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Isolation of Alcohol-tolerant Amylolytic *Saccharomyces cerevisiae* and Its Application to Alcohol Fermentation

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Abstract: An novel amylolytic yeast, *Saccharomyces cerevisiae* HA 27, isolated from *nuruk*, displayed resistance against high sugar (50% glucose) and alcohol (15%). Maximal production of amylolytic enzyme by *S. cerevisiae* HA 27 was achieved on 9 days of cultivation at the optimal temperature 20°C and pH 6.0. The activity of amylolytic enzyme produced by *S. cerevisiae* HA 27 was stable, even at 70°C, and over a broad pH range (4.0-11.0). Also, the amylolytic enzyme of *S. cerevisiae* HA 27 showed optimal activity in pH 5.0 at 50°C. *S. cerevisiae* HA 27 exhibited 6.2%(v/v) alcohol fermentation ability using starch as a carbon source.

Keywords: amylolytic yeast, *nuruk*, *Saccharomyces cerevisiae*, alcohol-tolerance

Introduction

Brewing is a 3-step process involving the liquefaction of starch by α-amylase, saccharification of the liquefied starch by glucoamylase, and the production of alcohol with glucose (1). The energy, time, equipment, and labor required for saccharification comprises a substantial proportion of the production costs. Therefore, the development of yeast that can produce amylolytic enzyme such as amylase and glucoamylase, and thus, ferment alcohol simultaneously is necessary to reduce the cost for brewing alcohol. Amylolytic yeasts were isolated from sweet potato, corn, and traditional *nuruk* in Korea (2-5), in order to ferment alcohol directly through a 1-step process without saccharification. Seu *et al.* (6) developed a recombinant amylase-producing alcohol-fermentative yeast strain though the fusion of *Saccharomyces diastaticus* and *Saccharomyces cerevisiae.* Moreover, research using amylase-integrating vectors has yielded monoploid yeast that simultaneously secretes α-amylase and glucoamylase (7). Cell fusion of *S. diastaticus* and *Candida tropicalis* has additionally been performed as a part of the general effort to augment amylase activity (8). However, these yeasts were not durable in the presence of ethanol or high concentration of sucrose. For production of highly concentrated alcohol using starch substrates, the yeasts must be significantly resistant to ethanol and grow efficiently under high sugar concentration.

In this study, *S. cerevisiae* HA 27, a novel yeast which can simultaneously ferment alcohol from starch, was isolated from the traditional *nuruk* used in the manufacture of *hwahyangju*, a Korean traditional liquor, and was tested for application to alcohol fermentation.

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Materials and Methods

Isolation of amylolytic yeasts Sterilized distilled water was added to *nuruk* manufactured for the fermentation of *hwahyangju* at the Youga area of Dalsung-gun, Daegu, Korea. One-hundred μL of the suspension was spread on yeast malt extract agar [2%(w/v) agar; 1%(w/v) glucose; 0.5%(w/v) peptone; 3%(w/v) yeast extract; 3%(w/v) malt extract, pH 6.0], and cultured for 24 hr at 28°C. Colonies formed on the agar were transferred onto starch agar [1.5%(w/v) agar; 1%(w/v) corn starch; 1%(w/v) peptone; 0.12%(v/v) bromocresol purple solution] with a toothpick, and incubated for 5 days at 30°C. A yellow halo caused by hydrolysis of starch was observed (9).

Sugar and ethanol tolerance of isolated yeasts The growth of amylolytic yeasts at high sugar concentrations was examined in culture broth. Amylolytic yeasts were cultured at 28°C for 24 hr in the yeast malt extract broth [2%(w/v) glucose; 0.5%(w/v) peptone; 3%(w/v) yeast extract; 3%(w/v) malt extract, pH 6.0] whose 2% glucose had been substituted with 40 or 50%(w/v) glucose. The yeasts were also incubated for 5 days in 10 mL of the yeast malt extract broth containing 5, 10, or 15%(v/v) ethanol, and the growth the yeasts were compared with a control incubated in the broth containing no ethanol to determine ethanol tolerance. Yeast growth was determined by measuring the optical density with a spectrophotometer (Ultraspec 2100; GE Healthcare Bio-Sciences Co., Piscatway, NJ, USA) at 600 nm (10).

