A Lactobacillus plantarum strain newly isolated from Chinese sauerkraut with high γ-aminobutyric acid productivity and its culture conditions optimization

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Abstract

The objective of this study was to screen GABA-producing strain from traditional Chinese fermented sauerkraut and optimize culture conditions of the isolated strain for GABA production. One out of 36 strains isolated from different Chinese sauerkraut samples was obtained with high GABA production, which was identified as Lactobacillus plantarum by 16S rDNA sequence and analysis phenotypic characterization together with its morphological and physiological characterization, it was nominated as L. plantarum CWQ-7. Optimal culture conditions for growth of L. plantarum CWQ-7 and accumulation of GABA were 55g/L L-glutamate and 0.10 g/L pyridoxine hydrochloride added in modified MRS medium at initial pH 5.5 and 32°C for 66 h. Under such conditions, the GABA production reached 6. 35 g/L, with an increase of 43.6% compared to that of the original fermentation conditions. The results of the study indicated this newly isolated strain could be applied in GABA food production.
Keywords: γ-AMINOBUTYRIC ACID, PRODUCTION, LACTOBACILLUS PLANTARUM, CHINESE SAUERKRAUT, ISOLATING, OPTIMIZATION

1. Introduction

γ-Aminobutyric acid (GABA), a non-protein-constituted amino acid, is known as a major neurotransmitter which could regulates inhibitory neurotransmission in mammalian central nervous systems. It has been proved to be effective for enhancing sleep, lowering blood pressure and improving relaxation[1]. Therefore, the effects of GABA on human health are also of current interest in medical and food production[2,3]. Studies have proved that some fermented products, such as sauerkraut, pickle, cheese and yoghurt have rich GABA content, owing to the starter cultures of these fermented foods contained GABA-producing microorganisms, lactic acid bacteria (LAB) [4,5]. Generally, LAB are regarded as safe microorganisms and known as probiotics. Currently, the screening of GABA-producing LAB and the production of food enriched with GABA by LAB are being studied actively[6-8]. GABA-producing LAB have high glutamate decarboxylase (GAD) [EC 4.1.1.15] activity and could convert glutamate (Glu) into GABA, while pyridoxine hydrochloride acts as its coenzyme[9]. In this work, a GABA-producing strain was screened from traditional Chinese sauerkraut samples. Subsequently, based on 16S rDNA sequence determination and blast analysis together with its morphological and physiological characterization, this strain was preliminarily identified as Lactobacillus plantarum. Optimized culture conditions of the isolated strain for GABA production were investigated.

2. Materials and Methodology

2.1. Chinese Sauerkraut Samples

The Chinese sauerkraut samples produced in 10 companies were randomly bought from markets in Hangzhou City, China. The sauerkraut samples (100g/ sample) were ground separately in a household stainless steel blender and filtered with filter paper to get the juice, then kept in a refrigerator (4°C) until subsequent use and analysis.

2.2. Analysis of GABA

Previously, a paper chromatography method with high sensitivity, veracity and low cost for quantitative analysis of GABA was established according to reference [10] with modification. After spreading in n-butanol/acetic acid/water (4:1:3, by volume) containing 0.4% (w/v) ninhydrin, the paper was dried for color yield. GABA spots were scratched out from the paper and extracted with 4 ml 0.6 % (w/v) cupric sulfate/75 % (v/v) ethanol (2:38, v/v). The absorbance of the supernatant was read at 520nm (UV-2450 PC ultraviolet-visible spectrophotometer, Shimadzu, Japan). The content of GABA produced was calculated using the standard curve.

2.3. Screening Procedure

Prepared as 2.1, we got ten juice samples of commercial Chinese sauerkraut products. 1mL of juice samples was added to 9 mL of sterilized saline (0.85 %) and allowed to stand for 20 min. 2 mL of the suspension was then inoculated into 48 mL of the MRS medium with 1 % glutamate and 0.1 g/L pyridoxine hydrochloride (w/v). MRS medium is composed of: casein peptone (tryptic digest) 10.0 g, meat extract 10.0 g, yeast extract 5.0 g, glucose 20.0 g, Tween-80 1.0 g, K2HPO 4 2.0 g, sodium acetate 5.0 g, diammonium citrate 2.0 g , MgSO4•7H2O 0.2 g , MnSO4•4H2O 50.0 mg, distilled water 1.0 L , adjust pH to 6.0.

