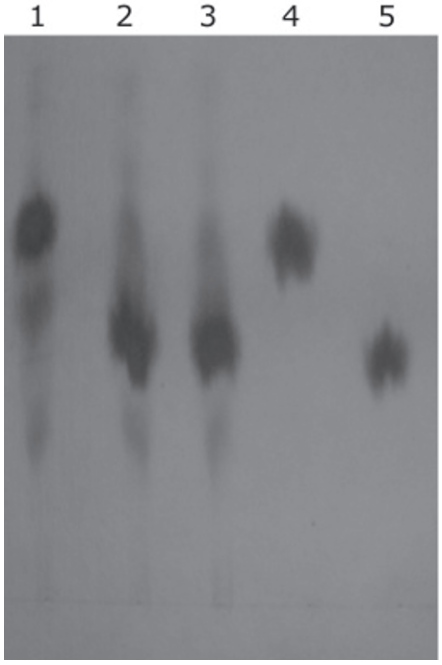

FIG. 1 - Paper chromatogram of strain NCL912 culture broth and control. Lane 1: strain NCL912, lane 2: GABA-negative producing LAB (*Lactobacillus brevis* ZDY26b), lane 3: fresh MRSS, lane 4: standard GABA (5 g l^{-1}), lane 5: standard sodium glutamate (5 g l^{-1}). All strains were grown in MRSS broth at 30 °C for 2 d. Paper chromatography using A *N*-butanol–acetic acid–water (5:3:2) solvent (adcending technique) was carried out, and paper was then sprayed with ninhydrin reagent. Sodium glutamate spot dissappeared and a new spot which has the same R_f value to standard GABA appeared in strain NCL912 culture broth. While sodium glutamate did not disappear in GABA-negative LAB culture broth and fresh MRSS broth.

g sodium acetate and 200 µl triethylamine in 1000 ml 20% (v/v) acetonitrile solvent. A reference strain *Lactobacillus brevis* ZDY26b was donated by Dr. Hua Wei in Nanchang University (China).

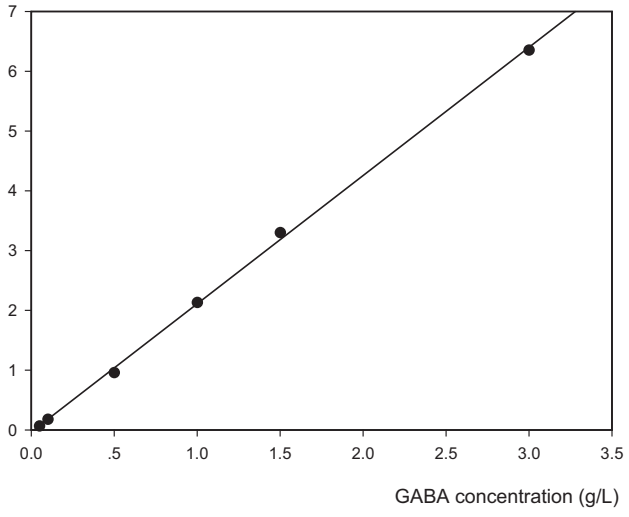

FIG. 2 - The calibration curve of standard GABA. The regression equation is y = 2.1452x - 0.0342 and R^2 is 0.9992.

Culture medium and conditions. Unless otherwise stated, the LAB strains were stationary cultivated in 250 ml flasks containing 100 ml medium at 30 °C for 2 d. Modified MRS medium contained 2.5% glucose, 0.6% yeast extract, 0.6% tryptone, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$ and 2 ml l^{-1} Tween 80. MRSS medium is prepared by adding 1% (w/v) sodium glutamate to the modified MRS medium. The initial pH of the medium was adjusted to 5.0 and not controlled during the course of fermentation. The medium was sterilised at 121 °C for 20 min.