Identification of the selected amylolytic yeast The Microlog TM 4.0 identification system (Biolog Inc., Hayward, CA, USA) was used to identify high alcohol tolerant amylolytic yeast. Also, the internal transcribed spacer (ITS) region sequence homology of the yeast was additionally examined. Specifically, genomic DNA was isolated with a Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), and the ITS was amplified by polymerase chain reaction (PCR) using the ITS 1

universal primer (5'-TCCGT AGG TGAACCTGCGG-3') and ITS 4 universal primer (5'-TCCTCCGCTTATTGAT ATGC-3'). The partial ITS sequence was determined using the sequencing service of Solgent Co., Ltd. (Daejon, Korea), and the homology was evaluated with a BLAST search (NCBI, http://www.ncbi.nlm.nih.gov) (11). A phylogenetic tree was constructed with the neighbor-joining method using Mega2.

Culture conditions for growth and amylolytic enzyme production of yeast The selected yeast was incubated in starch broth [1%(w/v) corn starch; 1%(w/v) peptone], the supernatant of the culture was harvested after 2 days of incubation, and the optimal growth time was established. In order to determine the optimal culture temperature for cell growth and amylolytic enzyme activity, the selected yeast strains were incubated for 4 days in starch broth at 10, 20, 30, and 40°C. The effects of pH were determined by adjusting the pH of starch broth from pH 3.0 to 11.0 with 1 N HCl or 1 N NaOH. Cell growth was spectrophotometrically measured at 600 nm, and amylase activity was measured with the dinitrosalicylic acid (DNS) method using 1% soluble starch as substrate (12).

pH and thermal stability of amylolytic enzyme produced by yeast After the culture supernatant fluid of yeast was heated at 10-70°C for 30 min, the residual activity of the amylolytic enzyme was determined to evaluate the thermal stability. The pH stability was also determined by measuring the residual activity in lyophilized culture supernatant after incubating at pH 4.0-11.0 and 4°C for 12 hr. Specifically, 3 mL of culture supernatant of yeast was lyophilized and dissolved in 3 mL of 50 mM citrate-phosphate buffer (pH 4.0-6.0), 50 mM Na-phosphate buffer (pH 6.0-8.0), 50 mM Tris-HCl buffer (pH 8.0-9.0), or 50 mM glycine-NaOH buffer (pH 9.0-11.0).

Effects of temperature and pH on amylolytic enzyme activity The effect of temperature on amylolytic enzyme activity, starch digesting enzyme, was determined at 20-70°C. The optimal of pH on amylolytic enzyme activity was determined in the range of pH 4-11. The buffers used were 50 mM citrate-phosphate buffer (pH 4.0-6.0), 50 mM Na-phosphate buffer (pH 6.0-8.0), 50 mM Tris-HCl buffer (pH 8.0-9.0), and 50 mM glycine-NaOH buffer (pH 9.0-11.0) instead of sodium acetate buffer (pH 5.0).

Thin layer chromatography (TLC) TLC plate (Kiesel Gel 60; Merck, Darmstadt, Germany), was developed with a solvent system of *n*-butanol-pyridine-water (6:4:3 vol.), and spots were visualized by treating with an α-naphthol solution [0.5%(w/v) α-naphthol and 5%(v/v) sulfuric acid in ethanol] and heating at 120°C for 10 min.

Alcohol fermentation from starch by amylolytic yeast The selected amylolytic yeast were also incubated in a nitrogen-base medium without amino acid and ammonium sulfate (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 2%(w/v) starch as the carbon source for 7 days. The alcohol concentration in the culture broth was determined to verify alcohol fermentation by amylolytic yeast with the specific gravity method according to the guidelines provided by the regulation of alcoholic beverages analysis of the National Tax Service Technical Service Institute (13).

