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Production of γ-aminobutyric acid by *Streptococcus salivarius* subsp. *thermophilus* Y2 under submerged fermentation

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Summary. γ-Aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system, has several well-known physiological functions and has been applied to the production of many drugs and functional foods. The technology of GABA production via submerged fermentation by *Streptococcus salivarius* subsp. *thermophilus* Y2 was investigated in this paper. It indicated that the GABA production was related to the biochemical characteristics of glutamate decarboxylase (GAD) of *S. salivarius* subsp. *thermophilus* Y2. After 24 h of fermentation at 37 °C, which is the suitable culture conditions for GAD-production, then the culture condition were adjusted to the optimal temperature (40 °C) and pH (4.5) for the GAD reaction activity in biotransformation of cells and pyridoxal 5′-phosphate (0.02 mmol/l) were added to the broth at the 48 h, the GABA production was increased up to 1.76-fold, reaching 7984.75 ± 293.33 mg/l. The strain shows great potential use as a starter for GABA-containing yoghurt, cheese and other functional fermented food productions.

Keywords: γ-Aminobutyric acid – Glutamate decarboxylase – *Streptococcus salivarius* subsp. *thermophilus*

1. Introduction

γ-Aminobutyric acid (GABA), a non-protein amino acid, is an efficient neurotransmitter of inhibition in the central nervous system (Gulati and Santon, 1960) and plays an important role in cardiovascular function (Santon, 1963). GABA has been proved to be effective on dieresis, tranquilization, antihypertension and prevention of diabetic conditions (Hagiwara et al., 2004; Hayakawa et al., 2004; Okada et al., 2000; Santon, 1963). Therefore, the effects of GABA on human health are also of current interest in food production.

Currently, the screening of GABA-producing lactic acid bacteria (LAB) and the production of GABA-enriched food by LAB are being investigated actively. Several GABA-producing LAB have been reported, including *Lactobacillus brevis* isolated from alcohol distillery lees (Yokoyama et al., 2002), *Lactococcus lactis* from cheese starters (Nomura et al., 1998), and *Lactobacillus paracasei* from fermented fish (Komatsuzaki et al., 2005). 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, taste and flavor formation ability. These profiles are considered as important factors in the use of LAB as starters in the production of fermented foods (Gran et al., 2003).

Streptococcus salivarius subsp. *thermophilus* is Gram-positive coccus that forms chains and produces *L*-lactate from glucose. It is essential to milk fermentation, especially in the yoghurt and cheese making processes to provide optimal conditions for the development of texture and flavour (Zoon and Allersma, 1996). In the previous work, we have found that *S. salivarius* subsp. *thermophilus* Y2 showed a great GABA-producing ability. The culture conditions for GABA-production via submerged fermentation by *Streptococcus salivarius* subsp. *thermophilus* Y2 were firstly investigated in this paper, and the results indicated that *S. salivarius* subsp. *thermophilus* Y2 had great potential use as starter in the production of GABA-containing yoghurt, cheese and other functional fermented foods.

2. Materials and methods

2.1 Culture medium and conditions

The *S. salivarius* subsp. *thermophilus* Y2 was grown (without shaking) at 37 °C in 11 of flask containing 400 ml of nutrient medium with the

following compositions: 15.0 g/l of peptone, 12.5 g/l of beef extract, 12.5 g/l of sucrose, 1.03 g/l of dipotassium hydrogen phosphate, 5 g/l of sodium acetate, 2 g/l of ammonium dibasic citrate, 2.12 g/l of calcium chloride, 1 g/l of tween 80, and initial pH 6.79. Sodium glutamate was added to culture at concentration as indicated in figure legends. In pH-regulated cultivation, pH was adjusted every 12 h by adding 1 mol/l of NaOH or 6 mol/l of HCl during cultivation.