Preliminary screening with paper chromatography. Paper chromatography was performed according to the method described by Sethi (1999). *Paocai* samples were collected aseptically from different areas of Jiangxi province, China; 10 g of each *paocai* sample was inoculated in 100 ml MRSS broth and incubated at 30 °C for 2 d. One ml of culture broths was centrifuged at 5 000 *x* g for 5 min and a 2 µl portion of the supernatant was taken and monitored by paper chromatography. The suspicious GABA-producing cultures were used for further studied as follows: 0.5 ml of the fermented broths were diluted 1:10 with 4.5 ml of 0.9% NaCl (w/v), 0.2 ml of serial dilutions were spread on MRSS agar and were incubated at 30 °C for 2 d. The colonies were picked up and subcultured in MRSS broth at 30 °C for 2 d and re-tested by paper chromatography. The GABA-producing strains were further confirmed by HPLC.

HPLC Analysis of GABA. GABA was derived by mixing 10 µl of the amino acid solution with 20 µl OPA-MCE and 100 µl boric acid buffer. The mixture was reacted at room temperature for 5 min. An S1121 Solvent Delivery System (Syknm) with UV/VIS Detector 3200 was used for separation and detection of amino acids. A Hypersil ODS colum (250 x 4.6 mm, 5 µm) was used for separation of amino acids. All samples and the mobile phase were filtered with 0.45 µm syringe filter discs and degassed by sonication. Sample aliquots of 20 µl were injected into the analytical column and the mobile phase flow-rate was fixed at 0.8 ml min^{-1} and at the wavelength of 338 nm.

Confirming high GABA-producing bacteria. The GABA-producing strains obtained from first screening were inoculated into 5 ml of modified MRS broth containing 3% sodium glutamate. The cells were removed by centrifugation at 5000 *x* g for 5 min after fermentation for 48 h. Two volumes of ethanol were added to the supernatants and stored at -20 °C for at least 1 h, then centrifuged again at 12000 *x g* for 10 min. The supernatant was sampled and analysed by HPLC method described above and the GABA content was tested to determine the GABA producing ability of the bacteria screened. The strain with highest GABA-producing ability was selected for further work.

HPLC-MS. The molecular weight of the production was determined by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The chromatographic analysis was carried out on a Hypersil ODS column (250 x 4.6 mm, 5 µm) with a flow rate of 0.8 ml min^{-1}. The ESI^+ was used to monitor ions with 3 kV capillary voltage.

Identification of the GABA-producing strain. The strain was routinely grown on the MRS medium at 30 °C unless otherwise stated. The Gram reaction was performed using a standard method. Gas production from glucose and gluconate was evaluated by inoculation of culture into 10 ml MRS broth and incubation at 30 °C for 5 d. Catalase activity was detected by placing

drops of 10% (v/v) hydrogen peroxide on the culture growing on MRS agar. Growth at different temperature and tolerance of NaCl were tested in MRS broth for 5 d after inoculation. Carbohydrate fermentation patterns were determined at 30 °C by using the API CHL 50 system according to the manufacturer's instruction (bioMérieux). The data were added into the software Apiweb (bioMérieux).

DNA was extracted according to the method of Van der Meulen *et al*. (2007). A 2 µl portion of the supernatant was used as a template in a PCR. The nearly complete 16S rDNA sequence was amplified by using oligonucleotides 5'-AGTTTGATCCTGGCTCAG-3' (primer 27FC; 5' end of the 16S rRNA gene) and 5'-GTTACCTTGTTACGACTTC-3' (primer 1490RC; 3' end of the 16S rRNA gene) described by Tanasupawat *et al*. (2000). The 16S rDNA sequence was analysed directly by using the purified PCR product as the sequencing template. The sequencing reactions were performed by automated fluorescent Taq cycle sequencing using the ABI PRISM 3730 automatic DNA sequencer (Applied Biosystems). Sequences determined in this study were compared with 16S rRNA gene sequences obtained from the GenBank database.