Results and Discussion

Selection and identification of amylolytic yeast HA 27 Three amylolytic yeast strains, HA 27, HA 28, and HA 29, were isolated from *nuruk* made in the Youga area (Fig. 1). The HA 27 strain showed the highest growth, even in the culture containing 50% glucose and 15% ethanol, among the 3 strains, and thus, was selected as the amylolytic yeast for 1-step alcohol fermentation (Table 1). The HA27 not only survived in the culture containing high concentrations of alcohol and glucose, but also possessed the starch-utilizing ability, implying its ability to produce alcohol from starch and to survive in high concentration of alcohol. This strain is considered to be an extraordinarily useful

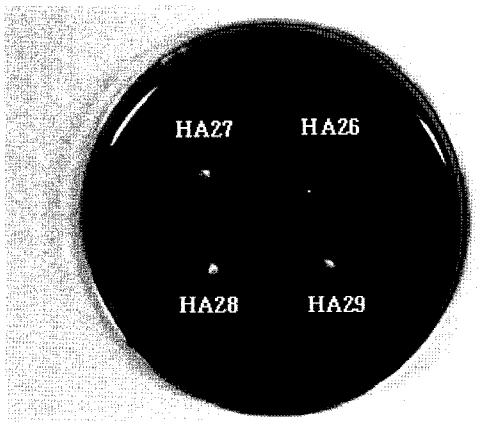

Fig. 1. Selection of amylolytic yeast from traditional *nuruk* used in the manufacture of *hwahyangju*.

Table 1. Effects of glucose and ethanol on growth of yeasts isolated from traditional *nuruk*

Strain No.	Cell growth[1)]							Enzyme activity (unit/mL)[2)]
	Glucose concentration (%)			Ethanol concentration (%)				
	2	40	50	0	5	10	15	
HA27	2.426	1.736	0.875	1.88	1.804	0.743	0.069	200.1
HA28	2.363	1.305	0.832	1.825	1.767	0.679	0.058	183.2
HA29	2.287	1.255	0.883	1.83	1.744	0.532	0.039	112.1

[1)]Measured with spectrophotometer at 600 nm.
[2)]One amylase activity unit was determined as the amount of amylase which was produced 1 μg of glucose from soluble starch at 40°C.

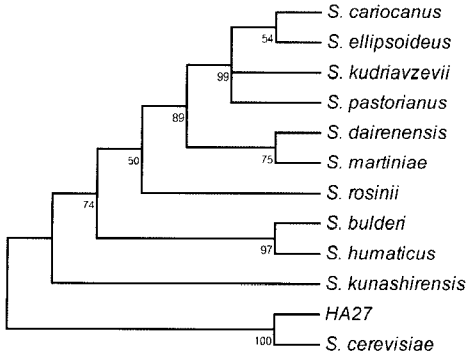

Fig. 2. Phylogenetic tree of *S. cerevisiae* HA 27 with related species. The marker bar denotes the relative branch length. Bootstrap values, expressed as percentages of 1,000 replications, are given at the branch points.

strain, which may allow a general reduction of production cost and an increase of production yield in the alcohol fermentation industry.

The HA 27 was identified as *S. cerevisiae*, based on 98% homology of its partial ITS sequence with the yeast. Also, HA 27 showed the similarity of 99% on Microlog TM 4.0 identification system. A phylogenetic tree was constructed to verify the relationship with other *Saccharomyces* species (Fig. 2).

Optimal culture conditions for amylolytic enzyme production by *S. cerevisiae* HA 27 Amylolytic enzyme production cell growth of *S. cerevisiae* HA 27 were maximal on 9 days, but decreased gradually afterwards (Fig. 3A). Moreover, the optimal growth temperature of *S. cerevisiae* HA 27 and enzyme production was 20°C (Fig. 3B). The growth of *S. cerevisiae* HA 27 decreased rapidly at the higher temperatures than 30°C, while amylolytic enzyme production was not seriously affected by increasing temperature to 40°C. This finding was different from a previous study reporting an optimal temperature of 30°C for growth and amylase production of *S. diastaticus* (14). Within a pH range of 3 to 11, maximal growth and enzyme production of *S. cerevisiae* HA 27 were recorded at pH 5-6, which was in accordance with the earlier reports (14) (Fig. 3C). However, *S. cerevisiae* HA 27 growth declined rapidly at the pH higher than 7, similar to *Sporobolomyces holsaticus* FRI Y-52, the amylolytic yeast previously reported by Park *et al.* (15). Similarly, amylolytic enzyme production was decreased rapidly at the higher pH than 8.