2.2 GABA analysis

Extracellular GABA accumulated in the culture medium was prepared as follows. First, the culture broth was separated from cells by centrifugation ($8000 \times g$ for 15 min at 4 °C), and the supernatant was 100-fold diluted with 7% (V/V) of acetic acid. Then, the diluted sample was centrifugated at $8000 \times g$ for 15 min at 4 °C, and the supernatant was collected for further analysis.

Intracellular GABA was prepared as follows. First, the cells were washed with 0.9% of NaCl for three times, and resuspended in 20 ml of phosphate buffer (pH 8.0). Then, the cell suspension was treated with sonication (500 W, 20 min) in ice bath. The homogenate was centrifuged at $8000 \times g$ for 15 min at 4 °C, and the supernatant was collected for further analysis.

100 µl of the preparations of extracellular GABA or intracellular GABA were derivatized to phenylthiocarbamyl-GABA and analyzed by high-performance liquid chromatography (HPLC) according to Rossetti and Lombard (1996) with some modifications. The derivatized samples were dissolved in 200 µl of initial mobile phase, consisting of a mixture of 60% solution A (aqueous solution of 10.254 g sodium acetate, 0.5 ml triethylamine and 0.7 ml acetic acid in 1000 ml, pH 5.8), 12% solution B (acetonitrile) and 28% solution C (water). HPLC separations were performed on the instrument of Agilent 1100 Series (United State) with ZORBAX. Eclips XDB-C_{18} column (Agilent, 4.6×250 mm, 5 µm) at 27 °C and 254 nm. The elution programs were shown in Table 1.

2.3 Assay for GAD activity of the cells

The cells of *S. salivarius* subsp. *thermophilus* Y2 were collected by centrifugation ($8000 \times g$ for 15 min at 4 °C) and washed with 0.9% of NaCl for three times, then resuspended in 10-fold (V/W) 0.9% of NaCl and stirred with a magnetic stirrer for 15 min. One millilitre of the suspension was then incubated with 1 ml of monosodium glutamate solution (150 mmol/l) and 2 ml of acetate buffer (150 mmol/l) for 30 min. The reaction temperature and pH were indicated in figure legends. Eight millilitre absolute ethanol was added at −20 °C to terminate the reaction. The suspension was centrifuged at $8000 \times g$ for 15 min at 4 °C. Then, GABA content in the suspension was measured by HPLC following the indications in the steps of GABA analysis. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of GABA in 1 min.

2.4 Determination of cell viability

Cell viability was determined as colony forming units (CFU) by the plate dilution method using modified TJA agar (Huang et al., 2006), consisting of yeast extract 5 g/l, beef extract 10 g/l, lactose 20 g/l, sucrose 2 g/l, sodium acetate 5 g/l, K_2HPO_4 2 g/l, tween 80 1 g/l, tomato juice 50 ml/l, agar 10 g/l, and initial pH 6.8 ± 0.2. Serial dilutions of each sample were plated in triplicate, and the plates were incubated at 40 °C for 48 h. The results were expressed as log CFU/ml.

Table 1. Elution programs of high-performance liquid chromatography

Time/ min	Solution A/ %	Solution B/ %	Solution C/ %	Flow/ ml/min
0.00	60.00	12.00	28.00	0.60
6.00	60.00	12.00	28.00	0.60
6.10	20.00	13.50	66.50	0.40
22.00	20.00	13.50	66.50	0.40

3. Results

3.1 Time course study of extracellular and intracellular GABA content

The GABA-producing ability of *S. salivarius* subsp. *thermophilus* Y2 was determined by conducting a time course analysis of extracellular and intracellular GABA content in culture medium. Figure 1 showed that the content of intracellular GABA was extremely low compared with that of extracellular GABA, and intracellular GABA content peak (at 24 h) occurred earlier than that of the extracellular GABA content peak (at 84 h). The results, complied with the reports that GAD was the sole enzyme that catalyses the irreversible α-decarboxylation of *L*-glutamate to GABA and CO_2 in biology and was localized in cytoplasm (Higuchi et al., 1997), suggested that GABA might be synthesized in cytoplasm and then secreted into the culture medium. The extracellular GABA was largely synthesized when the pH of culture medium was dropped to acid range (pH = 4.5–5.0), and was continuing synthesized even if the viable cells begun markedly decrease after 48 h. The results, along with the reported optimum pH for bacterial GAD activity (Yang et al., 2006), indicated that the GABA-synthesis under submerged fermentation was related to the biochemical

