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Food Microbiology 22 (2005) 497–504

FOOD MICROBIOLOGY

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Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods

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Received 10 September 2004; accepted 5 January 2005

Abstract

Lactobacillus strains that accumulated γ-aminobutyric acid (GABA) in culture medium were screened to determine strains with high GABA-producing ability. One strain, NFRI 7415, which was isolated from a Japanese traditional fermented fish (funa-sushi), showed the highest GABA-producing ability among the screened strains. Identification tests (i.e., 16S rDNA sequencing and sugar assimilation ability) indicated that NFRI 7415 belongs to *Lb. paracasei*. The GABA production was further improved by the addition of pyridoxal phosphate to the culture medium and pH regulation of culture medium at pH 5.0. Under optimal cultivation conditions, strain NFRI 7415 produced GABA at a concentration of 302 mM when the glutamate concentration in the culture medium was 500 mM.

Keywords: γ-aminobutyric acid; *Lactobacillus*; glutamate decarboxylase

1. Introduction

γ-aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature (Manyam et al., 1981). GABA has several well-known physiological functions, such as neurotransmission, induction of hypotensive, diuretic effects, and tranquilizer effects (Jakobs et al., 1993; Guin Ting 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). Due to the physiological functions of GABA, development of functional foods containing GABA at high concentration has been actively pursued (Tsushida and Murai, 1987; Saikusa et al., 1994). GABA enrichment has been achieved in anaerobic-incubated tea (gabaron tea) (Tsushida and Murai, 1987) and in rice germ soaked in water (Saikusa et al., 1994). GABA production by various micro-organisms has been reported, including bacteria (Smith et al., 1992; Maras et al., 1992), fungi (Kono and Himeno, 2000), and yeasts (Hao and Schmit, 1993).

In this study, we focused on the GABA production ability of lactic acid bacteria (LAB), because LAB show potential for commercial use as starters of production in fermented foods, such as pickled vegetables and fermented meats and fishes. Several GABA-producing LAB have been reported, including *Lactobacillus brevis* isolated from kimuchi (Ueno et al., 1997) and from alcohol distillery lees (Yokoyama et al., 2002), and *Lactococcus lactis* from cheese starters (Nomura et al.,

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0740-0020/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2005.01.002

1998). Screening various types of LAB that have GABA-producing ability is important for the food industry, because individual LAB have specific fermentation profiles, such as acid production and flavor formation ability. Because quality of fermented foods, such as taste and flavor, depends on the fermentation profiles of the LAB, such profiles are considered an important factor in the use of LAB as starters in the production of fermented foods (Kato et al., 2001; Gran et al., 2003).

Glutamate decarboxylase (GAD) [EC: 4.1.1.15] is considered responsible for GABA production in GABA-producing strains of LAB, such as *Lactobacillus* and *Lactococcus*. GAD has been isolated from a wide variety of sources, and its biochemical properties have been characterized (Nomura et al., 1999, 2000). Ueno et al. (1997) purified GAD from *Lb. brevis* and then determined its biochemical characterization. Nomura et al. (1998) characterized GAD and cloned the gene responsible for GAD activity in *L. lactis*, and found that *L. lactis* contained only one GAD gene. Although GAD is widely distributed in LAB, GABA-producing ability varies widely among LAB.

The aim of this study was to screen various types of GABA-producing LAB from unique resources, such as traditional fermented foods and silage, as possible starters in the production of fermented foods. GABA-producing LAB is expected to enhance development of functional fermented foods containing GABA. Based on our hypothesis that GABA production during cultivation of the strains can be improved by adjusting the culture conditions suitable for GAD reaction, we then adjusted culture conditions, and added a coenzyme of GAD during cultivation of screened LAB that had high GABA-producing ability. Results showed that *Lb. paracasei* isolated from a traditional fermented fish (funa sushi) produced GABA at high level (>300 mM), and that cultivation conditions suitable for GABA production can be improved by utilizing the biochemical characteristics of GAD.

2. Materials and methods

2.1. Culture medium and conditions

Lactobacilli MRS broth (Difco, Detroit, USA) was used for GABA production and maintenance of *Lactobacillus* strains. Unless otherwise stated, the LAB strains were grown (without shaking) in capped test tubes at 30 °C. Sodium glutamate was added to MRS broth at concentrations indicated in figure legends. In pH-regulated cultivation, pH was adjusted every 24 h by adding NaOH or HCl during cultivation.

