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INCREASED OSMOTOLERANCE OF GENETICALLY MODIFIED ETHANOL PRODUCING STRAINS OF *Saccharomyces Sp.*

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SUMMARY

A stable spheroplast fusion product of the polyploid brewing strain *Saccharomyces uvarum (carlsbergensis)*, strain 21 and a genetically constructed diploid *Saccharomyces diastaticus*, strain 1384 has been shown to have improved ethanol producing capability in defined media (Panchal *et al.*, 1982). This fusion product, strain 1400 was further subjected to fermentations in defined media containing glucose substrate and varying concentrations of the non-metabolized sugars sorbitol or mannitol.

While the fermentation efficiencies of all the three strains decreased with increasing osmotic pressure imparted by sorbitol or mannitol, the detrimental effect was least apparent with the fusion product than with either of the fusion partners. This attribute of the stable fusion product has major significance in relation to its potential for industrial ethanol production.

INTRODUCTION

The production of fermentation ethanol for fuel usage has been a major venture in many parts of the world since the crude oil prices soared to the present high levels. In spite of a recent slowdown in North America, ethanol production from renewable resources has been receiving ever-increasing attention in many of the so-called Third World countries (Hammond, 1978; Maiorella *et al.*, 1981). Also receiving close scrutiny has been the process of producing ethanol, particularly the improvement of yeast strains to increase the efficiency of the fermentation process.

In a previous publication from this laboratory (Panchal *et al.*, 1982) it was reported that the techniques of hybridization and spheroplast fusion were employed to construct novel yeast strains which were capable of producing elevated ethanol levels in fermentations with defined media or whole corn mashes. These strains were further investigated to determine their ability to withstand osmotic pressure, a situation that would exist in commercial fermentations using high sugar concentrations.

MATERIALS AND METHODS

Yeast Strains

The polyploid brewing strain of *Saccharomyces uvarum (carlsbergensis)* (strain 21) was obtained from the Labatt culture collection. The construction of the hybrid strain 1384 (*a/α, DEX1/DEX1, DEX2/DEX2, STA3/STA3, malo/malo*) and strain 1400, a fusion product of 1384 and 21, have been described previously (Stewart *et al.*, 1982).

Growth Media and Fermentation Conditions

Yeast strains were subcultured in PYN medium which consisted of: peptone (Difco), 3.5g; yeast extract (Difco), 3.0g; KH_2PO_4, 2.0g; $MgSO_4 \cdot 7H_2O$, 1.0g; $(NH_4)_2SO_4$, 1.0g; glucose, 20g; all dissolved in one litre of distilled water and adjusted to pH 5.0.

Fermentations were conducted in PYN medium containing 100g/l of glucose and supplemented with sorbitol or mannitol varying in concentration from 0 to 300g/l. All fermentations were carried out at 30°C in 300 ml Erlenmeyer flasks containing 200 ml of media and placed on a rotary shaker. The inoculum was 1.0% w/v in sorbitol supplementation studies and 0.8% w/v in mannitol supplementation studies.

Analytical Assays

Five millilitre samples were sequentially removed from the shake flasks, centrifuged immediately and the supernatants frozen for subsequent analysis. Glucose and ethanol concentrations were determined enzymatically as reported previously (Bergmeyer, 1974).

