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Fungal Invertase as an Aid for Fermentation of Cane Molasses into Ethanol

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Comparative studies of the fermentation of cane molasses into ethanol by *Saccharomyces cerevisiae* in the presence or absence of fungal invertase were performed. When cane molasses was fermented by the yeast at 30°C and pH 5.0, the presence of the enzyme had no effect on ethanol production. At pH 3.5, ethanol production was increased by the addition of invertase. At 40°C, the addition of invertase increased ethanol production by 5.5% at pH 5.0 and by 20.9% at pH 3.5.

The current interest in ethanol as a liquid fuel or fuel supplement has stimulated research into the improvement of traditional fermentation processes. Various techniques for improving the production of ethanol, including continuous culturing, continuous fermentation with cell recycling, vacuum distillation with cell recycling (2), and immobilization of yeast cells (3), have been evaluated. Other research has involved the examination of different strains of microorganisms for alcoholic fermentation (6, 7).

Common distillery practice is to dilute molasses to contain fermentable sugars at ca. 12 to 15% (wt/vol); the sugars are fermented to ethanol at 6 to 7% (vol/vol). Invertase and fermentation activities of yeasts are quite important for efficient fermentation. Our laboratory demonstrated that yeast strains must contain adequately balanced invertase and fermentation activities for good production of ethanol.

Saccharomyces cerevisiae UNICAMP 224 was grown on agar slants containing cane molasses (20% reducing substances) and 1.5% agar at pH 6.0, and *Aspergillus oryzae* UNICAMP 27 was grown on Sabouraud dextrose agar slants at 30°C.

Purified fungal invertase was prepared as follows. A 1-ml portion of an *A. oryzae* spore suspension was used to inoculate 100 ml of liquid culture medium in an Erlenmeyer flask (500 ml), which was incubated at 30°C and 250 rpm on a rotary shaker for 5 days. The liquid culture medium consisted of 2% peptone (Difco Laboratories), 0.5% yeast extract (Difco), 2% soybean extract (commercial soybean), 0.5% KCl, and 0.5% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.0. The spore suspension was prepared from an *A. oryzae* slant culture. A 10-ml portion of sterilized water was added to the slant culture, and the surface was gently rubbed with a sterilized wire loop to obtain a spore suspension. After incubation, the liquid medium was filtered to eliminate insoluble materials. The enzyme in the filtrates was further purified by using ammonium sulfate fractionation, amberlite IR 45, and DEAE-cellulose column chromatography with 0.05 M acetate buffer at pH 5.0. Invertase activity was measured by incubation of 9 ml of the substrate (4% sucrose; pH 5.0) with 1 ml of the enzyme solution at 40°C for 30 min. After incubation, total amounts of reducing substances were measured by previously described methods (5, 8). One unit of invertase activity was defined as the amount of enzyme which liberated 1 μmol of reducing substance per min under these conditions.

For alcohol fermentation, concentrated commercial cane molasses was diluted to 15.5° brix (~23% of the reducing sugars), and ammonium phosphate dibasic (0.1%) was added. We added 175-ml portions to Erlenmeyer flasks (500 ml), which we then autoclaved. For obtaining inocula, 50-ml portions of molasses (diluted as described above) in Erlenmeyer flasks (250 ml) were inoculated with yeast strains and incubated at 30°C and 250 rpm on a rotary shaker. After 24 h of incubation, 25-ml portions of culture medium were added to Erlenmeyer flasks (500 ml) as inocula, and the pH values were adjusted to 3.5 or 5.0 with 2 N H_2SO_4. The flasks were immediately protected from the atmosphere with a tube containing concentrated sulfuric acid. The flasks

