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Technical Note

Increasing Alcohol Yield by Selected Yeast Fermentation of Sweet Sorghum. II. Isolation and Evaluation of Mutants and Wild Types for Ethanol Production*

ABSTRACT

Three yeast strains, which gave over 93% sugar conversion efficiency (SCE) in sweet sorghum juice containing 20% total sugar in a previous study, were submitted to 3 and 6% ethylmethanesulfonate (EMS) to induce mutations. Several mutants produced by the EMS treatment and their respective wild types grew well in media containing up to 40% sugar. Some mutants tolerated 2·5% more alcohol than their respective wild types. Saccharomyces cerevisiae *IZ 1716 Mutant 10 produced a significantly greater yield of ethanol from sweet sorghum juice containing 26% sugar than the wild type. Under large-scale fermentation, this mutant had an SCE of 89·4% after 36 h in sweet sorghum juice containing 28% sugar. The final alcohol concentration reached 13·28% (w/v) after 48 h, corresponding to an SCE of 93·57%.*

INTRODUCTION

An earlier study in our laboratory demonstrated that sweet sorghum juice is suitable for selected yeast fermentation and ethanol production (deMancilha *et al.*, 1984). Therefore, the present investigation was undertaken to induce mutations of selected yeast strains (deMancilha *et al.*, 1984) by treating them with ethylmethanesulfonate (EMS), and to compare their ethanol production with the selected parent strains.

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MATERIALS AND METHODS

The sources of sweet sorghum and yeasts, evaluation for ethanol production, fermentation media and analytical methods were the same as described earlier (deMancilha *et al.*, 1984).

Isolation of mutants

Mutants were derived by treatment with ethylmethanesulfonate (EMS). Three wild yeast strains with high sugar efficiency conversion in 20% (w/v) total sugar medium were inoculated into 10 ml of YEPD medium and grown at 30 °C for 24 h to yield 10^8–10^9 cells/ml.

The cells were centrifuged at 1500 rpm for 10 min and the supernatant discarded; they were then resuspended in 10 ml phosphate buffer (pH 8·00), and recentrifuged at 1500 rpm for 10 min. The cells were again resuspended in 10 ml of buffer and either 0·3 or 0·6 ml of EMS was added. The mixture was incubated for 50 min at 30 °C without agitation. The cells were then alternately washed and centrifuged three times in 10 ml phosphate buffer. After the third washing, the cells were resuspended in 10 ml of phosphate buffer and 1 ml of the suspension was diluted with 9 ml of liquid (YEPD) medium and placed in the shaker for 48 h at 30 °C.

Cell numbers were determined by plating on YEPD agar medium before and after the EMS treatment to determine the killing effect. After the culture reached 10^8–10^9 cells/ml, the suspension was diluted to 100–300 cells/plate, spread on YEPD agar medium and incubated at 30 °C for several days. Cells showing good growth were selected and tested for sugar tolerance by transferring to YEPD agar media containing 25, 30, 35 and 40% (w/v) glucose, and incubating at 30 °C for several days. Controls were carried through the same procedures using non-mutagenized cells.

Cells with high sugar tolerance were tested for alcohol tolerance by transferring to YEPD agar media containing 10·0, 12·5, 15·0, 17·5 and 20·0% (v/v) ethanol, and incubating at 30 °C for several days. Controls were treated the same way using non-mutagenized cells.

Evaluation of mutants for ethanol production

Mutants showing good tolerance to sugar and alcohol (five from each EMS treatment) and their respective wild types were inoculated into 30 ml

of fermentation medium containing 30% (w/v) total sugar, placed in 125 ml Erlenmeyer flasks, and incubated at 30 °C with constant agitation for 48 h in a gyratory shaker. Following incubation the alcohol content and SCE were determined. Mutants with more than 80% SCE and the respective wild types were tested in 26% (w/v) total sugar medium.

A final test using the best mutant was conducted in a 6-liter fermentor drive assembly (New Brunswick Co., New Brunswick, NJ) containing 28% total sugar medium, at 30 °C and 100 rpm agitation. Alcohol content and SCE were determined every 12 h up to a maximum of 48 h.

RESULTS AND DISCUSSION

The strains tested included *S. uvarum* NRRL Y-6004, *S. boulardii* IZ 1904 and *S. cerevisiae* IZ 1716. Results demonstrated that different yeast strains vary in their resistance to EMS. *S. uvarum* NRRL Y-6004 suffered the most from both levels of EMS, with *S. cerevisiae* IZ 1716 being affected the least and *S. boulardii* IZ 1904 being intermediate. Variation in killing effects ranged from 60·7 to 69·7% for 3% EMS and from 88·1 to 97·8% in 6% EMS, which is similar to results reported by Lindegren *et al.* (1965).

Several of the mutants and wild types grew well on media containing up to 40% sugar, showing that both wild types and mutants can have good sugar tolerance. Sugar tolerance tests using both wild types and mutants gave results equal to, or even better than, those reported elsewhere (Tarkow *et al.*, 1942; Onishi, 1963; Jones *et al.*, 1982).

