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Technical paper

Utilization of *Lactobacillus amylovorus* as an Alternative Microorganism for Saccharifying Boiled Rice

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The growth condition of *Lactobacillus amylovorus* strain JCM 10628, an amylolytic lactic acid bacterium, was tested as a source of enzymes for saccharifying boiled rice to produce a beverage similar to *Amazake*, a traditional non-alcoholic beverage. The composition of a medium that should leave no unpleasant taste in the *Amazake*-like product was confined to 1.0% raw cornstarch, 2.0% skim milk, and 0.1% yeast extract. α-Amylase activity and cell growth decreased in the medium lacking any of the three ingredients. Production of α-amylase was stimulated by soluble starch as well as raw cornstarch, but neither by glucose nor sucrose. When 100 ml of the culture broth was mixed with 16 g of rice after boiling and incubated at 55°C for 24 h, the obtained fluid had an acceptable sweet and sour taste.

Keywords: *Lactobacillus amylovorus*, α-amylase, saccharification, boiled rice

Amazake, a traditional non-alcoholic beverage in Japan, is produced from a mix of rice *koji* and boiled rice that is incubated at 55 to 60°C over 8 h. In this process, amylases produced by the fungus *Aspergillus oryzae* grown in rice *koji* hydrolyze starch to dextrin and glucose. Historically, *Amazake* was an important sweet food before the common use of purified sugar from cane or beets; however, current consumers are not always tolerant of the characteristic flavor derived from rice *koji*. Thus, some other microorganisms capable of hydrolyzing starch might possibly be used to improve the quality of *Amazake*. Therefore, we have examined the potential of a lactic acid bacterium as a safe food-grade microorganism.

Lactic acid bacteria usually ferment monosaccharides and disaccharides, but certain species secrete amylases, which degrade starch to fermentable monosaccharides (Morlon-Guyot *et al*., 1998). *Lactobacillus amylovorus*, a member of the *L. acidophilus*-group, is one of these amylolytic species (Nakamura, 1981). α-Amylase and glucoamylase produced by the type strain of *L. amylovorus* have been characterized in detail (Giraud & Cuny, 1997; James *et al*., 1997). We previously compared ten strains of *L. amylovorus* with respect to lactic acid fermentation from starch and found that strain JCM 10628 produced lactic acid from raw cornstarch at a much higher rate than other strains (Oda *et al*., 2000).

The present paper reports optimal culture conditions for *L. amylovorus* to synthesize α-amylase efficiently and how to apply the culture broth for saccharifying boiled rice to produce a beverage similar to *Amazake*.

Materials and Methods

Bacterial strain and growth conditions *Lactobacillus amylovorus* strain JCM 10628 was originally isolated from Chinese cabbage (Oda *et al*., 2000) and deposited at the Institute of Physical Research (Wako-shi, Saitama). The bacterial cells grown in 10 ml of MRS medium (De Man *et al*., 1960) were collected by centrifugation and resuspended in 5.0 ml of a 2.0% (w/v) skim-milk solution. Basal medium (100 ml) composed of 2.0% (w/v) skim milk and 0.1% (w/v) yeast extract (pH 6.8) supplemented with 1.0 % (w/v) of either glucose, sucrose, soluble starch or raw cornstarch was inoculated with 0.5 ml of the cell suspension. The initial cell count after inoculation was about 5 to 7×10^6 cfu/ml. Sterilization was conducted by autoclaving at 105°C for 30 min except for the medium containing raw cornstarch which was separately treated at 150°C for 1 h and added to the autoclaved medium. Culture broth after static incubation at 37°C was used in the following experiments.

High performance liquid chromatography Lactic acid and sugars were analyzed using a high performance liquid chromatograph (Model SCL-10A, Shimadzu Co., Kyoto), equipped with a Shodex RS pack KC-811 column (Showa Denko Co., Tokyo) and a refraction index detector (Model RID-10A, Shimadzu Co.). Phosphoric acid (0.1%) was used in the mobile phase at a flow rate of 1.0 ml/min.

