

1 **Short title**

2 **GABA fermentation with date residue**

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4 **Gamma-aminobutyric acid fermentation with date residue by a lactic acid**
5 **bacterium, *Lactobacillus brevis***
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7 Momoko Hasegawa,^{1,2} Daisuke Yamane,¹ Kouichi Funato,¹ Atsushi Yoshida,² and Yoshihiro
8 Sambongi^{1,*}
9

10 *Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-*
11 *Hiroshima, Hiroshima 739-8528, Japan,¹ and Otafuku Sauce Co., Ltd., 7-4-27 Shoko Center, Nishi-*
12 *ku, Hiroshima 733-8670, Japan²*

13 *Corresponding author. Tel./fax: +81 82 424 7924.

14 *E-mail address:* sambongi@hiroshima-u.ac.jp (Y. Sambongi)
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Dates are commercially consumed as semi-dried fruit or processed into juice and puree for further food production. However, the date residue after juice and puree production is not used, although it appears to be nutrient enriched. Here, date residue was fermented by a lactic acid bacterium, *Lactobacillus brevis*, which has been generally recognized as safe. Through degradation of sodium glutamate added to the residue during the fermentation, γ -aminobutyric acid (GABA), which reduces neuronal excitability, was produced at the conversion rate of 80 – 90% from glutamate. In order to achieve this GABA production level, pretreatment of the date residue with carbohydrate-degrading enzymes, i.e., cellulase and pectinase, was necessary. All ingredients used for this GABA fermentation were known as being edible. These results provide us with a solution for the increasing commercial demand for GABA in food industry with the use of date residue that has been often discarded.

Palm tree *Phoenix dactylifera* is a dioecious plant mainly growing in the arid and semi-arid regions of desert countries such as the United Arab Emirates, Egypt, Saudi Arabia and Iran, which have average annual temperatures of 21 to 27 °C (1). The plant bears fruit known as dates, more than 7,600,000 tons of which are produced as of 2014 (FAOSTAT, <http://www.fao.org/faostat/en/#home>). Dates are consumed as semi-dried fruit or processed into juice and puree for further production of food such as sauces.

Dates are rich in nutrition: the semi-dried and dried forms contain 50 – 80% (w/w) carbohydrates, predominantly glucose and fructose, and ranges of minerals and vitamins (2,3). In particular, carbohydrates in dates are promising sources of fermentable carbon, which can be converted by microorganisms. Date residue after juice and puree production appears to be nutrient enriched. Therefore, the total utilization of dates is regarded as one of the promising technologies for the food, chemical, and pharmaceutical industries (3).

Many strains of lactic acid bacteria, generally recognized as safe, that had been isolated from traditional fermented foods have been used for production of γ -aminobutyric acid (GABA), a non-protein amino acid (4). GABA has been found to be an inhibitory neurotransmitter in the animal nervous system (5), thus having potential as a bioactive component when GABA-containing foods and pharmaceuticals are consumed. Recently, GABA production involving date puree fermented by autochthonous lactic acid bacteria has been reported for the first time (6), but to the best of our knowledge, date residue after juice and puree production has not been examined as to the GABA fermentation aiming at food industry.

In the present study, a lactic acid bacterium, *Lactobacillus brevis*, was used to ferment date residue with other edible ingredients such as sodium glutamate and acetic acid for the production of GABA. Our present results will provide a solution for the increasing commercial demand for GABA with the use of date residue that has been often discarded.

MATERIALS AND METHODS

Bacterial strains used and culture medium *Lactobacillus brevis* JCM 1059^T and JCM

1061 were used for the fermentation of date residue in this study. The strains were statically cultivated in deMan, Rogosa and Sharpe (MRS) broth (Difco, USA) at 30 °C for one day. The resulting cells were suspended in a sterile physiological salt solution [0.85% (w/v) NaCl] at the cell concentration of 10⁹ to 10¹⁰ cfu/ml before inoculation as described below.