Heat and pH stability of amylolytic enzyme produced by *S. cerevisiae* HA 27 The activity of amylolytic enzyme produced by *S. cerevisiae* HA 27 tended to decrease to some extent with the increases in temperature. However, 95% of the activity was retained, even at 70°C, signifying remarkable heat stability (Fig. 4A). The activity of the amylolytic enzyme decreased at the pH lower than 5 and higher than 8, however, was retained more than 90% residual activity at pH 4-11 (Fig. 4B). Amylolytic enzyme activity was particularly stable at the pH 5-7. These results were similar to pH and heat stability of an amylase produced from *Bacillus substilis* JS-2004 (16) and showed to be suitable for application in starch processing.

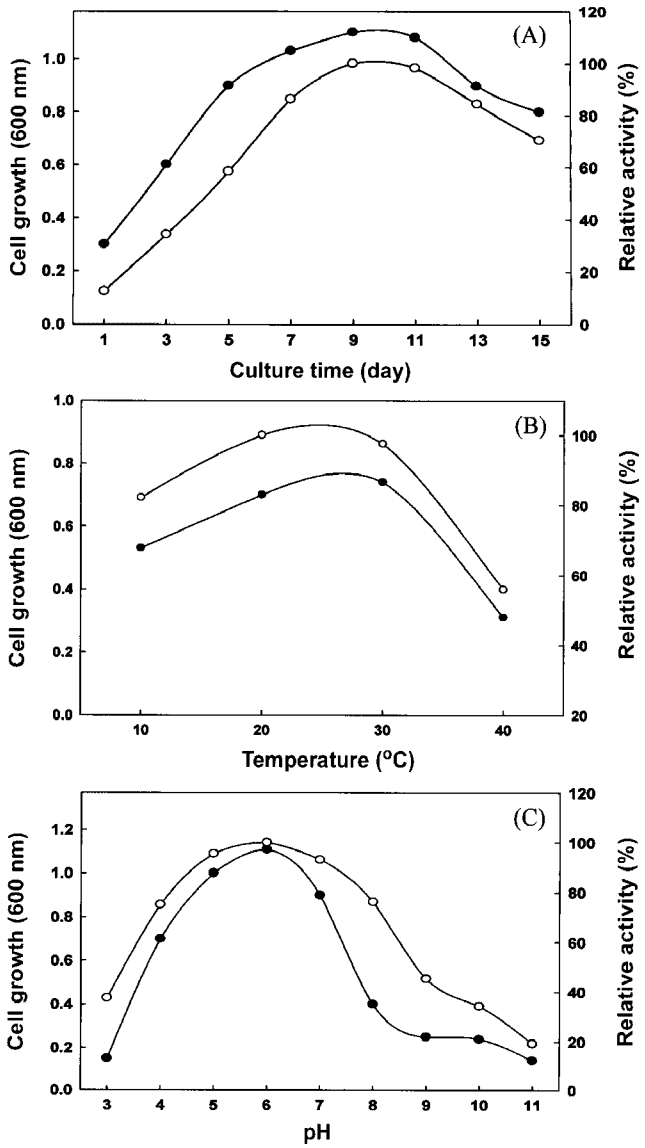

Fig. 3. Effects of culture conditions (culture time: A, temperature: B, pH: C) on amylolytic enzyme production of *S. cerevisiae* HA 27. -●- Cell growth, -○- amylolytic enzyme activity.

Optimal reaction temperature and pH for the amylolytic enzyme of *S. cerevisiae* HA 27 As the temperature was raised up to 50°C, the amylolytic enzyme activity of *S. cerevisiae* HA 27 was observed to increase, but no significant increases occurred at temperatures higher than that (Fig. 5A). In addition, at temperatures of approximately 70°C, the amylolytic enzyme generated by *S. cerevisiae* HA 27 evidenced sufficient activity, which did not substantially differ from the activity at the optimal reaction temperature, 50°C. As compared to the amylase generated by *S. holsaticus*, which retained only 30% of maximal activity levels at 50°C (3), the amylolytic enzyme generated by *S. cerevisiase* HA 27 was demonstrated to be markedly heat-stable.