Cultivation was carried out in an incubator where temperature could be controlled automatically. It was cultivated at 35 °C for about 12 h. After microbial growth was observed, 2 mL of the culture was transferred into a fresh medium. This procedure was performed three times. Tenfold serial dilutions of the resulting cultures were prepared in sterilized distilled water, and 0.1 mL diluted sample was spread on MRS medium plates to isolate single colonies. The colonies were cultured on MRS agar and observed after 24 h at 35°C. Morphologically different colonies on the plates were purified on the MRS medium, and the isolated pure colonies were grown on the test tube slant of the MRS medium with 1% glutamate and then preserved at 4 °C.

Strains with higher GABA producing ability were subsequently screened among these isolated strains. Transferred a loop of cells from a slant into a 250 mL flask containing 50 mL MRS medium with 2 % glutamate and cultivated at 35°C for 12 h, with A600 reaching 0.4-0.5, this was seed culture. Subsequently, transferred the seed culture (1.0 mL) to a 250 mL flask containing 50 mL fermentation medium (sterilized MRS broth containing 5% glutamate and 0.10 g/L pyridoxine hydrochloride), and cultivated at 35°C for 24h and 48h. 1mL of the culture was transferred into 1.5-mL eppendorf tube and centrifuged at 5, 000g, 4°C for 5 min. 10 µL of the supernatant resulting from the centrifugation was prepared for GABA quantitative determining, which showed the ability of converting glutamate to GABA.

2.4. Characterization of GABA-producing Bacteria

Morphological characterization was performed after 24 h incubation on MRS agar. Cell morphology was observed with a light microscope (Leica DMLB2, Germany). Some conventional physiological and biochemical assays were carried out.

The genomic DNA of selected strain was extracted by DNA extraction kit (Fermentas, China) according to the manufacturer's recommended procedure. DNA was precipitated by adding ethanol and sodium acetate, resuspended in 50 µL of Tris-EDTA buffer (pH 8.0) and stored at −20°C.

The 16S rDNA gene was amplified using the universal primers (F: 5'-AGAGTTTGATCCTG GCTCAG-3' and R: 5'- ACGGCTACCTTGT TACGACTT -3'). A PCR cycler (Bio-Rad) was used for this amplication. Amplication reactions contained 1.0µL of each primer(50 µmol/L), dNTP (10mmol/L) 1.0 µL, PCR buffer 5.0 µL, MgCl2 (50 mmmol/L) 1.5 µL, template DNA 1 µL, taq DNA polymerase (Fermentas, China) 1.0 µL, and ddH2O 37.5 µL, in a total volume of 50µL. The following conditions were used in the amplification of 16S rDNA gene: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 45 s and 72°C for 90 s, with final 10 min extension at 72°C. The PCR products were then checked on agarose gel with SYBR Green I staining. PCR product purification was conducted using PCR purification kit (UNIQ-10, China). The purified PCR product was sequenced in both directions using an automated sequencer by Shanghai Shenggong Co., Ltd (China).

As an underlying basis to identify the strain, the sequence was manually edited and compared with available data from GenBank databases (National Centre for Biotechnology Information website; http://www.ncbi.nlm.nih.gov/) using the BLASTN

program. The 16S rDNA sequence and reference sequences from Genbank database were aligned using DNAMAN ver. 5.0 (Lynnon Biosoft, USA). Phylogenetic tree was made with the neighbor-joining and bootstrap methods in MEGA 5.0.

2.5. Culture Conditions Optimization

After the GABA-producing strain screened, the effects of culture conditions were investigated. MRS media containing 5% (w/v) glutamate at various initial pHs from 4.5 to 8.5 (at intervals of 0.5 pH unit) were used for cultivation of selected strain at 35°C to test pH effect on cell growth and GABA production, the effect of temperature was measured from 26 to 40°C (at intervals of 2°C) at pH 5.5.