Preparation of date residue The variety of semi-dry date fruit used in this study was

Sayer, one of the top commercial varieties (7), most of which had been imported from Al Foah L.L.C. of the United Arab Emirates. After preparing juice and puree from the dates, the resulting date residue, which would have been routinely discarded during food processing at Otafuku Sauce Co., Ltd., Japan, was mixed with tap water in a ratio of 1:1 (w/w). Our preliminary survey indicated that this ratio provided the highest GABA production. The resulting mixture was then homogenized with a mixer, Masscolloider (MKCA6-2J, MASUKO SANGYO Co., Ltd.), before examining GABA production.

Fermentation of date residue The resulting homogenized date residue was incubated

with carbohydrate-degrading enzymes [0.3 % cellulase A and 0.3 % pectinase G (w/w) (Amano Enzyme Inc., Aichi, Japan)] at 35 °C for six hours. Our preliminary survey indicated that 0.3 % concentration of these enzymes were minimally required for the highest GABA production. After incubation with these carbohydrate-degrading enzymes, 32.2 mM edible sodium glutamate (Ajinomoto Co., Inc., Tokyo, Japan) and 0.14% (v/v) edible acetate (Otafuku Vinegar Brewery Co., Ltd., Hiroshima, Japan) were added to the homogenized date residue, which was further heated at 70 °C for 20 minutes. The enzyme-treated and heated date residue (10 g) was then inoculated with

the *L. brevis* cell suspension to give a final cell concentration of 10^8 cfu/gram of date residue, followed by fermentation at 25 °C up to eleven days without shaking. The cell number inoculated, acetate concentration added, and temperature of heat treatment for date residue after enzyme treatment were also optimized in our preliminary experiments.

Measurement of GABA and other contents One gram of the fermented date residue described above was mixed with 4 ml of the 1st lithium citrate buffer, pH 2.98 (JEOL, Tokyo, Japan), the mixture then being mixed with an equal volume of 3% (w/v) 5-sulfosalicylic acid dihydrate (Nacalai Tesque, Kyoto, Japan) in order to precipitate proteins. After spinning down the precipitated proteins, the supernatant was filtrated through a polyethersulfone membrane filter (pore size: 0.22 μ m; Starlab Scientific, Xian, Shaanxi Province, China) and then diluted five times with the 1st lithium citrate buffer. The resulting solution was analyzed for the concentrations of amino acids including GABA and glutamate with an amino acid analyzer (JEOL JLC-500/V, Tokyo, Japan). This procedure only gave the contents of extracellular amino acids including GABA, which appeared to represent the whole GABA production in the present *L. brevis* cells, because that of intracellular GABA was negligible in *Lactobacillus paracasei* (8). The concentrations of glucose and lactic acid in the same solution were also determined using an automated biochemical analyzer (AU480; Beckman Coulter, Brea, CA, USA). At the same time, the viable cell number and pH of the fermented date residue were also recorded.

RESULTS AND DISCUSSION

Conditions for GABA fermentation with date residue

We first attempted to determine whether GABA could be produced in the date residue on the six-day's fermentation using *L. brevis* JCM 1059^T. The fermentation temperature of 25 °C was adopted throughout this study, because the GABA production at 25 °C was 1.2- and 1.8-fold increase compared with those at 30 and 37 °C, respectively (data not shown). The concentration of sodium glutamate added was also fixed at 32.2 mM (0.5 % w/w) and the initial pH was adjusted at ~4.4 to 4.5 with 0.14% (v/v) of acetate, which gave the highest GABA productivity (see below). Furthermore, the 0.14% (v/v) concentration of acetate adopted in this study turned out to be an indispensable constraint from the standpoint of avoiding contamination.