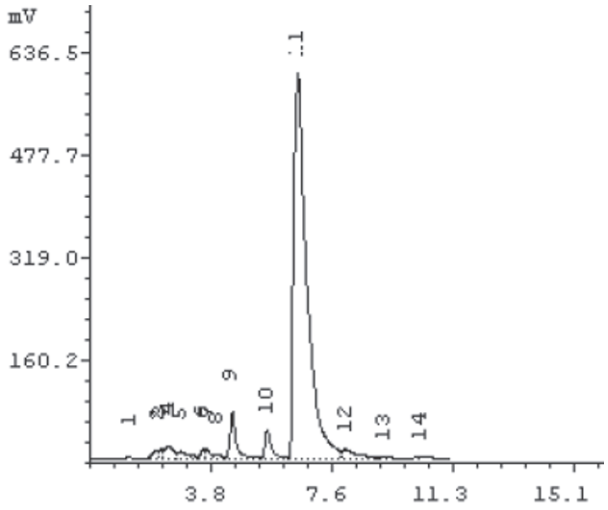

FIG. 3 - HPLC chromatogram of strain NCL912 fermenting liquor. A new peak (the 11th peak) (retention time is the same to that of standard GABA) appeared while the peak corresponding to sodium glutamate disappeared.

RESULTS AND DISCUSSION

Preliminary screening by paper chromatography

More than 1000 LAB strains were isolated from *paocai* samples. All the strains were screened firstly by paper chromatography. The results showed that 23 LAB strains can convert almost all of the 1% sodium glutamate added in the medium to GABA after 2 d of cultivation. In this paper, only the paper chromatogram of the strain NCL912 was shown (Fig. 1) due to the paper chromatograms of other 22 strains were the same to that of NCL912. These strains were selected for further HPLC analysis.

Paper chromatography was applied to routine screening of GABA-producing LAB from *paocai* samples. In comparison to other methods, paper chromatography is simple, convenient and less expensive, and it allowed us to directly visualise amino acid products, which were separated on paper with a good resolution and high sensitivity.

Further analysis by HPLC

To verify the results of paper chromatography and preliminarily determine the GABA-producing ability of the strains, a HPLC method was used to analyse the culture broth. The retention time of standard sodium glutamate and GABA is 2.38 min and 6.63 min, respectively. So GABA and sodium glutamate could be completely separated in this study. The calibration curve was linear in the interval of concentration studied for GABA (Fig. 2). The concentration of the GABA can be determined using the calibration curve of the standard solutions. The HPLC chromatogram of the culture supernatants of all presumptive GABA-producing strains exhibited a new peak that showed the same retention time to that of standard GABA, while the peak of sodium glutamate disappeared. Only the HPLC chromatogram of the strain NCL912 was shown in Fig. 3 due to the HPLC chromatograms of other 22 strains were identical to that of NCL912.

The presumptive GABA-producing strains were stationary cultivated for 2 d in 250 ml flasks containing 100 ml modified MRS medium supplemented with 3% of sodium glutamate, and GABA concentrations in the culture supernatants were measured. The levels of GABA produced by all 23 strains isolated from *paocai* and the reference strain *Lactobacillus brevis* ZDY26b in the same culture condition were demonstrated in Table 1. Screening results revealed that all the 23 strains produced GABA at a concentration higher than 109 mM. Among these strains, NCL912 showed the highest yield of GABA that reached 149.05 mM after 2 d cultivation, and have the identical morphology to the other 22 LAB strains, so only the strain NCL912 was selected for further research.