2.2. Measurement of extracellular and intracellular GABA content

Extracellular GABA accumulated in the culture medium was measured as follows. First, the culture broth was separated from cells by centrifugation (8000*g* for 10 min at 4 °C). Then, the supernatant (20 μl) was 50-fold diluted with sample application buffer (pH 2.2), which consisted of 1.5 g of tri-lithium citrate, 19.8 g of citric acid, 12.0 g of LiCl, and 20.0 g of 2,2-thiodiethanol (per liter). Portions (10 μl) of the diluted samples were directly injected into an amino acids analyzer (Yanako Ltd, Japan, LC-11A). Intracellular GABA was measured as follows. First, the cells were washed with PBS (pH 7.0) containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 (per liter), and then the cells were suspended in 0.5 ml of 75% (v/v) ethanol. The cell suspension was vigorously mixed for 1 min at room temperature and centrifuged at 8000*g* for 10 min at 4 °C. The supernatant was dried using a centrifugation evaporator (EYELA, Tokyo, Japan). The residual substance was dissolved in 0.5 ml of the sample application buffer. Portions (20 μl) of the dissolved samples were injected into the amino acids analyzer. Assay of extracellular and intracellular GABA content was carried out as two or three independent experiments and GABA content was measured in triplicate in each independent experiment.

2.3. Screening of GABA-producing LAB

A total of 72 strains of LAB obtained from the Microbiological Bank of the National Food Research Institute (NFRI) were screened for their GABA-producing ability. These strains consisted of strains isolated from fermented foods, silage, and strains purchased from other culture collections such as IFO (Institute for Fermentation, Osaka). All strains were grown in MRS medium containing 50 mM of glutamate for 7 d at 30 °C. GABA content in the supernatants was measured using the amino acids analyzer.

2.4. Identification of GABA-producing LAB

Identification of the GABA-producing strains was done using fermentation potential tests and 16S ribosomal DNA (rDNA) analysis. Fermentation potential of various sugars listed in Table 1 was determined using API rapid CH fermentation strips (Bio Mérieux, Lyon, France) as follows. The LAB strains were grown in 15 ml of MRS medium at 30 °C for 24 h. The cells were collected by centrifugation (5000*g* for 10 min at 4 °C) and washed twice with PBS. The washed cells were suspended in 2 ml of suspension medium (Bio Mérieux) and inoculated into the API CH strips. The strips were incubated at 30 °C for 48 h, and then acid formation was

Table 1
Genetical and phenotypic characteristics of strain NFRI 7415

Parameter or test	Characteristics of strain NFRI 7415
Homology of 16S rDNA sequences	99.8% with *Lb. paracasei* JCM 8130Ta 99.0% with *Lb. casei* subsp. *casei* JCM 1134^{T}
Utilizable sugar	Ribose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, mannitol, sorbitol, *N*-acetyl glucosamine, amygdaline, arbutine, salicine, cellubiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-lyxose, *β*-gentiobiose, D-turanose, D-tagatose, and gluconate
Non-utilizable sugar	Glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, *β*-methyl-xyloside, rhamnose, dulcitol, α-methyl-D-mannoside, α-methyl-D-glucoside, D-arabitol, esculin, D-rafinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, 2-keto-gluconate, and 5-keto-gluconate

[a]JCM: Japan Collection of Microorganisms.

determined by monitoring the change in color of bromocresol purple used as an indicator. The entire 16S rDNA of the strain was amplified by PCR and directly sequenced using the method of Mori et al. (1997). Total DNA of the strain was extracted from cells grown in 1.0 ml of MRS medium using an InstaGene purification matrix (Bio-Rad Laboratories, Richmond, USA) according to the manufacturer's instructions. The 16S rDNA fragments in the total DNA were amplified by PCR and sequenced using a DNA sequencer (ABI 310, Perkin Elmer, Wellesley, USA) using oligonucleotide primers described by Mori et al. (1997). The 16S rDNA sequences of related micro-organisms were obtained from the DNA Data Bank of Japan (DDBJ) and then aligned using GenetyxMac software (Software Development, Tokyo, Japan).