RESULTS AND DISCUSSION

The techniques of hybridization and spheroplast fusion, although less specific than DNA transformation, are very useful tools for genetically modifying yeast strains, particularly industrial yeast strains of undefined genetic makeup. Spheroplast fusion has been successfully used in improving antibiotic producing strains of *Streptomyces* (Wesseling, 1982). It was reported previously (Panchal *et al.*, 1982) that when spheroplasts of a brewing yeast *Saccharomyces uvarum (carlsbergensis)* strain 21 and a genetically modified diploid *Saccharomyces diastaticus*, strain 1384 were fused in the presence of polyethylene glycol (PEG), stable transformants were obtained. One of the fusion products, strain 1400, was found to possess enhanced ethanol producing capability in defined media with glucose substrate as well as in whole corn mash medium containing maltose as a major substrate. In peptone-yeast extract medium containing 300g/l glucose, strain 1400 outperformed all the other strains of *Saccharomyces* investigated. Thus, it was found to be capable of producing in excess of 12% w/v ethanol in the medium and the rate of ethanol production was very rapid. In order for a genetically modified strain to be useful industrially, it has to demonstrate stability to various physical and physiological parameters encountered in industrial fermentations. It has been shown previously that one of the parameters of great significance in ethanol fermentation is the osmotic pressure of the medium (Panchal and Stewart, 1980). An efficient ethanol producing strain has to be tolerant to elevated ethanol concentrations as well as be osmotolerant to high concentrations of sugars present in the medium and needed to produce high concentrations of ethanol. A systematic study of stability to osmotic pressures of the genetically modified strains was thus warranted.

Fermentation media were prepared in PYN containing 100g/l glucose and varying concentrations of sorbitol or mannitol, sugars which are not utilized or taken up by yeast but which impart osmotic pressure to the media (Kuo and Lampen,1971; Arnold,1981).

Figure 1 shows the effect of sorbitol induced osmotic pressure upon fermentation ability of strain 21, a polyploid brewing *Saccharomyces uvarum (carlsbergensis)* strain. As can be seen, after 45 hours, while 80% of the

FIGURE 1
Effect of sorbitol induced osmotic pressure on fermentation characteristics of *Sacch. uvarum (carlsbergensis)* strain 21. Sorbitol supplement(g/l): 0 (○), 100 (■), 200 (▲), 300 (●).

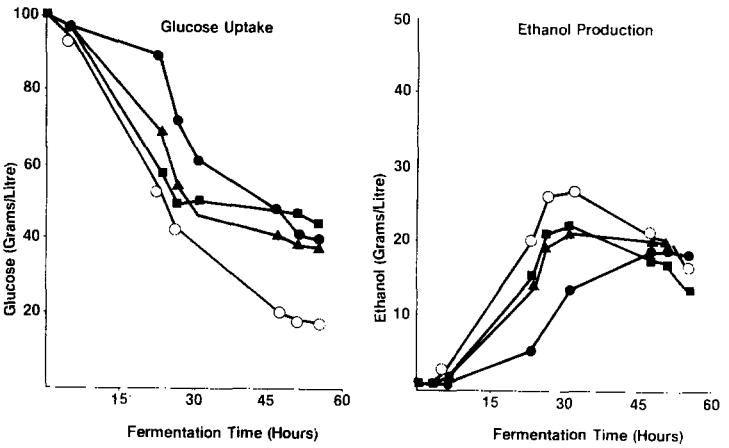


FIGURE 2
Effect of sorbitol induced osmotic pressure on fermentation characteristics of *Sacch. diastaticus* strain 1384. Sorbitol supplement(g/l): 0 (○), 100 (■), 200 (▲), 300 (●).

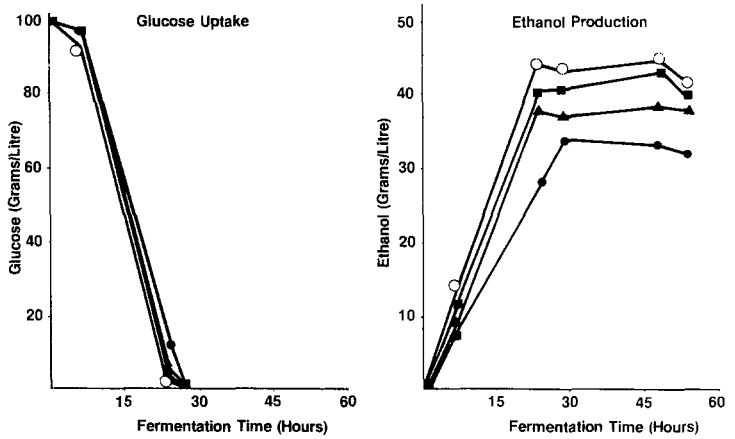


FIGURE 3
Effect of sorbitol induced osmotic pressure on fermentation characteristics of *Saccharomyces sp.*strain 1400. Sorbitol supplement(g/l): 0 (○), 100 (■), 200 (▲), 300 (●).