The optimal pH for amylolytic enzyme reaction was generally weakly acidic with findings similar to those of a study reporting (12,17). The amylolytic enzyme generated by *S. cerevisiase* HA 27 evidenced maximal activity levels at a pH 6.0 (50 mM citrate phosphate buffer), and the

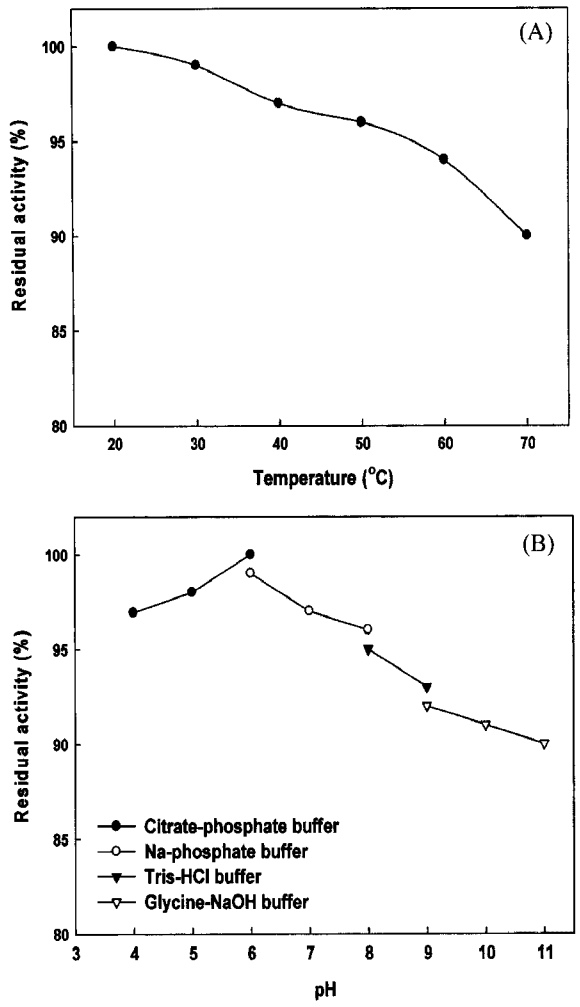

Fig. 4. Temperature (A) and pH (B) stability of amylolytic enzyme produced by *S. cerevisiae* HA 27.

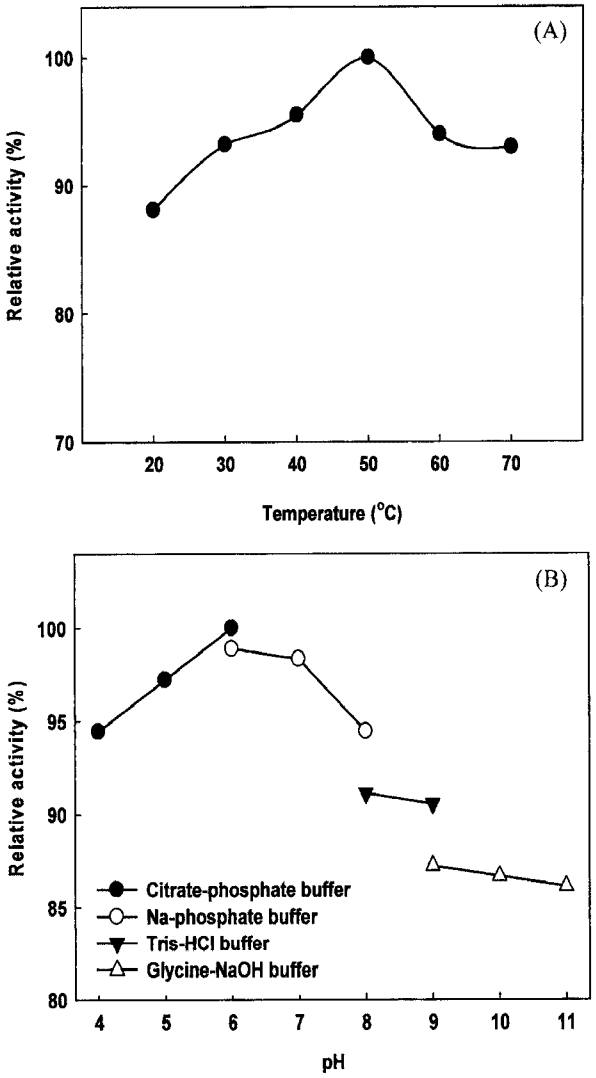

Fig. 5. Effect of temperature (A) and pH (B) on the activity of amylolytic enzyme produced from *S. cerevisiae* HA 27.