For the effect of glutamate concentration, MRS media containing 2–8 % (w/v) glutamate (at intervals of 0.5%) were used [11]. For the effect of carbon sources for GABA productivity, MRS media containing 5 % (w/v) glutamate with glucose, lactose, sucrose, and soluble starch at 2 % (w/v) were prepared. To compare GABA productivity for various nitrogen, nitrogen source in MRS medium, i.e., casein peptone (tryptic digest), meat extract and yeast extract were removed, while 1-3 % nitrogen sources of casein peptone, meat extract, yeast extract and milk were added. Different pyridoxine hydrochloride (0~0.30%, w/v, at intervals of 0.05 %) and time course (0~84h, at intervals of 6h), together with NaCl concentration (0~3.0%, w/v) for GABA production were also tested.

3. RESULTS

3.1. Isolation And Identification of High GABA-Producing Strain From Chinese Sauerkraut.

The level of GABA production by all of the selected 36 strains from 10 Chinese sauerkraut samples ranged from 0.01 to 4.42 g/L. 7 out of 36 strains was obtained with obvious GABA-producing capacity, a strain numbered CWQ-7, turned out to be the most powerful and used for further studies (Table1). After cultivated in modified MRS medium (with added 5 % glutamate and 0.1g/L pyridoxine hydrochloride) for 48h, it accumulated 4.42 g/L GABA in broth.

Table 1. GABA-producing Strains Isolated from Chinese Sauerkraut Samples

Strains number	CWQ-2	CWQ-7	CWQ-16	CWQ-22	CWQ-30
GABA/(g/L)	2.34	4.42	1.49	3.61	2. 28

The morphological characteristics and some physiological and biochemical characteristics of this strain were as follows: Colonies on MRS agar were smooth, wettish, not transparent, middle uplift and milk white (Fig. 1). It's gram-positive, nonspore-forming and non- movement bacillus (Fig. 2). It produced lactic acid, showed hydrogen peroxide enzyme reaction negative. Biochemical characterization revealed that isolated CWQ-7 might be similar to Lactobacillus plantarum according to the description in Bergey's Manual of Determinative Bacteriology [12].

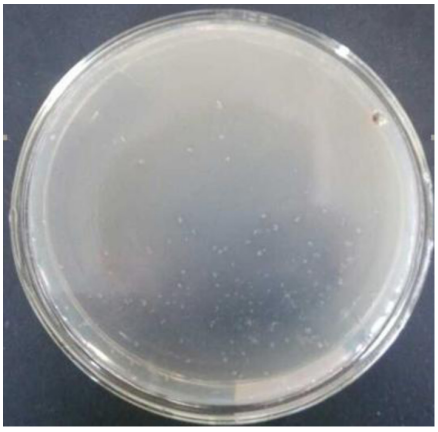

Figure 1. Colony's configuration of Strain CWQ-7

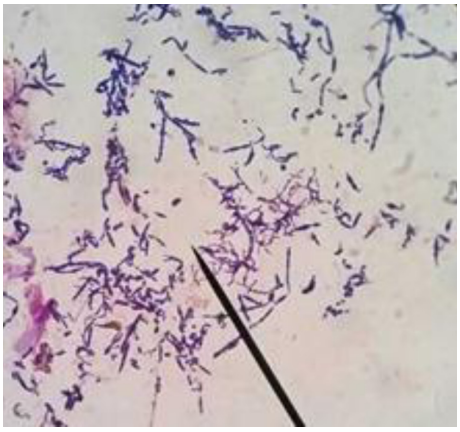

Figure 2. The micrograph of Strain CWQ-7

Fig. 3 revealed agarose gel electrophoresis of 16S rDNA PCR product for strain CWQ-7. For a more reliable identification, 16S rDNA gene sequences were partially sequenced and a phylogenetic tree was constructed (Fig. 4). The 16S rDNA sequence of strain CWQ-7 showed 99% similarity with the corresponding sequences of some Lactobacillus plantarum strains such as L. plantarum JCM 1149, when compared with available data from GenBank databases using the BLASTN program. Multiple alignment and phylogenetic analysis revealed the strain was closely clustered with L. plantarum JCM 1149. Based on 16S rDNA sequence determination and blast analysis together with its morphological and physiological characterization, this newly isolated GABA-producing strain was preliminarily identified and nominated as L. plantarum CWQ-7, which belongs to the category of probiotics.