2.5. Assay for GAD activity

The GABA-producing LAB strains were cultivated in 15 ml of MRS medium containing 100 mM of glutamate for 4 d at 37 °C. The cells were collected by centrifugation (5000*g* for 10 min at 4 °C) and washed twice with PBS. The washed cells were suspended in 5 ml of 20 mM sodium phosphate buffer (pH 7.0) and 1 g of glass beads (100 μm diameter, Merck, Darmstadt, Germany). The cells in the suspension were disrupted by shaking (30 s × 5 times) using a homogenizer (type MSK, Braun, Frankfurt, Germany) at 4 °C. The crude extracts were obtained by centrifugation (10,000*g* for 10 min at 4 °C). Protein concentrations of the crude extracts were measured by using a protein assay kit (Pierce, Rockford, USA). The crude extracts were then incubated with 50 mM of glutamate and 50 mM of pyridoxal 5-phosphate (PLP) in 0.2 M pyridine–HCl buffer (pH 5.0) for 30 min. Enzyme reactions were stopped by boiling the enzyme assay mixture for 5 min. Then, GABA content in the enzyme mixture was measured using the amino acids analyzer. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA in 1 min. The assay for GAD activity was done in triplicate.

3. Results and discussion

3.1. Screening of GABA-producing LAB

To screen LAB strains that produce GABA at high-level concentration in culture medium, we cultivated 72 strains in MRS medium containing 50 mM of glutamate and measured GABA concentrations in the culture supernatants. Screening results revealed that three strains produced GABA at a concentration higher than 1.8 mM: *Lactobacillus* sp. NFRI 7415, *Lactobacillus plantarum* NFRI 7313 (Kiuchi et al., 1990), and *L. brevis* NFRI 7340 (IFO 3960). *Lactobacillus* sp. NFRI 7415 was originally isolated from traditional fermented crucians (funa-sushi) in Japan as described previously (Momose et al., 1999). Crucians (*Carassius auratus*) are a freshwater fish and used as food in Japan. Fig. 1 shows the measured growth rate expressed as optical density at 600 nm and pH change for these three LAB strains cultured in MRS medium containing 100 mM glutamate. Both the growth rate and pH change differed among the strains. The change in pH might reflect the acid-producing ability, which is an important factor in the production of quality-fermented foods.

3.2. Identification of strain NFRI 7415

Taxonomic identification of strain NFRI 7415 was done here for the first time. Identification involved 16S rDNA sequencing and assimilation potential of various sugars. Table 1 summarizes the results. The sequence of 16S rDNA of strain NFRI 7415 showed 99.8% homology to *Lb. paracasei* JCM 8130^{T}. The sequence was deposited in DDBJ under accession no. AB182642.