Approximately 28 mM GABA was reproducibly detected in the fermented date residue that had been treated with carbohydrate-degrading enzymes (Fig. 1A). However, only 0.3, 0.6, and 0.8 mM GABA were detected without the cell inoculation, date residue, and treatment with carbohydrate-degrading enzymes, respectively. These results together indicated that GABA was produced from glutamate due to the cellular activity of *L. brevis* JCM 1059^T and that the treatment of the date residue with the carbohydrate-degrading enzymes was critical for the GABA production. The enzyme treatment resulted in a slight increase in the variety of amino acids (less than 1 mM, data not shown), but there is presently no explanation of how the enzyme treatment works. In addition, another strain of *L. brevis*, JCM 1061 only produced 0.2 mM GABA in the same experimental setup with date residue (Fig. 1A). The reason for this also remains unknown, which will be examined in future together with the function of enzyme treatment.

Even with the cell inoculation of *L. brevis* JCM 1059^T and enzyme treatment of the date residue, only 1.0 mM GABA was detected without the addition of sodium glutamate (Fig. 1B), thus the GABA production of less than ~1 mM observed in Fig. 1A appeared to be the basal level with the

present experimental setup. The concentration of sodium glutamate added to the date residue for the optimal GABA production was ~30 mM and increased concentrations of more than 150 mM inhibited the GABA production (Fig. 1B). The inhibitory effect of excess addition of sodium glutamate was likely due to the pH increase to more than 5.0 (Fig. 1B) that could not be controlled with 0.14% (v/v) acetate initially added in the present study from the **standpoint of avoiding contamination**. In contrast, the initial pH lower than 4.0 caused by the excess addition of acetic acid resulted in lowering of GABA production (data not shown), consistent with the previous observations showing decreased GABA production under the same pH conditions (8,9). Our present results and previous observations on the optimal pH conditions for the GABA production by lactic acid bacteria are consistent with the optimal pH range of 4.0 – 5.0 for the activity of glutamate decarboxylases, which catalyze GABA production from glutamate (5).

Time course of GABA production Under the optimal conditions for GABA production in the present study, i.e., 10^8 cfu/g *L. brevis* JCM 1059^T cell inoculation, presence of date residue, carbohydrate-degrading enzyme treatment, and 32 mM sodium glutamate addition (Fig.1), we further investigated the time course of GABA production in order to determine the change in the rate of conversion of GABA from glutamate. The GABA concentration rapidly increased in two days after the cell inoculation, and afterwards remained constant at approximately 28 mM (Fig. 2), consistent with the results in Fig. 1A. At the same time, the concentration of glutamate in the fermentation batch decreased from ~30 to ~5 mM during the four-day's fermentation. After the four day's fermentation, the rate of conversion of GABA from glutamate was nearly 80 – 90%. This conversion rate was comparable to that obtained previously using *Lactobacillus brevis* IFO-12005 in rice liquor distillery lees containing about 10 mM free glutamate (10), and was two-fold of that obtained previously using *Lactobacillus lactis* in MRS broth with 1% (w/v) sodium glutamate (11).

In this study, $101.0 \pm 0.3\%$ (means \pm standard errors for three independent experiments)

conversion rate of GABA from 32 mM glutamate was observed at three-day's fermentation using *L. brevis* JCM 1059^T with MRS broth instead of date residue, while $78.6 \pm 8.3\%$ (means \pm standard errors for three independent experiments) conversion rate was found with date residue (Fig. 2). Although *L. brevis* JCM 1059^T produced GABA with MRS slightly efficiently compared with date residue, the edible date residue can be used for sufficient GABA production, which may be advantageous for the future food application. Expensive and inedible MRS broth may not be used for this purpose.