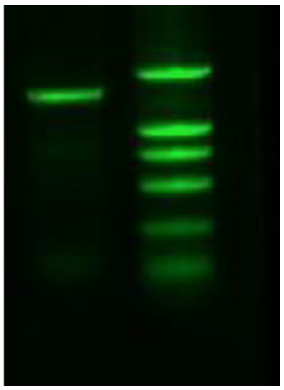

Figure 3. Agarose gel electrophoresis of 16S rDNA PCR product for Strain CWQ-7

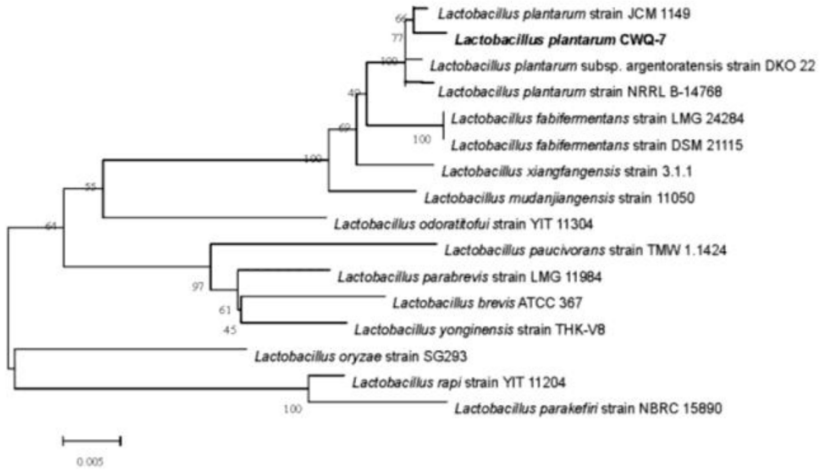

Figure 4. Phylogenetic Tree Based on 16S rDNA Sequences of Strain CWQ-7

3.2. Culture Conditions Optimization Results

3.2.1. Effect of Incubation Tmperature on GABA Production of L. plantarum CWQ-7

Temperature could strongly influence culture growth and GABA-producing. Since we had determined CWQ-7 as a L. plantarum strain, the effect of incubation temperature on GABA-producing was investigated during 26~40°C, at intervals of 2°C (Fig. 5). The relatively high GABA value was gained in 32°C for 48h, while the growth density almost simultaneously showed the highest value compared to other incubation temperatures, subsequent optimization studies were carried out at this incubation temperature.

The highest GABA concentration was achieved at 32 °C. All of the samples were cultivated for 48h.

3.2.2. Effect of initial media pH on GABA production of L. plantarum CWQ-7

To investigate the effect of initial culture pH on GABA production, culture media was adjusted to different pH (4.5~8.5) with 100 mmol/L McIlvaine buffer. The results revealed that the optimum pH of the medium for GABA production was pH 5.5 (Fig. 6).

3.2.3. Effect of Glutamate Concentration In Initial Media on GABA Production of L. plantarum CWQ-7

The GABA production by L. plantarum CWQ-7 was affected by the concentration of glutamate in media. When glutamate concentration increased from 4.0 to 5.5 % (w/v), GABA production increased significantly.

However, above 7.0 %, GABA production decreased (Fig. 7). The extra high concentration of glutamate was inhibitory for the bacterium growth and for GAD activity.

The highest GABA production was achieved at pH5.5. The strain L. plantarum CWQ-7 was also cultured at 32 °C for 48 h for GABA production.

The highest GABA production was achieved at glutamate concentration increased to 5.5 % (w/v).