Mutants of *S. uvarum* NRRL Y-6004 from the 6% EMS treatment only and of *S. bouldarii* IZ 1904 from both the 3 and 6% EMS treatments showed good growth on media containing up to 17·5% (v/v) ethanol, while mutants of *S. cerevisiae* IZ 1716 at both the 3 and 6% EMS levels grew well on media containing up to 20·0% (v/v) ethanol. Wild types only grew well on media containing up to 15·0% (v/v) ethanol. Therefore, the mutants tolerated about 2·5% more alcohol than the wild types. High or low alcohol tolerance apparently is not peculiar to any particular genus or species, since both high and low alcohol tolerance have been reported in yeast strains belonging to the same genus (Gray, 1941). Likewise, one strain of a species may exhibit high alcohol tolerance while another may have low alcohol tolerance (Gray, 1941).

A number of workers (Gray, 1941; Nosiro & Ouchi, 1962; Gray &

Sova, 1968) have observed that different yeast strains vary in alcohol tolerance. The manner in which alcohol kills yeasts is unclear, although the most likely explanation is that death results from denaturation of the intracellular enzymes upon passage of ethanol through the plasma membrane, as suggested by Thomas *et al.* (1978).

After completion of initial tests for alcohol and sugar tolerance, 10 mutants (five from each EMS treatment, except for *S. uvarum* NRRL Y-6004) were retested to confirm their sugar and alcohol tolerance. Alcohol yields and SCE tests were also performed with the same mutants and their respective wild types, using sweet sorghum juice containing 30 % total sugar. It was demonstrated that ethanol yields and the SCE varied for the wild types and their respective mutants, with alcohol yields varying from 9·81 to 12·77 % (w/v) and SCE values varying from 64·25 to 83·67 %, respectively. Thus, the mutation process resulted in a significant change ($P < 0{\cdot}01$) in alcohol yield for the three wild types tested.

The mutants, which gave around 80 % SCE on 30 % total sugar medium, and their respective wild types were tested using 26 % total sugar media. The results are shown in Table 1. Alcohol yields ranged from 11·42 to 12·06 % (w/v) ethanol with the SCE values varying from 86·98 to 91·81 %, respectively. Mutation resulted in a significant increase in ethanol yields

TABLE 1
Ethanol Production by Wild Type and Yeast Mutants in Sweet Sorghum Juice with 26 % (w/v) Total Sugar

Strain[a]	*Ethanol concentration*[b] (% *w/v*)	*SCE*[c] (%)
S. uvarum NRRL Y-6004	11·45	87·21
S. uvarum NRRL Y-6004 mut. 5.	11·43	87·06
S. cerevisiae IZ 1716	11·52	87·72
S. cerevisiae IZ 1716 mut. 3	11·85	90·23
S. cerevisiae IZ 1716 mut. 7	11·84	90·03
S. cerevisiae IZ 1716 mut. 10	12·06[d]	91·81
S. boulardii IZ 1904	11·80	89·88
S. boulardii IZ 1904 mut. 2	11·51	87·62
S. boulardii IZ 1904 mut. 9	11·42	86·98

[a] Mutants numbered 1–5 were derived from the 3 % EMS treatment; those numbered 6–10 were from the 6 % EMS treatment.
[b] Each value represents the average of four observations.
[c] Sugar Conversion Efficiency.
[d] Different from wild type ($P < 0{\cdot}01$).

($P < 0{\cdot}01$) only for *S. cerevisiae* IZ 1716. All three mutants of *S. cerevisiae* IZ 1716 tested produced more alcohol than the wild type. However, the higher alcohol production was statistically significant ($P < 0{\cdot}01$) only with mutant 10.

A final test with *S. cerevisiae* IZ 1716 mutant 10 was carried out in a 6-liter fermentor (working volume 5 liters) containing 28% total sugar medium, at 30 °C and 100 rpm agitation. The results are shown on Table 2. After 36 h, 95·8% of the sugar was consumed and the SCE was 89·34%. The final alcohol concentration reached was 13·28% (w/v) at the end of 48 h, which corresponds to 93·7% SCE.

The final SCE was higher for large-scale fermentation than for flask fermentation. Two possibilities may explain this observation. First, the inoculum for the large-scale fermentation was prepared under 200 rpm agitation, whereas, in flask fermentation it was prepared without agitation. Thus, a higher number of cells may have been obtained by agitation. Secondly, the large-scale fermentation was performed at lower speeds, which may offer an explanation for the differences. Nevertheless, more sugar was converted to ethanol under large-scale fermentation conditions than under flask fermentation.

Results obtained in the final test are similar to, or even better than, those reported in the literature (Rose, 1976; Huang, 1982; Jones *et al.*, 1982). The present study shows that EMS-induced mutation improved alcohol tolerance in all yeast strains tested. *S. cerevisiae* IZ 1716 Mutant 10 performed well in the final test and should be adaptable to commercial fermentation using sweet sorghum juice as substrate.

TABLE 2

Ethanol Production, Sugar Consumption and Sugar Conversion Efficiency for *S. cerevisiae* IZ 1716 mut. 10 in a 6-Liter Fermentor Using Sweet Sorghum Juice Containing 28% Sugar

Time (*h*)	*Sugar concentration*[a] (%)	*Ethanol concentration*[a] (% *w/v*)	*SCE*[b] (%)
0	28·16	—	—
12	20·31	2·90	20·43
24	6·12	10·14	71·44
36	1·18	12·68	89·34
48	—	13·28	93·57

[a] Each value represents the average of four observations.

[b] Sugar Conversion Efficiency.

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I. M. deMancilha*, A. M. Pearson, H. Momose† & J. J. Pestka
Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, USA

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* Present address: Departmento de Tecnologia de Alimentos, Universidade Federal de Viçosa, 36.570 Viçosa, Brazil.

† Present address: Basic Research Department, Central Research Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki, Japan.