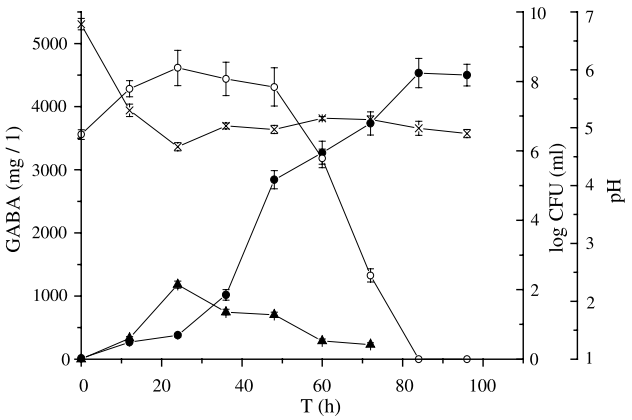

Fig. 1. Change in extracellular and intracellular GABA content produced by *S. salivarius* subsp. *thermophilus* Y2 cultured in the medium containing 10 g/l of monosodium glutamate at 37 °C. (●) Extracellular GABA content; (▲) 10-fold of intracellular GABA content; (○) log CFU/ml; (×) pH. Data expressed as mean ± SD from three independent experiments

characteristics of GAD and the GAD was remain the activity even though the cells died. Therefore, production of GABA would be increased by adjusting the fermentation conditions to the optimal conditions of enzyme reaction.

3.2 Effect of pH and temperature on the GAD activity in biotransformation of cells

To determine the optimal culture conditions for GABA production, GAD activities under various pHs and temperatures were measured. Results showed that the optimal pH and temperature for GAD activity in the biotransformation by the cells of *S. salivarius* subsp. *thermophilus* Y2 was 4.5 and 40–45 °C, respectively (Figs. 2 and 3). Since GAD activity was highest at pH 4.5 and 40–45 °C, we hypothesized that optimal GABA production required regulation of the pH and temperature of culture broth to pH 4.5 and 40 °C when the cells with GAD activity was reaching the peak during cultivation.

3.3 Effect of culture temperature and time on the GAD production

The optimal pH and temperature for GAD activity was 4.5 (Fig. 2) and 40 °C (Fig. 3), respectively. However, pH 4.5 and 40 °C might not be suit for the growth and GAD production of *S. salivarius* subsp. *thermophilus*. Therefore, the fermentation must be conducted at the suitable conditions for the cells growth and the GAD production

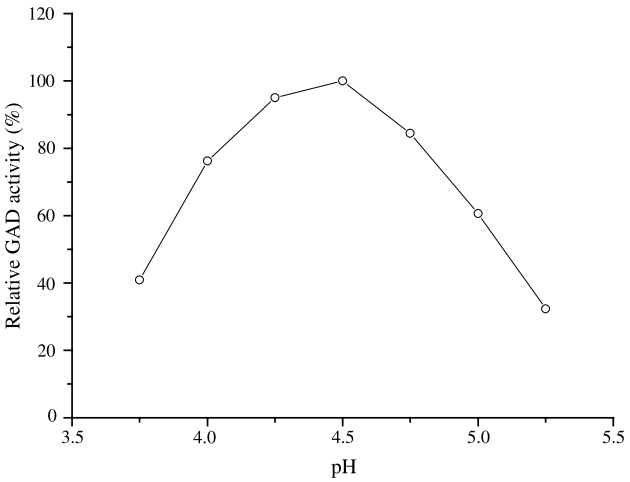

Fig. 2. Effects of pH on the GAD activity in biotransformation of cells. GAD activity was measured under various pHs at 40 °C for 30 min. The highest GAD activity was defined as 100% for calculation of relative activity, and the data were expressed as mean from three independent experiments