3.2.4. Effect of Carbon Sources and Nitrogen Sources In Initial Media on GABA Production of L. plantarum CWQ-7

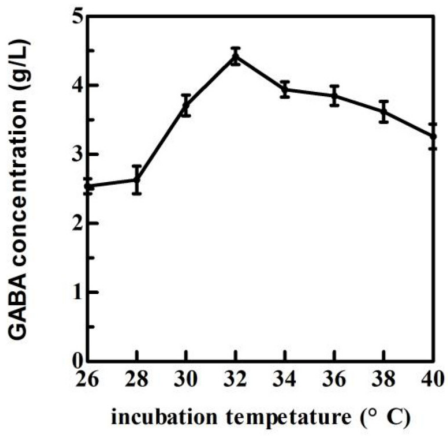

Figure 5. Effect of glutamate concentration in medium on GABA production

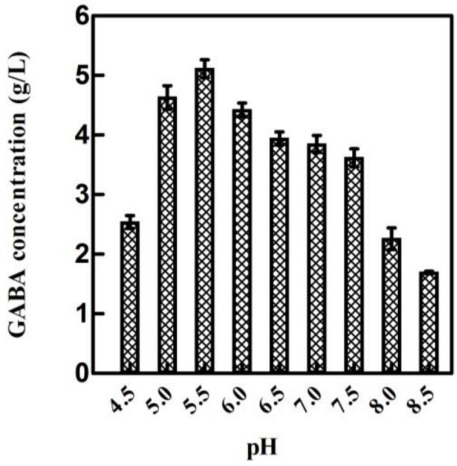

Figure 6. Effect of medium pH on GABA production

Fig. 8 showed that glucose was the best carbon source for GABA production of L. plantarum CWQ-7 compared to lactose, sucrose and soluble starch at the same concentration. 2 % Glucose is one of the standard MRS medium components, hence the change of carbon source was not effective for GABA production improvement.

The highest GABA production was achieved when the carbon source was glucose. It was exactly right the standard MRS medium components.

GABA production was affected by the nitrogen sources and the varied concentrations. GABA production increased according to the concentration at 1, 1.5, 2 and 2.5 % (w/v) of yeast extract respectively. However, at 3% yeast extract, it decreased. The similar patterns of GABA production were observed for casein peptone, meat extract and milk (Fig. 9). However, all of the single nitrogen source could not compete to the complex nitrogen sources in the standard MRS medium (1% of casein peptone (tryptic digest), 1% of meat extract, 0.5% of yeast extract). Therefore, the above combined nitrogen sources was the optimal one for GABA production with L. plantarum CWQ-7.

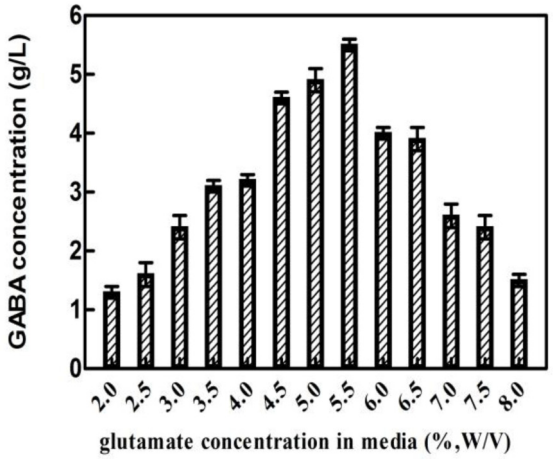

Figure 7. Effect of glutamate concentration in medium on GABA production

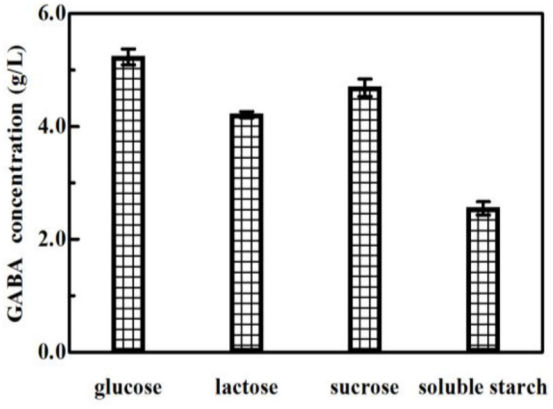

Figure 8. Effect of carbon source in medium on GABA Production of L. plantarum CWQ-7