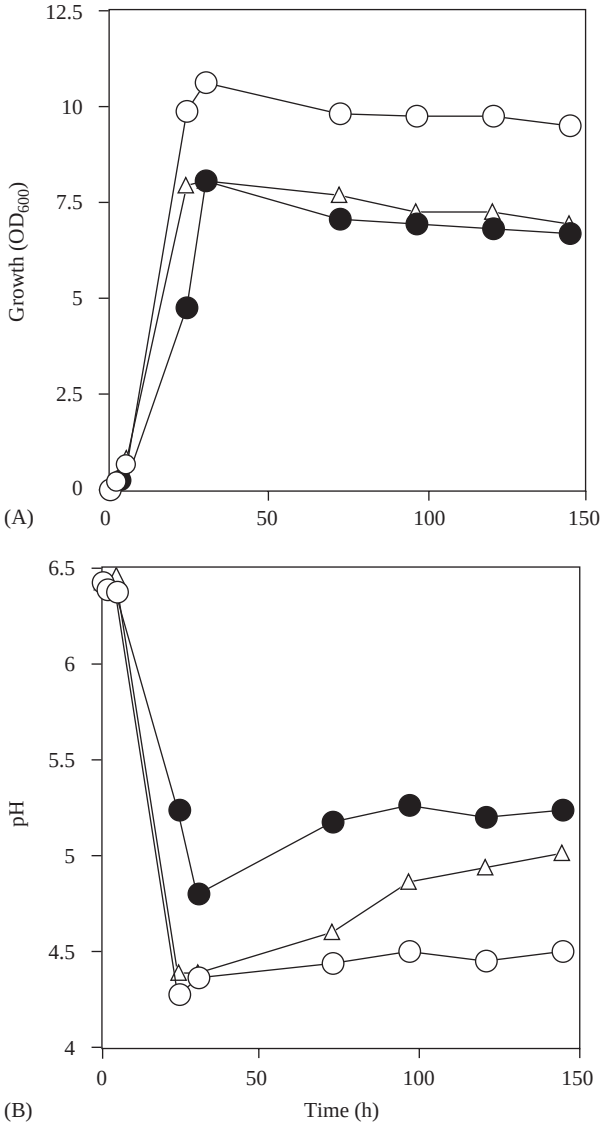

Fig. 1. Growth characteristics (A) and pH change (B) for three strains with high GABA-producing ability (>1.8 mM), *Lb. plantarum* NFRI 7313, *Lb. brevis* NFRI 7340, and *Lb. paracasei* NFRI 7415, grown in MRS medium containing 100 mM of glutamate at 30 °C. Symbols: (○) *Lb. plantarum* NFRI 7313; (●) *Lb. brevis* NFRI 7340; and (△) *Lb. paracasei* NFRI 7415. Data expressed as mean ± SD from three independent experiments.

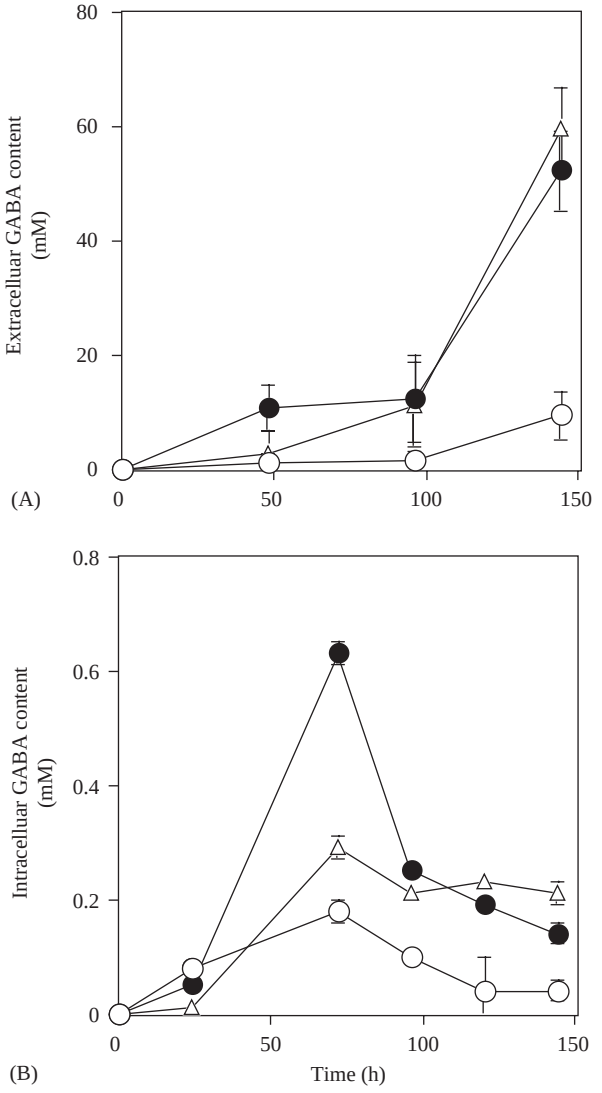

Fig 2. Change in extracellular (A) and intracellular (B) GABA content produced by *Lb. plantarum* NFRI 7313, *Lb. brevis* NFRI 7340, and *Lb. paracasei* NFRI 7415 cultured in MRS medium containing 100 mM of glutamate at 30 °C. Symbols: (○) *Lb. plantarum* NFRI 7313; (●) *Lb. brevis* NFRI 7340; and (△) *Lb. paracasei* NFRI 7415. Data expressed as mean ± SD from three independent experiments.

Pattern of the assimilation ability of sugars in strain NFRI 7415 agreed well with the metabolic characteristics of *Lb. paracasei* described by Kandler and Weiss (1989), Dicks et al. (1996), and Mori et al. (1997). These results suggest that strain NFRI 7415 belongs to *Lb. paracasei*.