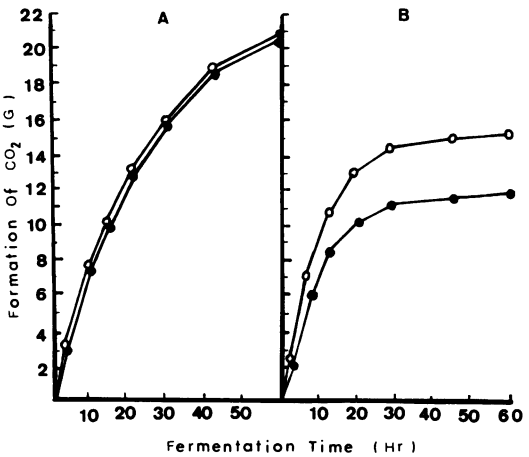

FIG. 1. Time course of fermentation of cane molasses by *S. cerevisiae* at 30°C and pH 5 (A) and 40°C and pH 3.5 (B) with (○) and without (●) invertase.

were divided into two groups. One group was incubated at 30°C with frequent stirring, and the other group was incubated at 40°C. In this system, only CO_2 can pass out of the flasks, but water vapor is absorbed by the sulfuric acid; thus, the weight loss of the contents represents the formation of CO_2. After 60 h, the fermented mashes were distilled, and the ethanol contents of the distillates were determined by measurement of specific gravity.

At pH 5.0, invertase did not affect CO_2 formation when the yeast cells fermented molasses (Fig. 1). After 60 h of fermentation, the yield of ethanol was 93.0% in the presence of invertase and 92.3% in the absence of invertase (Table 1). After 60 h of fermentation at pH 3.5, the yield of ethanol was 89.8% in the presence of invertase and 81.3% in the absence of invertase (Table 1).

When cane molasses was fermented by yeast cells at 40°C and pH 3.5, there was a remarkable increase in CO_2 formation in the presence of invertase, as compared with the rate observed when the enzyme was absent (Fig. 1). After 60 h, the yield of ethanol was 69.8% in the presence of invertase and 48.9% in the absence of invertase (Table 1). At 40°C and pH 5.0, fermentation in the presence of invertase slightly increased the ethanol yield over that obtained in the absence of invertase (Table 1). Fermentation of cane molasses at pH 3.5 in the presence of invertase effectively increased the ethanol yield. These results agree with an observation by Myrbäck and Willstaed (4), who found that yeast invertase activity is more affected by low pH values than is fermentation ability, presumably because invertase is located near the cell surface. Our data also indicate that the inhibitory effects of temperature and low pH on ethanol yield appear to be synergistic. The addition of fungal invertase reduced these inhibitory effects.

TABLE 1. Fermentation of cane molasses into ethanol by *S. cerevisiae* in the presence or absence of fungal invertase

Invertase (23.6 U/flask) added[a]	Fermentation temp (°C)	Fermentation pH	CO_2 formed (g)	Ethanol yield (%)
No	30	5.0	20.8	92.3
Yes	30	5.0	21.0	93.0
No	30	3.5	18.3	81.3
Yes	30	3.5	20.2	89.8
No	40	5.0	12.7	56.6
Yes	40	5.0	14.0	62.1
No	40	3.5	11.0	48.9
Yes	40	3.5	15.7	69.8

[a] The total amount of reducing sugars in each experiment was 46 g.

LITERATURE CITED

1. **Cook, A. H. (ed.).** 1958. The chemistry and biology of yeast, p. 379. Academic Press, Inc., New York.
2. **Cysewski, G. R., and C. R. Wilke.** 1978. Process design and economic studies of alternative fermentation methods for the production of ethanol. Biotechnol. Bioengr. **20**:1421–1444.
3. **Kolot, F. B.** 1980. New trends in yeast technology: immobilized cells. Process Biochem. **15**:2–8.
4. **Myrbäck, K., and E. Willstaed.** 1955. Ark. Kemi. **8**:367.
5. **Nelson, N.** 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**:375–380.
6. **Rose, D.** 1976. Yeast for molasses alcohol. Process Biochem. **11**:10–12.
7. **Rogers, P. L., K. J. Lee, and D. E. Tribe.** 1980. High productivity ethanol fermentation with *Zymomonas mobilis*. Process Biochem. **15**:7–11.
8. **Somogyi, M.** 1945. A new reagent for the determination of sugars. J. Biol. Chem. **160**:61–73.