3.2.5. Optimization of Other Factors and The Final Results

The optimal concentration of pyridoxine hydrochloride was defined as 0.10g/L, when we use the media without added pyridoxine hydrochloride, it decreased. This strain seemed not sensitive for NaCl concentration, it grew well when NaCl was between 0-3.0%.

Different incubation time (0~84 h) was employed to study the effect on L. plantarum CWQ-7. The fermentation was carried out keeping all other conditions at the above optimal levels. Sampling per 2 or 6 h was prepared for assay. It was depicted in Fig.10. The maximum GABA value was found on the 66th hour of fermentation, which was reached to 6.35g/L, with an increase of 43.6% compared to that of the original fermentation conditions (4.42g/L).

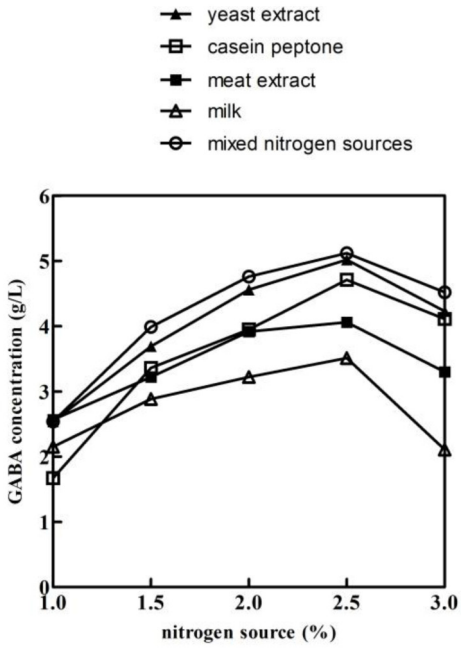

Figure 9. Effect of nitrogen source in medium on GABA production of L. plantarum CWQ-7

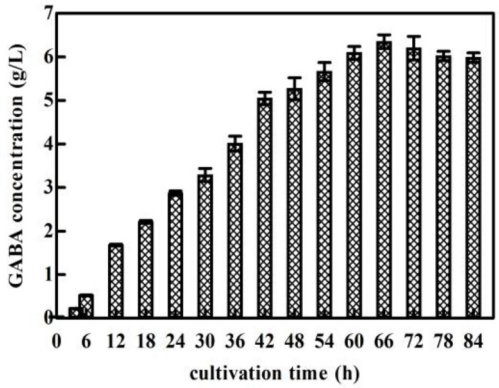

Figure 10. Effect of cultivation time with optimal conditions on GABA production

Any of the single nitrogen source, yeast extract (black uppointing triangle), casein peptone (white square), meat extract (blackfilled square) and milk (white uppointing triangle) could not compete to the complex nitrogen sources in the standard MRS medium (white circle) on production of GABA. 1% of casein peptone (tryptic digest), 1% of meat extract, 0.5% of yeast extract was the best nitrogen source combination. When the concentration of combined nitrogen source increased to 3.0%, production of GABA also decreased.

The highest GABA production was achieved at 66h (6.35g/L). After that, it decreased.

4. Conclusions

A strain with GABA biosynthesis capacity was isolated from Chinese sauerkraut and identified as Lactobacillus plantarum CWQ-7 by phenotypic characterization and 16S rDNA sequence analysis. Optimal culture conditions for growth of L. plantarum CWQ-7 and accumulation of GABA were 55g/L L-glutamate and 0.10g/L pyridoxine hydrochloride added in modified MRS medium at initial pH 5.5 and 32°C for 66 h. Under such conditions, the GABA production reached 6. 35 g/L, with an increase of 43.6% compared to that of the original fermentation conditions. The results of the study indicated this newly isolated strain could be applied in GABA food production.

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