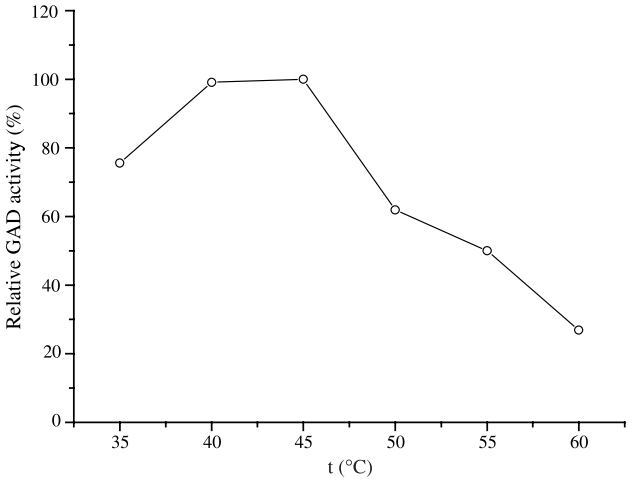

Fig. 3. Effect of temperature on the activity of GAD in biotransformation by cells. GAD activity was measured under pH 4.5 at various temperatures for 30 min. The highest GAD activity was defined as 100% for calculation of relative activity, and the data were expressed as mean from three independent experiments

before pH and temperature were regulated to the optimal pH and temperature for GAD activity.

Figure 4 shows that GAD activity increased while the culture temperature increased from 34 to 37 °C, and decreased while the culture temperature increased from 37 to 46 °C. Thus, the optimal culture temperature for GAD production was 37 °C.

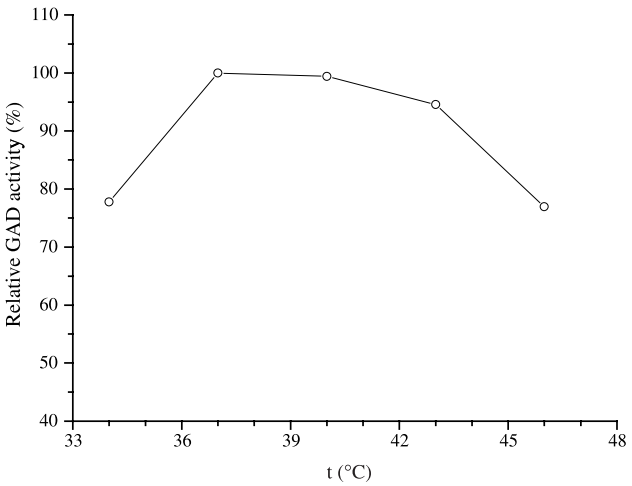

Fig. 4. Effect of culture temperature on the production of GAD. *S. salivarius* subsp. *thermophilus* Y2 was incubated in nutrient medium containing 10 g/l of monosodium glutamate at various temperatures for 24 h. The cells were collected by centrifugation (8000 × *g* for 15 min at 4 °C) and washed with 0.9% of NaCl for three times, respectively. Then the cells were used for the GAD activity determination under pH 4.5 at 40 °C, respectively. The highest GAD activity was defined as 100% for calculation of relative activity, and the data were expressed as mean from three independent experiments

