Rapid Paper

High Concentration-Ethanol Fermentation of Raw Ground Corn

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Fermentation tests were carried out at 30°C in batch cultures using a newly isolated ethanol tolerant yeast, *Saccharomyces* sp. w-y-2. The addition of fungal mycelia to the chemically defined medium stepwisely fed with sucrose increased the final ethanol concentration from 18.6 to 20.1% (v/v) within 3 days at 30°C. When the liquid *koji* of the high raw starch-digestive amylase producing mutant HF-15 of *Aspergillus awamori* var. *kawachi* was used in the scaled-up fermentation coordinated with saccharification of raw ground corn, 20.1% (v/v) ethanol was produced within 5 days at 30°C.

A distinctive feature of *saké* fermentation is the accumulation of more than 20% (v/v) ethanol in the mash. The fermentation needs to be coordinated with saccharification by *koji* at 8~15°C to form such a high concentration of ethanol. The high concentration ethanol-producing factor was identified to be a proteolipid.¹⁾ On the other hand, we^{2~4)} clarified the mechanism for formation of multiple types of glucoamylase in relation to their raw starch-digestibility. Recently, we⁵⁾ isolated a protease- and glycosidase-less mutant of *Aspergillus awamori* var. *kawachi*. This mutant was found to produce high amount of raw starch-digestive amylase. Ueda and Koga⁶⁾ employed the liquid *koji* of *Aspergillus awamori* and *saké* yeast in a simultaneous saccharification and fermentation of raw polished rice.

The present paper describes the use of the above fungal mutant and a newly isolated ethanol tolerant yeast for high concentration-

ethanol fermentation of raw ground corn.

MATERIALS AND METHODS

Organisms. A protease- and glycosidase-less mutant HF-15⁵⁾ of *Aspergillus awamori* var. *kawachi* was used for the production of high amount of raw starch-digestive glucoamylase. *Saccharomyces* sp. M-1 and M-2 adopted for industrial fermentation and a newly selected yeast strain isolated from fermenting rice wine mash were used for comparison of ethanol productivity. These organisms were maintained in GPY medium (glucose, 50 g; peptone, 5 g; yeast extract 3 g; KH₂PO₄, 1 g; agar, 20 g; pure water, 1000 ml) and kept at 4°C.

Assay of amylase activity. Amylase activity was determined according to the methods described previously.⁵⁾ One unit of amylase activity was defined as that with which 1 mg of glucose was released in one ml of reaction mixture in 100 min.

Preparation of high raw starch-digestive liquid koji. For seed cultures, portions (1 liter) of the synthetic medium (Table I) were dispensed into 5-liter round, flat-bottomed flasks, autoclaved at 121°C for 20 min and inoculated with spores from slant cultures of mutant HF-15. Incubation of the inoculated medium was carried out on a reciprocating shaker at 30°C for 2 days. For main submerged culture, 120 liters of the synthetic medium (Table I) were sterilized at 121°C for 20 min in a 200-liter stainless steel fermentor (Marubishi Laboratory Equipment Co.), inoculated with the previously prepared seed culture (5%, v/v), cultivated at 32°C with agitation at 200~250 rpm and aerated at a

TABLE I. COMPOSITION OF CULTURE MEDIA FOR ENZYME PRODUCTION

The initial pH was adjusted to 7.0 with ammonia water.

Ingredients	Seed culture	Main culture
Potato starch (g)	40	40
Glucose (g)	1	—
Potassium phosphate, monobasic (g)	3	5
Magnesium sulfate (g)	1	1
Sodium chloride (mg)	100	10
Ferric sulfate (mg)	100	100
Zinc sulfate (μg)	10	100
Manganese sulfate (mg)	0.5	1
Cobalt sulfate (μg)	10	—
Copper sulfate (μg)	10	10
Calcium chloride (mg)	10	10
Ammonium citrate (g)	10	10
Tap water (liter)	1	1

rate of 1 volume of air per volume of medium per min. The activities of the liquid *koji* harvested from the 48- and 72-hr culture were 30 and 42 U/ml, respectively.

Fermentation tests. Fermentation tests for selecting high ethanol tolerant yeasts were carried out by stepwise additions of sucrose to the synthetic medium at 30°C, as previously reported.⁷⁾ For fermentation coordinated with saccharification, 150 ml submerged culture of mutant HF-15 was dispensed into 500-ml culture bottles fitted with fermentation bungs and supplemented with 90 g ground corn, 0.45 g $(\text{NH}_4)_2\text{SO}_4$ and 0.15 g KH_2PO_4 . The initial pH of the medium was adjusted to 4.0. For inoculum, yeast from a freshly grown slope culture was inoculated into a 250-ml culture bottle containing 100 ml of synthetic medium, which after propagation for 30 hr at 30°C was used to inoculate the above-described medium to the extent of 1×10^8 cells per ml of medium. Each culture was incubated statically at 30°C until fermentation ceased. Additional ground corn (30 g) was added to the culture after 24 hr of incubation. The fermentation was followed by daily measurements of CO_2 gas evolved which was monitored by the decrease in weight of the whole culture. At the end of fermentation, the ethanol concentration (% v/v) of the culture liquid after removal of solids by centrifugation was determined with an alcohol hydrometer after distillation.⁸⁾ The reducing sugars were determined by the micro-Bertrand method.