Activities of *L. brevis* cells during fermentation of date residue We further analyzed the activities of *L. brevis* JCM 1059^T cells during the same fermentation process as shown in Fig. 2 in order to determine the cellular physiology. The amino acid concentrations before and after six-day's fermentation did not differ significantly (data not shown) except those of GABA and glutamate. The viable cell number increased up to three times after two-day's fermentation and then gradually decreased, the decrease being prominent after seven-day's fermentation (Fig. 3A). Glucose remained in the fermentation batch even after 11-day's fermentation (Fig. 3B), which appeared to be advantageous for sweet taste enhancement in the aspect of sensory profile. During the fermentation, the glucose concentration tended to decrease (Fig. 3B), indicating that the *L. brevis* cells were actively utilizing glucose.

During the same fermentation process, the pH decreased from 4.4 to 3.7 (Fig. 3C), which appeared to be due to the increase in the lactic acid concentration (Fig. 3D). The cell number started to decrease prominently after seven-day's fermentation (Fig. 3A), which correlated with the pH decrease to 3.7. This pH appeared to be the physiological limit for *L. brevis* JCM 1059^T growth with the present experimental setup, consistent with the previous observation with another strain of the same bacterium (4). These results together indicated that the GABA production from glutamate was due to the lactic acid fermentation by *L. brevis* JCM 1059^T.

Conclusion

We succeeded in GABA fermentation with date residue and other edible ingredients such as glutamate and acetate by using *L. brevis* JCM 1059^T. The preparations of date residue including carbohydrate-degrading enzyme treatment and fermentation conditions were designed in this study. The resulting fermented date residue extract containing GABA developed in this study will be useful for further health-oriented food production because only edible ingredients were used. Some of this work has been included in a patent (Hasegawa, M., Atsushi, Y., Sambongi, Y., and Funato, K.: Japanese patent application, Patent No. 2017-088049, submitted on 27th April, 2017).

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Figure legends

FIG. 1. Conditions for GABA production. A. Effects of strains, date residue, and carbohydrate-degrading enzyme treatment on the GABA production. "+" and "-" denote "added or done" and "not added or done", respectively. The bars and error bars shown are means and standard errors, respectively, for triplicate experiments, the former values being also indicated. B. Effects of the concentration of sodium glutamate added on the GABA production and initial pH. The strain JCM 1059^T cells and enzyme-treated date residue were added in all experiments. The GABA concentrations (circles) and initial pH values (squares) are shown. Each symbol represents the mean value of at least three independent experiments, in which the standard error values are within the width of symbols.

FIG. 2. Time course of GABA fermentation. Concentration of GABA (circles) and glutamate (squares), and GABA conversion rate from glutamate (triangles) are shown. Values in this time course shown are means \pm standard errors for three independent experiments.

FIG. 3. Cellular physiology during fermentation of date residue with *L. brevis* JCM 1059^T. A. Cell number. B. Glucose concentration. C. pH. D. Lactic acid concentration. Values in these time courses shown are means \pm standard errors for three independent experiments.