3.3. Time course study of extracellular and intracellular GABA content of LAB strains

The GABA-producing ability of the three LAB strains (NFRI 7415, NFRI 7313, and NFRI 7340) was determined by conducting a time course analysis of intracellular and extracellular GABA content in these strains in culture medium. Fig. 2A shows the measured extracellular GABA content. Among the three strains, *Lb. paracasei* NFRI 7415 showed the highest GABA production, reaching 60 mM after 144 h (6 d) cultivation. Fig. 2B shows the measured intracellular GABA content of the strains. For all three strains, intracellular GABA content was extremely low compared with extracellular GABA, and intracellular GABA content peaked before the extracellular GABA content peaked. These results, along with the reported localization of GAD in cytoplasm of *Lactobacillus* (Higuchi et al., 1997), suggest that GABA was synthesized in cytoplasm and

then secreted into the culture medium, although the molecular mechanisms remain unclear. GABA production of *Lb. paracasei* NFRI 7415 was analysed in detail here for the first time, as discussed in the next Sections (3.4–3.6), because *Lb. paracasei* NFRI 7415 showed the highest GABA production among the strains.

3.4. Determination of glutamate concentration and cultivation temperature optimal for GABA production in Lb. paracasei NFRI 7415

Culture conditions optimal for GABA production were determined by measuring the extracellular GABA content in *Lb. paracasei* NFRI 7415 for various glutamate concentrations and cultivation temperatures in MRS medium. The measured extracellular GABA production increased with increasing glutamate concentration (Fig. 3A). However, when the glutamate concentration exceeded 500 mM, the cell growth was strongly inhibited (Fig. 3B). GABA concentration reached 161 mM after cultivation of 144 h (6 d) in the medium containing glutamate concentration of 500 mM (Fig. 3A). These results suggest that GABA concentration increased depending on glutamate addition to the culture medium, and that a glutamate concentration of 500 mM was optimal for GABA production by *Lb. paracasei* NFRI 7415.

GABA production by *Lb. paracasei* NFRI 7415 at various cultivation temperatures was assessed (Fig. 3C). GABA was produced rapidly at 37 °C, but GABA production and growth rate were drastically inhibited at 43 °C (Figs. 3C and D). These results suggest that 37 °C was optimal for GABA production.

3.5. Effect of PLP addition to culture medium on extracellular GABA production in Lb. paracasei NFRI 7415

To increase the extracellular GABA production by LAB, the optimal culture conditions were determined based on biochemical characteristics of GAD that catalyse glutamate to GABA. Because PLP is a necessary coenzyme of GAD (Tong et al., 2002; Kang et al., 2002), we hypothesized that addition of PLP in the culture medium might affect GABA production. We therefore measured extracellular GABA production by