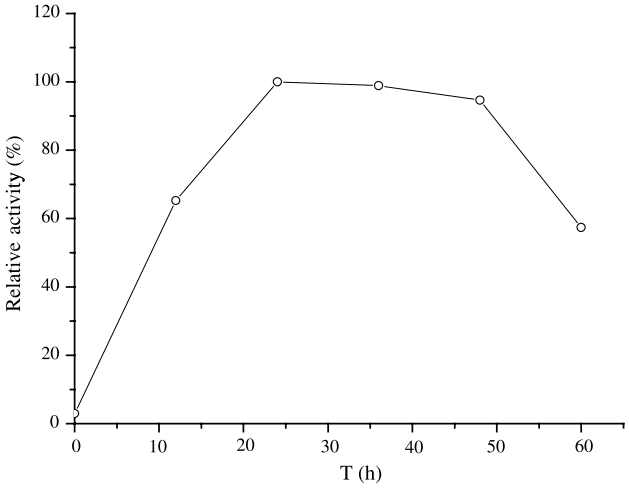

Fig. 5. Time course study of GAD production. *S. salivarius* subsp. *thermophilus* Y2 was incubated in nutrient medium containing 10 g/l of monosodium glutamate at 37 °C for 24 h. The cells of the same volume of broth that sampled at various culture time were collected by centrifugation (8000 × *g* for 15 min at 4 °C) and washed with 0.9% of NaCl for three times, respectively. Then the cells were used for the GAD activity determination under pH 4.5 at 40 °C, respectively. The highest GAD activity was defined as 100% for calculation of relative activity, and the data were expressed as mean from three independent experiments

Figure 5 shows that GAD activity increased with prolonging the culture time. However, the GAD activity became decreased when the cultivation after 24 h of cultivation. The results indicated that the optimal culture time for GAD production was 24 h.

3.4 Effect of pH and temperature regulation of culture medium on extracellular GABA production

GABA synthesis was found that it was related to the biochemical characteristics of GAD. We hypothesized that optimal GABA production of *S. salivarius* subsp. *thermophilus* Y2 required regulating the pH and temperature of the culture broth during cultivation to the optimal conditions for the biotransformation of cells, i.e., pH 4.5 and 40 °C respectively. Therefore, two-steps fermentation strategy was investigated, i.e., the fermentation was first carried out at 37 °C and pH-free for 24 h, then the temperature and pH of the culture broth were regulated to 40 °C and pH 4.5 respectively. As a result, GABA production was significantly enhanced under the optimal conditions, reaching 5770.35 ± 170.59 mg/l at 72 h (Fig. 6), 1.27-fold higher than that of none regulation of temperature and pH (Fig. 1, 4534.03 ± 231.52 mg/l). At the same time, 12 h of the fermentation time was saved compared with that of none regulation of temperature and pH (Fig. 1, 84 h).

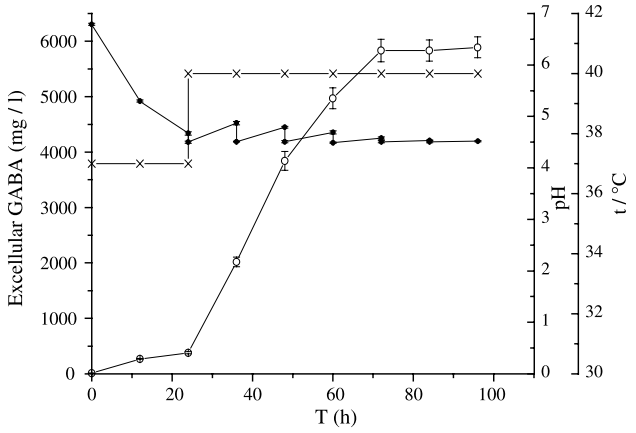

Fig. 6. Effect of pH and temperature regulation of culture medium on extracellular GABA production. *S. salivarius* subsp. *thermophilus* Y2 was incubated in nutrient medium containing 10 g/l of monosodium glutamate at 37 °C for 24 h, then the culture temperature was adjusted to 40 °C. The pH of culture medium was adjusted to pH 4.5 every 12 h by adding 1 mol/l of NaOH or 6 mol/l of HCl after free incubated for 24 h. (○) Extracellular GABA content; (●) pH; (×) culture temperature. Data expressed as mean ± SD from three independent experiments

3.5 Effect of monosodium glutamate concentration on extracellular GABA production

As a substrate of GAD, glutamate was an essential compound of the medium for GABA synthesis in fermentation. But, superfluous addition of glutamate would inhibit the cell growth and decrease the GABA production in some cases. Optimal culture conditions for GABA pro-