Scaled-up fermentation. The scaled-up fermentation test was carried out in a 200-liter capacity stainless steel cylindrical vessel with occasional agitation. The fermentation procedure involved the following sequential steps. To prepare inoculum, a loop of yeast culture was transfer-

red into a 100 ml sterile synthetic medium.⁷⁾ After 18 hr of incubation at 30°C on a reciprocating shaker, the contents of six flasks were transferred respectively, to six 5-liter round, flat-bottomed flasks containing 3 liters of fresh medium of the same composition. These cultures were permitted to grow statically for 30 hr at 30°C. On the other hand, portions (12 liters) of the 48-hr mold submerged culture were withdrawn and supplemented with 36 g $(\text{NH}_4)_2\text{SO}_4$, 12 g KH_2PO_4 , 3.6 kg raw ground corn and 2.4 kg sucrose. The liquid *koji* was adjusted to pH 4.0, then inoculated with the previously prepared yeast inoculum and incubated statically at 30°C for 24 hr. The seed culture was transferred to 108 liters of 72-hr mold submerged culture supplemented with 324 g $(\text{NH}_4)_2\text{SO}_4$, 108 g KH_2PO_4 , 64.8 kg raw ground corn and 5 kg sucrose. The initial pH of the mash was adjusted to 4.0 and the temperature was maintained at $30 \pm 1^\circ\text{C}$. After 24 hr of fermentation, 21.6 kg raw ground corn was added to the mash. The fermentation process was followed by measuring the residual sugar, ethanol concentration and cell population of the mash. During fermentation, samples were periodically removed from the mash and centrifuged, and the supernatant was collected and analyzed. The ethanol concentration and residual sugars were determined as previously described.^{5,8)} The cell population was determined by microscopic count, using Thoma's haemocytometer.

Substrate. Raw ground corn (66.2% w/w starch and 15.2% w/w moisture) was used throughout the experiment.

FIG. 1. Ethanol Fermentation by *Saccharomyces* sp. w-y-2 (A), *Saccharomyces* sp. M-1 (B) and *Saccharomyces* sp. M-2 (C) in Fungal Mycelia-supplemented Media.

All strains were cultivated at 30°C in basal synthetic medium (150 ml) supplemented with (○) or without (●) 10% (wet w/v) mycelia of HF-15. Inoculum size was 3×10^8 per ml. Percentages in parentheses indicate the final ethanol concentrations (v/v) produced.

RESULTS AND DISCUSSION

Selection of high ethanol tolerant yeast and effect of fungal mycelia on ethanol fermentation

Three strains were tested for ethanol fermentation in synthetic medium supplemented with or without the mycelia of the mutant HF-15. As shown in Fig. 1, *Saccharomyces* sp. w-y-2 produced 18.6% (v/v) ethanol in the basal medium and 20.1% (v/v) ethanol in the mycelia-supplemented medium in 5 days. Likewise, *Saccharomyces* sp. M-1 and M-2 produced 16.9 and 16.2% (v/v) ethanol in the

basal medium, and 18.2 and 18.1% (v/v) in the presence of mycelia, respectively, within 5 days. The addition of fungal mycelia to the basal medium enhanced the ethanol productivity of the yeast, which was close to the previously reported data.⁹⁾ Consequently *Saccharomyces* sp. w-y-2 was employed in all subsequent experiments.

Ethanol fermentation coordinated with saccharification from raw ground corn

Liquid *koji* was tested for use in saccharifying raw corn mashers for ethanol fermentation. Using 250-ml bottles with a fermentation bungs, ethanol production from raw ground corn was carried out with or without sucrose supplement. Although sucrose supplementation increased the rate of ferment-

FIG. 2. Effect of Sucrose Supplement on Ethanol Fermentation of Raw Ground Corn.

Fermentation tests by *Saccharomyces* sp. w-y-2 were carried out with or without sucrose supplement to 150 ml of liquid *koji* at 30°C. Inoculum size was 1×10^8 per ml.

	Sucrose supplement (g)	Final ethanol concentration (% v/v)	Ground corn added (g)
■	0	20.1	120
□	1.5	19.7	117
▲	4.5	19.9	113
△	7.5	20.1	108
●	15.0	20.1	95
○	30.0	19.7	95

FIG. 3. Scaled-up Fermentation of Raw Ground Corn for High Concentration-Ethanol Production.

△, cell population (/ml); ○, ethanol formed (% v/v); □, residual glucose (mg/ml). Experimental details are described in the text.

tation, the final ethanol concentration reached 20% (v/v) either with or without sucrose supplement (Fig. 2). No significant bacterial growth was observed microscopically during the fermentation process.

Scaled-up fermentation

During the first 4 days, the fermentation proceeded vigorously, and the sugars liberated from ground corn were consumed completely (Fig. 3). After this period, the residual sugars increased gradually. The fermentation was completed within 5 days, and the final ethanol concentration in the mash was 20.1% (v/v). The mash cell count increased from 3.2×10^7 per ml to 5×10^8 per ml in a day and became almost constant during the fermentation. The final pH of the mash was 4.4 and the final titrable acidity was 7.7 ml. No reports have been found on the rapid formation of high ethanol concentration as high as 20% (v/v) in the mash within 3 to 5 days at 30°C. The fermentation system reported here represents a saving of time, equipment and energy in addition to high ethanol productivity.

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