Yokoyama *et al*. (2002) reported that almost all of the free glutamic acid (10.50 mM) in Japanese *shochu kasu* was converted to GABA by *Lactobacillus brevis* IFO-12005 within 2 d of

TABLE 1 - The levels of GABA produced by all 23 strains isolated from *paocai* and certain reference strain in the same culture condition

Strains	Levels of GABA (mM)
NCL73	136.32
NCL83	125.00
NCL88	133.56
NCL94	109.32
NCL139	143.14
NCL234	111.75
NCL283	120.98
NCL332	129.03
NCL384	133.57
NCL414	140.90
NCL449	123.00
NCL505	132.59
NCL543	127.45
NCL612	134.09
NCL678	111.89
NCL697	109.37
NCL745	121.03
NCL788	142.36
NCL890	139.12
NCL876	125.00
NCL894	140.79
NCL906	123.58
NCL912	149.05
Lactobacillus brevis ZDY26b	Not be detected

stationary culture at 30 °C. The amount of GABA in the *kome shochu kasu* medium finally reached 10.18 mM. Park and Oh (2007) reported that GABA produced by *Lactobacillus brevis* OPY-1 was over 24.24 mM after it was incubated in MRS broth at 30 °C for 24 h. The GABA production ability of strain NCL912 is clearly higher than that of *Lactobacillus brevis* IFO-12005 and *Lactobacillus brevis* OPY-1. Although *Lactobacillus paracasei* NFRI 7415 showing higher GABA production of 302 mM reported by Komatsuzaki *et al*. (2005), the *Lactobacillus paracasei* NFRI 7415 was cultivated under optimal condition for 6 d, and the strain NCL912 was cultivated under initial culture condition for only 2 d. The GABA production ability of strain NCL912 could be improved by optimising fermentation conditions such as regulating pH and adding 5′-pyridoxal phosphate.

Determination of molecular weight of the presumptive GABA by HPLC-MS

Although the product was monitored by paper chromatography and HPLC method as GABA, it is necessary to determine the molecular weight of the product to confirm it. In this study, the HPLC-MS method was applied to determine the molecular weight of the product. The results showed that the molecular weight of the standard GABA and presumptive GABA derived by OPA is 267.73 and 267.80, respectively. The molecular weight of the product and standard GABA is almost the same. On the basis of the analysis data above, we could conclude that the strain NCL912 converted sodium glutamate into GABA.

Identification of the strain NCL912

The strain was Gram-positive and catalase-negative. Colonies of strain NCL912 grown on MRS agar plates for 3 d are very small (up to 1 mm in diameter), white, smooth, circular and with entire edges. Cells are non-motile and non-spore-forming, rod-shaped, though occasionally longer cells are observed, and occur mainly in pairs. The strain grows at 10, 20, 30, 37 and 40 °C, but not at 4 or 45 °C. The strain grows in a medium with 8% NaCl. The strain produces gas from glucose or gluconate. The API CHL 50 system was used to assess the metabolic profile of the new isolate. Carbohydrates could be fermented by strain NCL912 were L-arabinose, ribose, D-xylose, galactose, glucose, fructose, maltose, melibiose, α-methyl D-glucoside, *N*-acetyl D-glucosamine and gluconate. The carbohydrate fermentation profile was analysed with the software apiweb (bioMérieux). The strain NCL912 and *Lactobacillus brevis* ATCC 367, shared 99.9% metabolic profile similarity.

The almost complete sequence (1470 bp) of the 16S rRNA gene of strain NCL912 was amplified by PCR. The closest known relatives of the new isolates were determined by performing database searches. A comparison of the 16S rRNA gene amplified with homologous sequences from other related species indicated that the isolate exhibited the highest similarity value to *Lactobacillus brevis* (97% similarity). The next highest similarity value was to *Lactobacillus plantarum* (94%), followed by other species (≤ 92%). The results of metabolic pattern, other phenotypic characteristics, and phylogenic analysis clearly indicated that the strain NCL912 belongs to *Lactobacillus brevis* according to the available compilation of data by Stackebrandt and Goebel (1994). The result obtained by phenotypic characterisation is identical to that of genetic characterisation.

In conclusion, a high GABA-producing strain was screened from Chinese traditional *paocai*. On the basis of both phenotypic and phylogenetic findings, it is evident that the strain belongs to *Lactobacillus brevis*. This strain was designated *Lactobacillus brevis* NCL912(= CCTCCM 208054).

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