enzyme activity decreased with increases in pH (Fig. 5B). This result was similar to the finding that the activity of the glucoamylase as generated by *S. diastaticus* was reduced at pH levels in excess of pH 7.0 (18). However, in comparison with the finding that the pH range for the reaction of amylase generated by other yeasts was only pH 6.0-8.0 (2,12), the amylolytic enzyme generated by *S. cerevisiae* HA 27 showed greater than 80% remaining activity even at a pH 11.0 and 90% at pH 4.0. Thus, it was verified that the enzyme was capable of reacting within a wide pH range. In alcohol fermentation, the pH of the fermentation solution becomes generally acidic as the result of the growth of *Lactobacillus* sp. Therefore, for continuous direct ethanol fermentation from starch, saccharification must be achieved by sufficient amylolytic enzyme activity under acidic conditions.

In the case of the amylolytic enzyme generated by *S. cerevisiae* HA 27, 95% remaining activity was observed at a pH of 4.0-6.0, and thus it was considered that amylolytic enzyme may digest the starch as a substrate and induce saccharification in acidic fermentation solutions.

Product of soluble starch by the amylolytic enzyme of *S. cerevisiae* HA 27 The 1% soluble starch was digested by the cell-free supernatant as the crude amylolytic enzyme from *S. cerevisiae* HA 27 at 50°C for 4 hr. The end product of starch after hydrolysis was glucose as determined by TLC (Fig. 6). This result means that the amylolytic enzyme from *S. cerevisiae* HA 27 was glucoamylase (19). Accordingly the glucoamylase of *S. cerevisiae* HA 27 have α-(1, 4) and α-(1, 6)-debranching activity, and *S. cerevisiae* HA 27 is useful to an efficient starch hydrolysis for 1-step alcohol fermentation without saccharification.

Alcohol fermentation by the amylolytic enzyme producing *S. cerevisiae* HA 27 Alcohol production was attempted using starch as a carbon source and *S. cerevisiae* HA 27. *S. cerevisiae* HA 27 effectively fermented alcohol from starch to produce 6.2%(v/v) alcohol (Fig. 7), which is superior alcohol fermentation ability to the other *nuruk*-isolated and recombinant yeasts (20-23) producing 3-5.5%(v/v) alcohol from starch. Therefore, the amylolytic yeast isolated in this study, *S. cerevisiae* HA 27, produced alcohol from cheap carbon sources, such as sweet potato or corn, through 1-step fermentation and was resistant against high concentrations of alcohol and glucose, indicating that *S. cerevisiae* HA 27 can effectively reduce production cost

Fig. 6. Thin layer chromatography of the reaction product hydrolyzed by the amylolytic enzyme of *S. cerevisiae* HA 27 in soluble starch. The product of hydrolysis by the amylolytic enzyme of *S. cerevisiae* HA 27 (A), maltooligosaccharide (B), glucose (C), maltose (D).

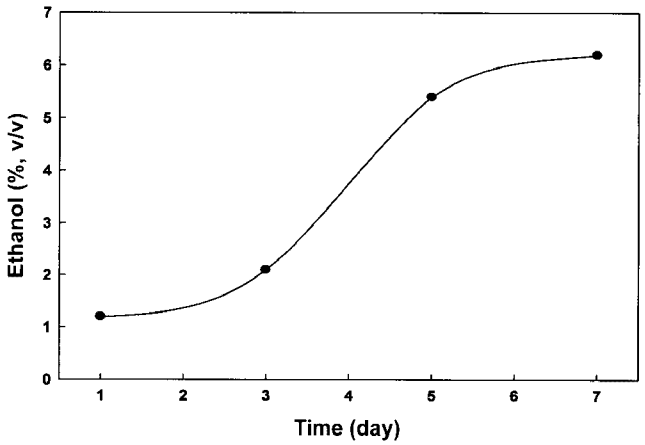

Fig. 7. The time course of ethanol production from starch by *S. cerevisiae* HA 27.

and increase yield in the large scale alcohol fermentation industry.

Acknowledgments

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