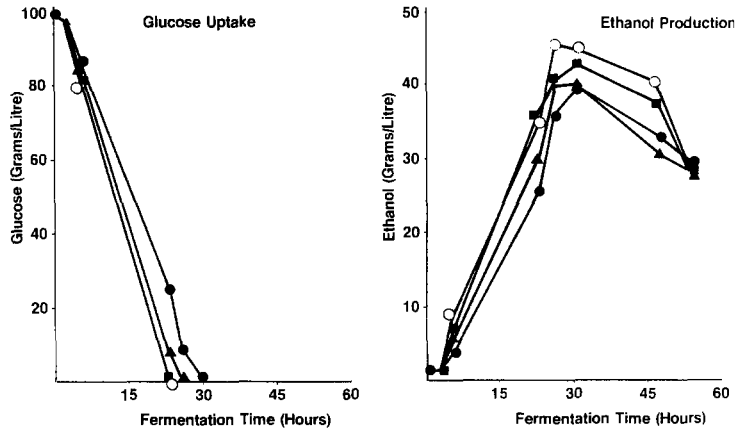


FIGURE 4
Effect of mannitol induced osmotic pressure on fermentation characteristics of *Sacch. uvarum (carlsbergensis)* strain 21. Mannitol supplement(g/l): 0 (○), 100 (■), 200 (▲), 300 (●).

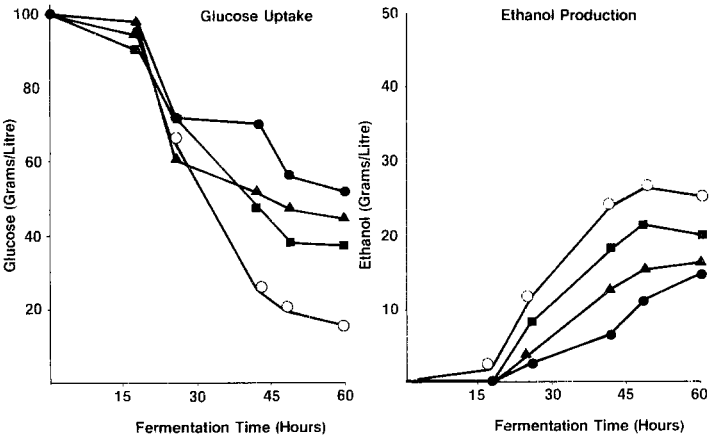


FIGURE 5
Effect of mannitol induced osmotic pressure on fermentation characteristics of *Sacch. diastaticus* strain 1384. Mannitol supplement(g/l): 0 (○), 100 (■), 200 (▲), 300 (●).