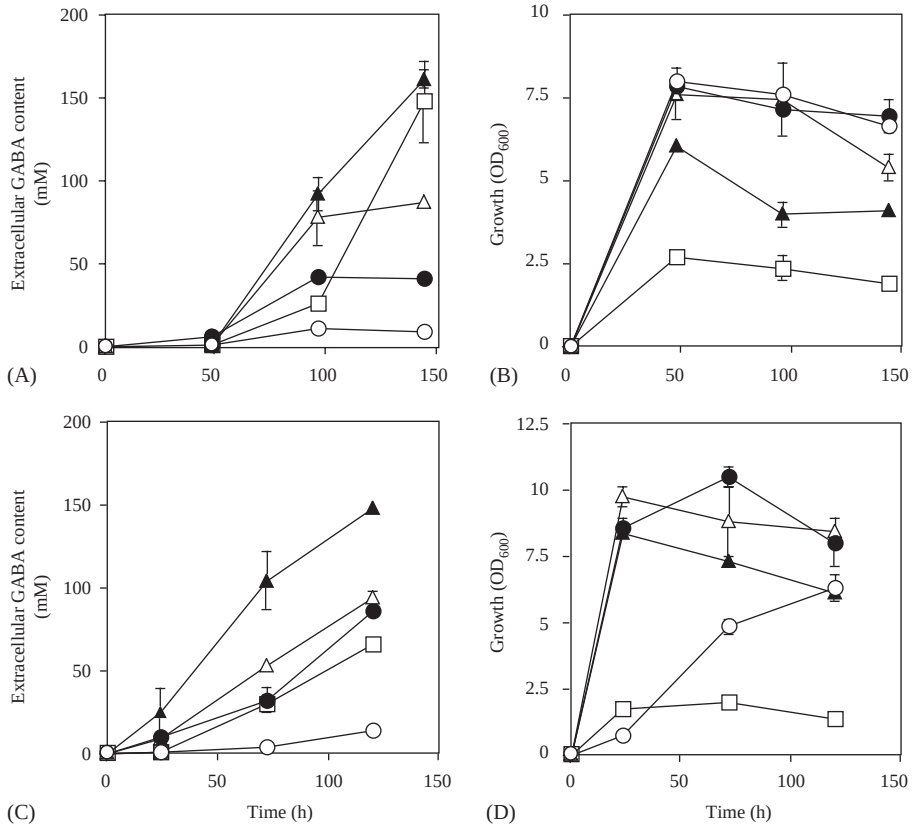

Fig. 3. Effect of glutamate concentration and cultivation temperature on GABA production by *Lb. paracasei* NFRI 7415. Extracellular GABA content (A) and growth rate (B) were monitored at various glutamate concentrations at 30 °C. In (A) and (B), symbols used were, (○) 10 mM; (●) 50 mM; (△) 100 mM; (▲) 500 mM; and (□) 1 M. Extracellular GABA content (C) and growth rate (D) were monitored at various cultivation temperatures in MRS medium containing 100 mM glutamate. In (C) and (D), symbols used were, (○) 10 °C; (●) 25 °C; (△) 30 °C; (▲) 37 °C; and (□) 43 °C. Data expressed as mean ± SD from two independent experiments.

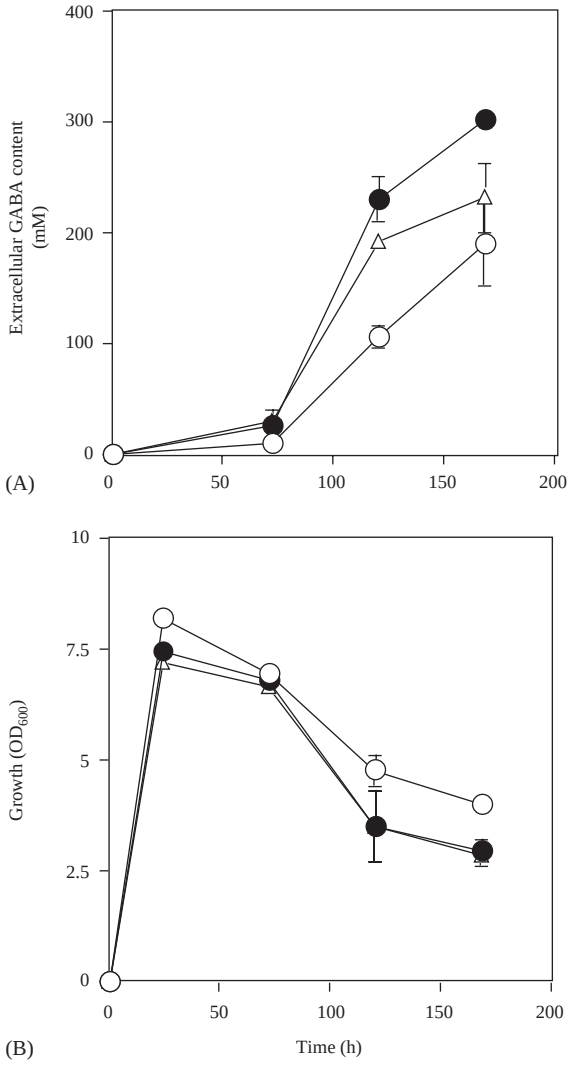

Fig. 4. Effect of PLP addition on GABA production (A) and growth rate (B) of *Lb. paracasei* NFRI 7415 grown in MRS medium containing 500 mM of glutamate at 37 °C. Symbols: (○) no-addition of PLP; (●) 10 μM PLP; and (△) 100 μM PLP. Data expressed as mean ± SD from three independent experiments.

Lb. paracasei NFRI 7415 in MRS medium containing PLP at various concentrations. Fig. 4 shows the results. As expected, PLP addition effectively increased the extracellular GABA production (Fig. 4A), but did not strongly inhibit the cell growth (Fig. 4B). These results suggest that PLP addition in culture medium enhanced the GAD activity.