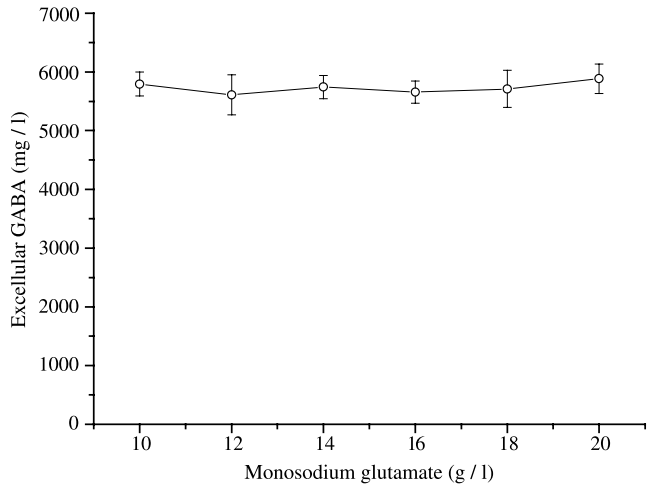

Fig. 7. Effect of monosodium glutamate concentration on extracellular GABA production. *S. salivarius* subsp. *thermophilus* Y2 was incubated in nutrient medium containing various concentration of monosodium glutamate at 37 °C for 24 h, then the culture temperature was adjusted to 40 °C. The pH of culture medium was adjusted to pH 4.5 every 12 h by adding 1 mol/l of NaOH or 6 mol/l of HCl after free incubated for 24 h. (○) Extracellular GABA content. Data expressed as mean ± SD from three independent experiments

duction were determined by measuring the extracellular GABA content in *S. salivarius* subsp. *thermophilus* Y2 for various monosodium glutamate concentrations in the medium. The extracellular GABA almost was the same even though monosodium glutamate concentration was increasing from 10 to 20 g/l (Fig. 7). The results indicated that GABA production was not inhibited by monosodium glutamate at the range from 10 to 20 g/l.

3.6 Effect of pyridoxal 5′-phosphate addition to culture medium on extracellular GABA production

Because pyridoxal 5′-phosphate (PLP) is a necessary coenzyme of GAD (Sandmeier et al., 1994), we hypothesized that addition of PLP in the culture medium might affect GABA production. To increase the extracellular GABA production by *S. salivarius* subsp. *thermophilus* Y2, the effects of PLP addition on extracellular GABA production were investigated. As expected, GABA production was increased in different degree with the addition of PLP at various culture times. Figure 8 shows that the extracellular GABA production at 72 h reached 6271.79 ± 229.27, 6570.35 ± 190.59 and 7333.11 ± 207.87 mg/l, respectively when PLP was added at 0, 24 and 48 h, and GABA was continued to be synthesised after 72 h when PLP was added at 48 h with 7984.75 ± 293.33 mg/l of GABA at 84 h. The GABA production was 1.76-fold higher than that of none optimization (Fig. 1, 4534.03 ± 231.52 mg/l).