α-Amylase assay The culture broth containing bacterial cells was directly mixed with an equal volume of 100 mM malate buffer (pH 5.5) and used as a crude enzyme. Formation of *p*-nitrophenyl maltosaccharide from blocked *p*-nitrophenyl maltoheptaoside as the substrate (Sheehan & McCleary, 1988) was recorded by the Ceralpha α-amylase assay kit (Megazyme International Ireland, Ltd., Co. Wicklow, Ireland). The reaction mixture containing 0.1 ml of the substrate solution and 0.1 ml of the crude enzyme was incubated at 37°C for 30 min. After the addition of 2.0 ml of 1% Tris and centrifugation at 5000×*g* for 10 min to remove precipitate, the absorbance at 410 nm of the obtained supernatant was determined. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophe-

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nol per minute.

Saccharification Polished rice (16 g) was steeped in 24 ml of distilled water for 30 min and boiled at 105°C for 20 min. The boiled rice after cooling was added to the culture broth and saccharified at 55°C for 24 h, unless otherwise stated. Concentration of soluble sugar liberated from starch in the mixture was monitored using a digital refractometer (Atago Co., Tokyo). Conventional *Amazake* was made by incubating 50 g of rice *koji* in 150 ml of distilled water at 55°C for 12 h and gave 17.2% of soluble sugar.

Reproducibility All the experiments were independently carried out at least three times. The data are reported as the average values obtained from these experiments.

Results and Discussion

MRS medium generally used for the cultivation of lactic acid bacteria contains yeast extract, meat extract, and peptone to support vigorous growth. Distinct flavor and tastes of these natural substances may influence the quality of the final product if MRS medium was used for saccharifiyng boiled rice. In the present study, a medium based on skim milk supplemented with yeast extract was used.

Table 1 shows growth and α-amylase activity when the lactic acid bacterium was grown in a medium containing one of the sugars. The addition of sugars enhanced the bacterial population and simultaneous production of lactic acid, with a lowering of the pH of the culture broth. Little α-amylase activity was found in the culture broth containing glucose and sucrose as carbon sources. Raw cornstarch and soluble starch efficiently stimulated the production of α-amylase. Hydrolysis of raw cornstarch by α-amylase does not proceed as quickly as soluble starch (Bergmann *et al*., 1988). The bacterial cells may produce as much α-amylase as they can because fermentable sugar from raw starch is limited for cell growth. In the absence of yeast extract, both α-amylase activity and growth were reduced (data not shown). An activity in the culture broth without addition of sugar (7.1 mU/ml) may be caused to some sugars included in skim milk because galactose has been shown to elicit the production of α-amylase by *L. amylovorus* (Pompeyo *et al*., 1993).

The bacterium was grown in the medium containing various amounts of skim milk (Fig. 1). Skim milk was essential for the synthesis of α-amylase and propagation of cells. The activity increased in proportion to the amount of skim milk and was almost constant at a level above 2.0%.

α-Amylase activity was followed in the medium composed of 1.0% raw cornstarch, 2.0% skim milk, and 0.1% yeast extract (Fig. 2). The activity increased in proportion to the culture period

Table 1. Effect of sugars in the medium on the growth and production of α-amylase

Sugar	Cell count ($\times 10^7$ cfu/ml)	Lactic acid (mg/ml)	pH	α-Amylase activity (mU/ml)
None	1.2	0.12	6.3	7.1
Glucose	3.7	2.55	3.7	1.9
Sucrose	4.4	2.32	3.7	3.4
Soluble starch	7.7	2.19	3.8	13.5
Raw cornstarch	6.9	1.03	4.9	34.9

The cells of *Lactobacillus amylovorus* strain JCM 10628 were grown for 24 h in a medium containing 2.0% (w/v) skim milk, 0.1% (w/v) yeast extract, and one each of the 1.0% (w/v) sugars. The initial pH of each medium was 6.8.