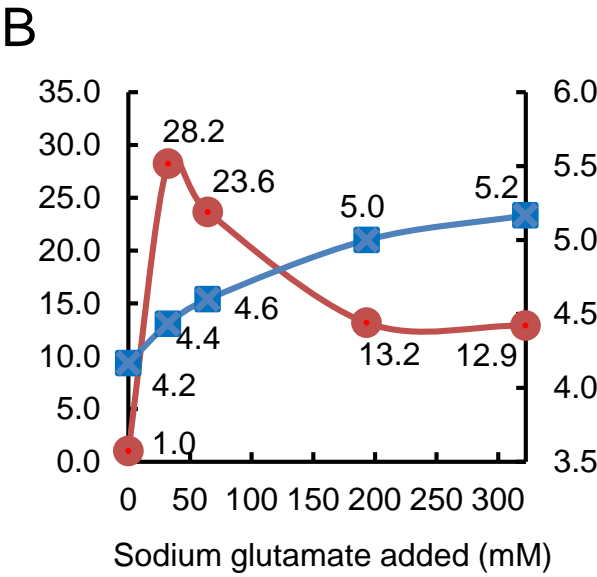
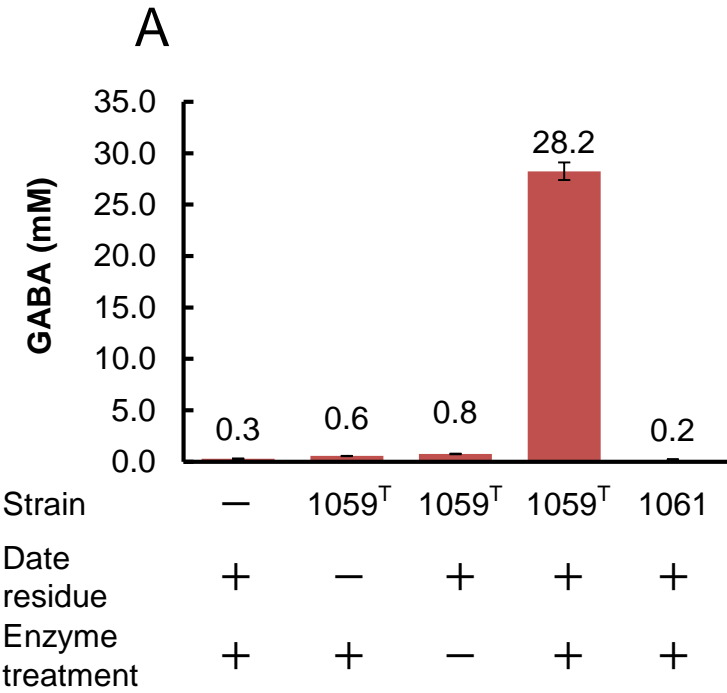


Fig. 1. Hasegawa et al.

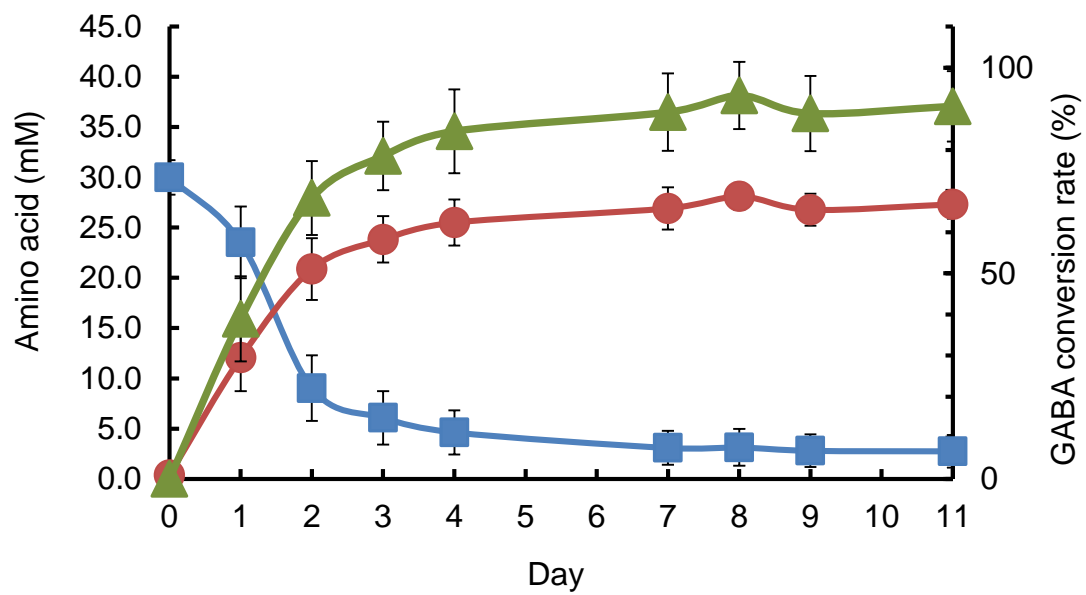


Fig. 2. Hasegawa et al.