3.6. Effect of pH regulation of culture medium on extracellular GABA production in Lb. paracasei NFRI 7415

To determine the optimal culture medium conditions for GABA production, GAD activity under various pH was measured for *Lb. paracasei* NFRI 7415. Results showed that the optimum pH for GAD activity was 5.0 (Fig. 5A). The GAD activity at pH 5.0 was twice that at pH 4.0. We hypothesized that optimal GABA production requires regulation of the pH of the culture broth during cultivation, because GAD activity was highest at pH 5.0. We therefore measured GABA production by *Lb. paracasei* NFRI 7415 under pH-regulated conditions (pH 4–6) (Fig. 5B). At pH 5.0, GABA production was significantly enhanced, reaching 210 mM. These results suggest that pH regulation at optimum pH for GAD effectively improved the GABA production. A major role of bacterial amino acids decarboxlase is

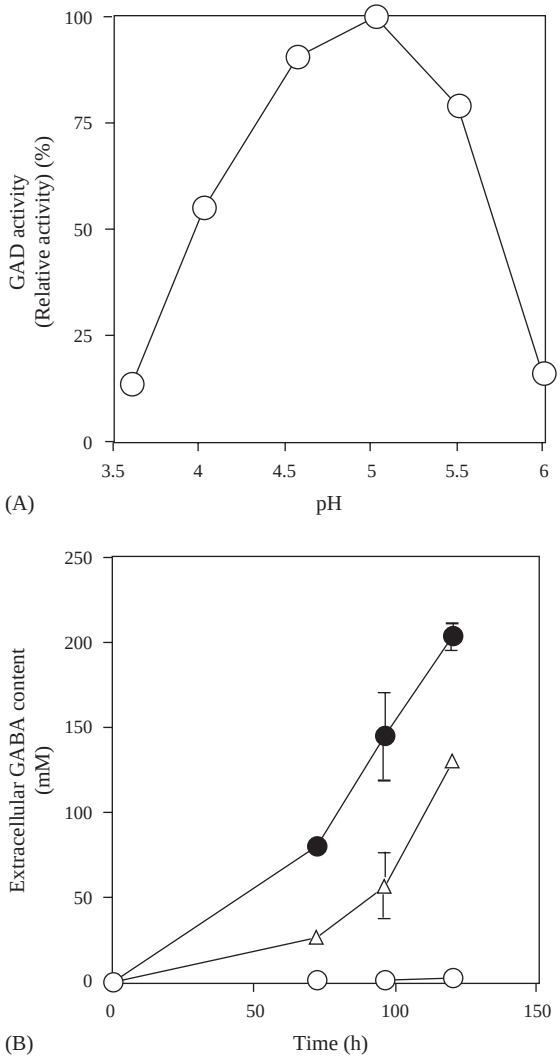

Fig. 5. Effect of pH regulation of culture medium on GAD activity (A) and growth rate (B) of *Lb. paracasei* NFRI 7415 grown in MRS medium containing 500 mM glutamate at 37 °C. GAD activity (A) of crude protein fraction extracted from *Lb. paracasei* NFRI 7415 was measured in 0.2 M pyridine–HCl buffer at various pH. Extracellular GABA content (B) was monitored under various pH-regulated cultivation conditions. In (B), symbols used were, (○) pH 4; (●) pH 5; and (△) pH 6. Data expressed as mean from two independent experiments.

considered maintenance of acidic pH by consumption of H^+ ions (Biase et al., 1999). In the experimental conditions in our study here, however, decarboxlase reaction was not enhanced under lower pH (<4) conditions.

In summary, GABA-producing LAB strains that had different physiological characteristics, such as growth rate and acid-producing ability, were screened for use as starters of various fermented foods. Acid-producing ability might be an important factor in the quality and taste of fermented foods. To develop fermented foods containing GABA with good taste, LAB strains with suitable acid and flavor production profiles should be chosen. To our knowledge, this is the first report of GABA production by *Lb. paracasei*. Although experimental conditions differed from those previously reported for GABA production by *Lb. brevis* (Yokoyama et al., 2002), GABA-producing ability of *Lb. paracasei* NFRI 7415 was apparently higher than that of *Lb. brevis* (IFO 12005). Our results show that PLP addition and pH regulation of culture medium can effectively increase the GABA content accumulated in other GABA-producing LAB as well. Future research will include screening of GABA-producing strains that have unique characteristics, such as acid production, flavor formation, growth rate, and higher GABA-producing ability. Such strains will accelerate the development of functional fermented foods.

Acknowledgements

This study was supported in part by a grant from the Ministry of Agriculture, Forestry and Fisheries (MAFF) Food Research Project "Integrated Research on Safety and Physiological Function of Food".

We thank Chise Suzuki (National Food Research Institute) for critical comments and Kikuichi Tsukahara (Domer, Inc.) for his advice on this work.

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