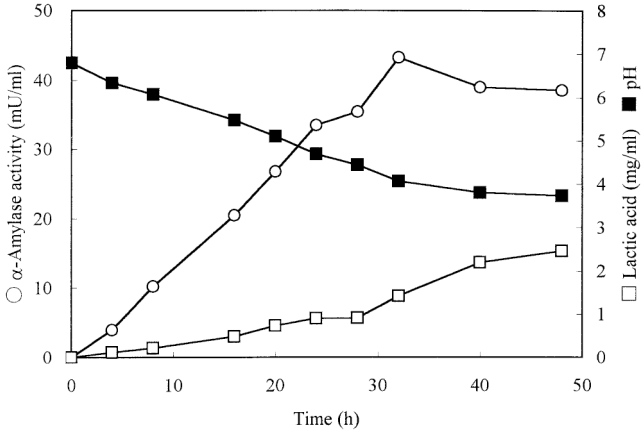

Fig. 2. Production of α-amylase and lactic acid by *Lactobacillus amylovorus* strain JCM 10628. ○, α-amylase activity; □, lactic acid; ■, pH.

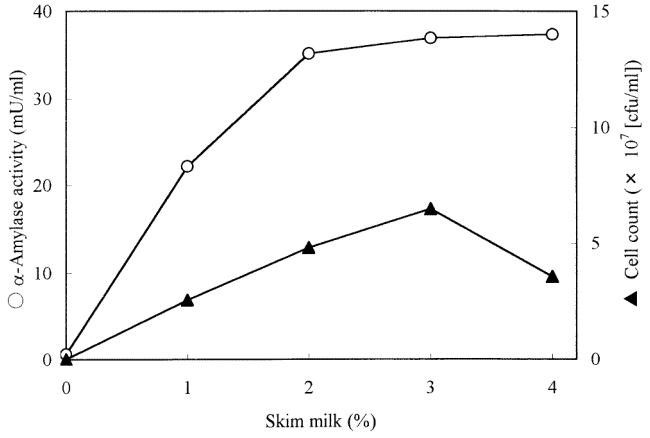

Fig. 1. Effect of skim milk on the growth and production of α-amylase. The cells of *Lactobacillus amylovorus* strain JCM 10628 were grown for 24 h in a medium containing 1.0% raw cornstarch, 0.1% yeast extract, and various concentrations of skim milk. ○, α-amylase activity; ▲, cell count (cfu/ml).

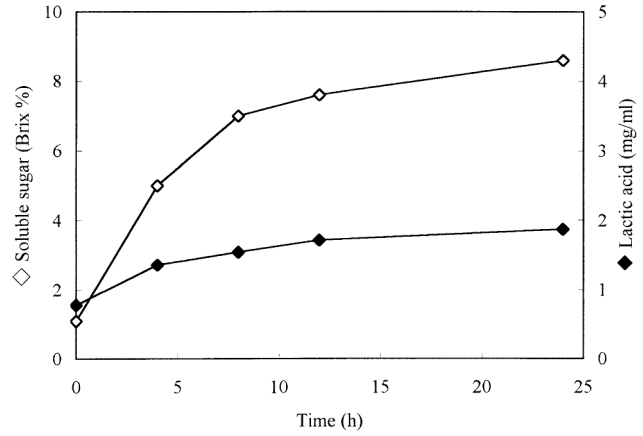

Fig. 3. Saccharification of boiled rice by the culture broth of *Lactobacillus amylovorus* strain JCM 10628. The amounts of soluble sugar and lactic acid were followed during incubation at 55°C in mixtures composed of the culture broth and boiled rice. ◇, soluble sugar; ◆, lactic acid.