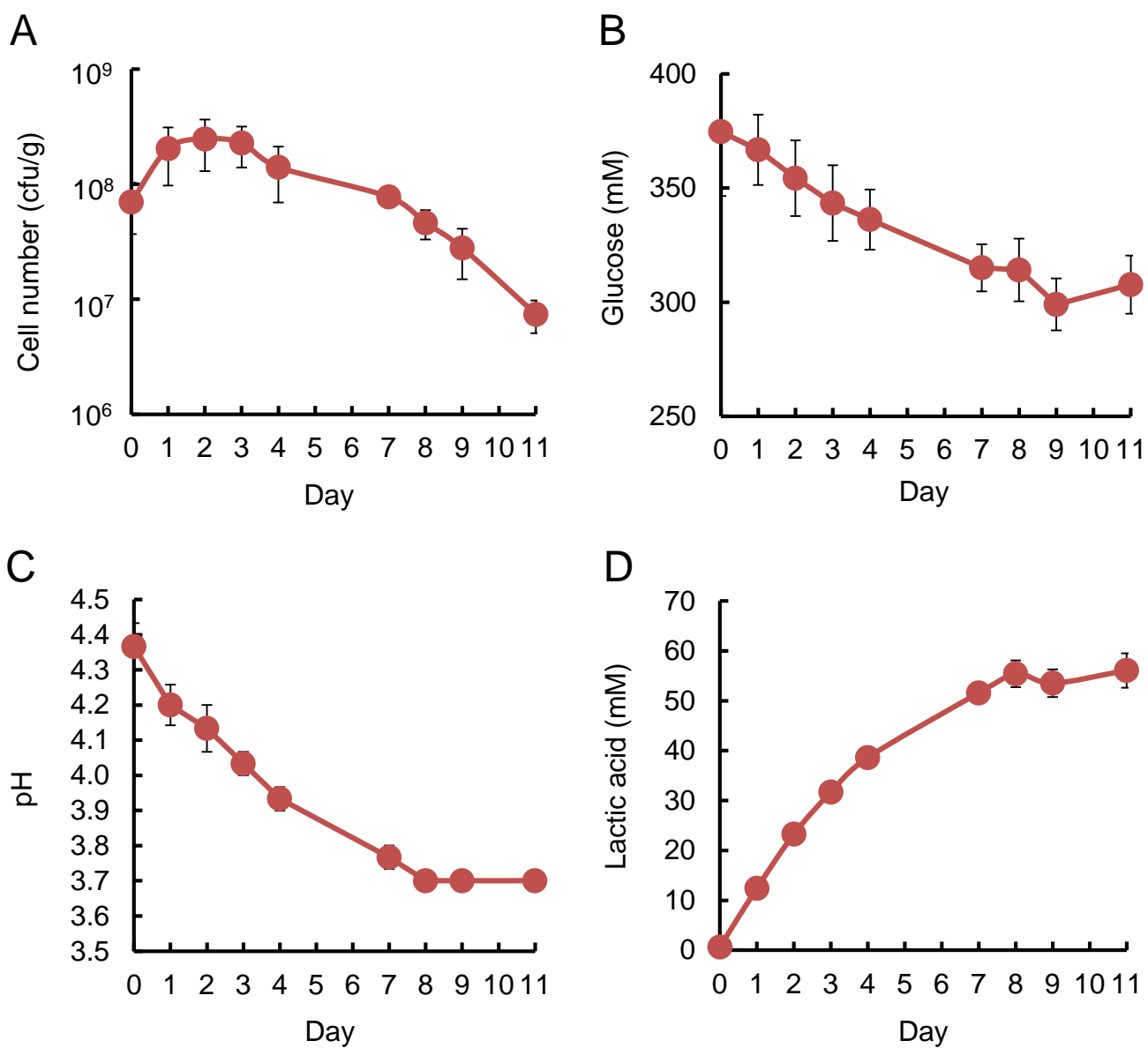


Fig. 3. Hasegawa et al.