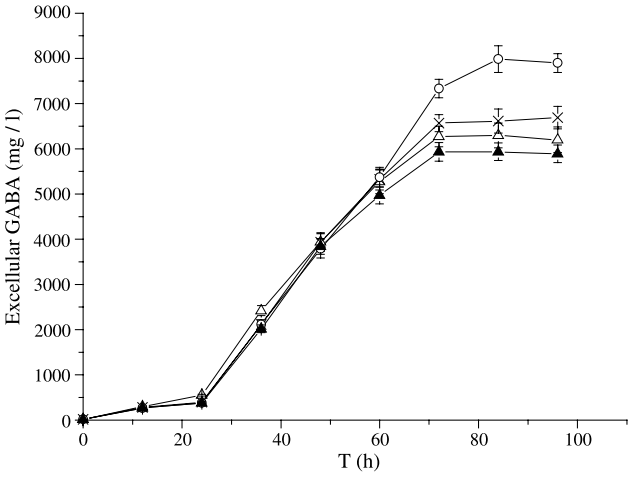

Fig. 8. Effect of PLP addition to culture medium on extracellular GABA production. *S. salivarius* subsp. *thermophilus* Y2 was incubated in nutrient medium containing 15 g/l of monosodium glutamate at 37 °C for 24 h, then the culture temperature was adjusted to 40 °C. The pH of culture medium was adjusted to pH 4.5 every 12 h by adding 1 mol/l of NaOH or 6 mol/l of HCl after free incubated for 24 h. PLP was added to the culture medium to 0.02 mmol/l (terminal concentration) at 0, 24 and 48 h, respectively. (▲) No PLP; (△) PLP was added at 0 h; (×) PLP was added at 24 h; (○) PLP was added at 48 h. Data expressed as mean ± SD from three independent experiments

When PLP was added at 48 h of fermentation, the GABA production was much higher than that of which PLP was added at the 0 and 24 h (Fig. 8). The results suggested that PLP could partly recover GAD activity. However, PLP could easily lose the role as coenzyme due to the denaturalization in the culture broth during the cultivation. Therefore, it may be more efficient to improve GABA production by supplementation of PLP at 48 h.

4. Discussion

GABA is synthesized by decarboxylation of *L*-glutamate, catalysed by GAD [EC 4.1.1.15]. GAD is localized in cytoplasm, and *L*-glutamate and GABA can permeate through membrane and exchange each other in LAB (Higuchi et al., 1997). In this paper, we found that GABA production during cultivation of *S. salivarius* subsp. *thermophilus* Y2 was related to the biochemical characteristics of GAD, and we hypothesized that the GABA-production during cultivation of the strain can be improved by adjusting the culture conditions suitable for the biotransformation conditions of the cells after the GAD-production had been reached the highest. Therefore, we firstly adjusted the culture conditions to the optimal culture conditions for GAD-production, and then turned to the optimal conditions of the GAD reaction activity in biotransformation by the cells of *S. salivarius* subsp. *thermophilus* Y2. Results showed that GABA production of *S. salivarius* subsp. *thermophilus* Y2 was greatly increased, reaching 7984.75 ± 293.33 mg/l, and which was 1.76-fold than that of none optimization.

Komatsuzaki et al. (2005) found that PLP could greatly promoted GABA production of *Lb. paracasei* when it was added to the initial culture medium. However, in our study, PLP could not effectively increase the GABA production of *S. salivarius* subsp. *thermophilus* Y2 when it was added at initial fermentation (Fig. 8). The results indicated that PLP could not increase the GAD production. But, PLP showed effectively role in increasing GABA production when it was added after the fermentation had been conducted for 48 h (Fig. 8). This may be due to that the free PLP is easily denatured during the cultivation and lost the role as the coenzyme of GAD. The complement of PLP would partly recover the GAD activity and increase the GABA production. Though *Streptococcus salivarius* subsp. *thermophilus* has been widely used in dairy for decades, to our knowledge, this is the first report of GABA

Table 2. γ-Aminobutyric acid productions via submerged fermentation by lactic acid bacteria

Microorganism	Fermentation time/h	GABA content/g/l	Reference
Lactobacillus brevis	48	1.05	Yokoyama et al. (2002)
Lactobacillus paracasei	168	31.11	Komatsuzaki et al. (2005)
Streptococcus salivarius subsp. *thermophilus* Y2	84	7.98	this paper

production by *Streptococcus salivarius* subsp. *thermophilus* via submerged fermentation.

The GABA-producing ability of *Streptococcus salivarius* subsp. *thermophilus* Y2 via submerged fermentation was intermediate compared with that of *Lactobacillus brevis* (Yokoyama et al., 2002) and *Lactobacillus paracasei* (Komatsuzaki et al., 2005) (Table 2). Because of the high GABA-producing ability, *S. salivarius* subsp. *thermophilus* Y2 has the great potential for use as a starter in the production of GABA-containing yoghurt, cheese and other functional fermented foods.

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