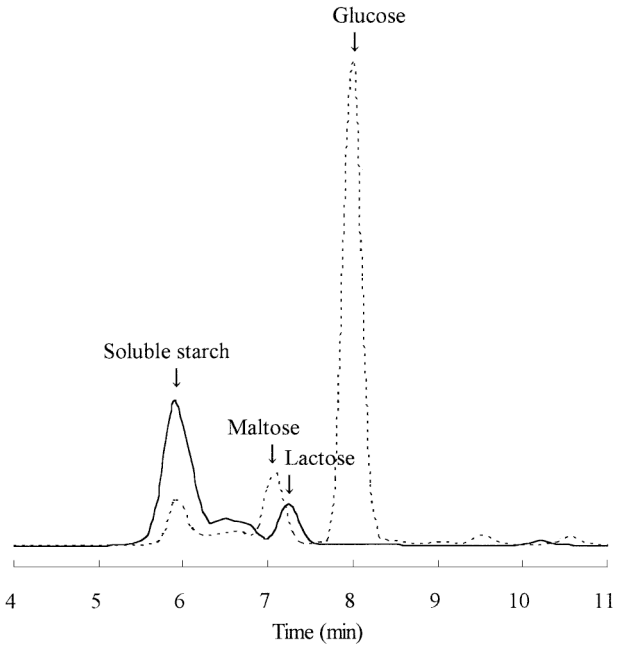

Fig. 4. High performance liquid chromatograms of the sugars in the fluid saccharified by *Lactobacillus amylovorus* JCM 10628 and the conventional *Amazake*. Solid and broken lines refer to chromatograms of the obtained fluid and *Amazake*, respectively. *Amazake* was made as described in Materials and Methods.

and became constant after 40 h. Glucoamylase activity was not detected by the method of James *et al*. (1997) throughout the experiments, suggesting that only a minimal amount of the enzyme was synthesized to supply glucose to the cells. Production of lactic acid and pH of the culture broth followed the change in α-amylase activity.

The culture broth incubated for 24 h was used for saccharifying boiled rice as described in Materials and Methods (Fig. 3). The amount of soluble sugar increased by the action of α-amylase secreted from the lactic acid bacterium with slight formation of lactic acid. The obtained fluid saccharified for 24 h was palatable with both slightly sweet and sour tastes but was free from the characteristic flavor of conventional *Amazake*. Figure 4 compares high performance liquid chromatograms of the obtained fluid and the conventional *Amazake*. Authentic sugars eluted according to molecular weight as shown by the arrows. The conventional *Amazake* was principally composed of glucose produced from starch by the action of amylases in rice *koji*. The fluid saccharified by the lactic acid bacterium contained soluble starch, oligosaccharides, and lactose, which may cause slight sweetness. Because *L. amylovorus* cannot ferment lactose (Nakamura, 1981), lactose included in skim milk was not consumed.

When saccharification was conducted at either 37°C or 45°C for 24 h, the bacterial cells efficiently produced lactic acid from sugars hydrolyzed from starch in boiled rice (Table 2). The resultant fluid containing a higher concentration of lactic acid was organoleptically worse and unacceptable with a strong sour taste. On the contrary, the fluid that was saccharified at 65°C did not taste sour because lactic acid fermentation was completely inhibited by the higher temperature.

Cereal grains are milled and used for the preparation of some foods fermented by indigenous lactic acid bacteria in Africa and

Table 2. The effect of temperature on pH and concentrations of lactic acid and soluble sugar of the obtained fluid.

	Incubation temperature			
	37°C	45°C	55°C	65°C
pH	3.6	3.4	4.2	5.0
Lactic acid (mg/ml)	3.79	4.72	1.89	0.93
Soluble sugar (Brix %)	7.4	7.9	8.7	8.2

The medium containing 2.0% (w/v) skim milk, 0.1% (w/v) yeast extract, and 1.0% (w/v) raw cornstarch was cultured for 24 h and used for saccharifying boiled rice.

in some areas of Asia. These cereal-based foods include various sour porridges, dumplings, and non-alcoholic beers (Salovaara, 1993). The general function of lactic acid fermentation is to improve the quality, safety, and nutritional value of cereals. From the Nigerian food "ogi," Johansson *et al*. (1995) isolated some strains of amylolytic lactobacilli, which enable low viscosity of the products and supply sugars for successive fermentation. Saccharification and lactic acid fermentation usually occur simultaneously in these cereal-based foods, while in the present experiments, high temperature for bacterial growth favored hydrolysis of starch and repressed the excess formation of lactic acid.

In conclusion, the culture broth of *L. amylovorus* can be used to saccharify boiled rice to produce a beverage similar to *Amazake*.

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