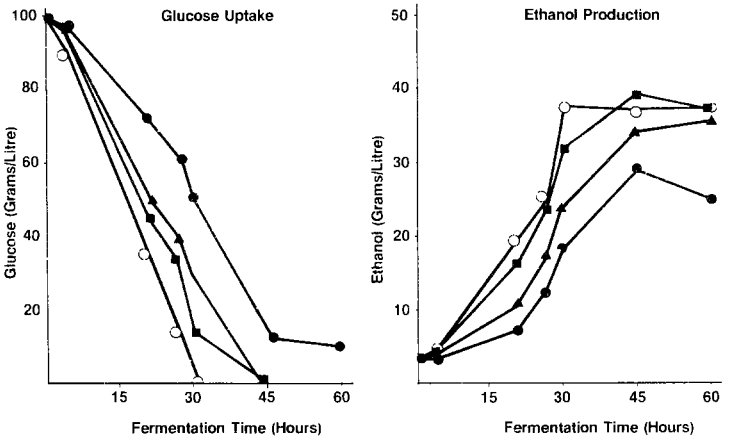
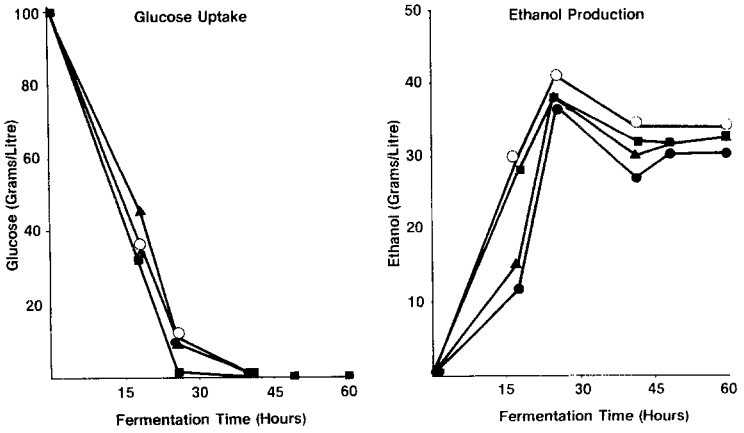


FIGURE 6
Effect of mannitol induced osmotic pressure on fermentation characteristics of *Saccharomyces sp.*strain 1400. Mannitol supplement(g/l): 0 (○), 100 (■), 200 (▲), 300 (●).



glucose was utilized when no sorbitol was present in the medium, 55% of the glucose was utilized with 10% sorbitol supplemented medium and only 50% of the glucose was utilized when the medium was supplemented with 20 or 30% sorbitol. This trend was also reflected in the total ethanol produced under the same conditions. While 2.8% w/v ethanol was produced in sorbitol unsupplemented medium, 2.2% w/v ethanol (21% reduction) was produced in medium supplemented with 10% sorbitol (7 atm. osmotic pressure), 2.1% w/v ethanol (25% reduction) was produced in 20% sorbitol supplemented medium and 1.8% w/v ethanol (36% reduction) was produced in 45 hours in 30% sorbitol supplemented medium. The strain 1384 was found to be more osmotolerant than strain 21 when subjected to similar fermentation conditions (Fig. 2). Thus, it is seen that in sorbitol supplemented as well as unsupplemented media, the glucose was rapidly utilized by strain 1384. However, the ethanol production profiles differed in each case of sorbitol supplementation. While the maximum ethanol produced in sorbitol lacking medium was 4.5% w/v, it was 4.25% in 10% sorbitol supplemented medium (10% reduction), 3.8% w/v in 20% sorbitol supplemented medium (16% reduction) and 3.4% w/v in 30% sorbitol supplemented medium (25% reduction).

The fusion product, strain 1400, was even less affected by osmotic pressure. As Figure 3 shows, all the glucose was utilized after 25 hours in sorbitol unsupplemented and supplemented media. In sorbitol unsupplemented medium, a maximum level of 4.6% w/v ethanol was reached while in 10% sorbitol supplemented medium, 4.3% w/v ethanol was produced (6% reduction) and in both 20% and 30% sorbitol supplemented media a maximum level of 4% w/v ethanol was reached (13% reduction).

The sugar D-mannitol, like D-sorbitol, is also not utilized by yeasts and has been used as an osmotic stabilizer for yeast spheroplasts. It has the same molecular weight as sorbitol (182.17) but its melting point (168°C) is higher than that of sorbitol (110°C, Merck Index, 1976). It was therefore of interest to see the effect of osmotic pressure imparted by mannitol on fermentation characteristics of the fusion partners, strains 21 and 1384 and the fusion product, strain 1400.

As Figure 4 shows, mannitol had a more detrimental effect on ethanol production by strain 21 than did sorbitol (compare Figures 1 and 4). Thus, while 2.7% w/v ethanol was produced in medium containing only glucose, 2.5% w/v ethanol was produced in 10% mannitol supplemented medium (22% reduction), 1.6% w/v ethanol was produced in 20% mannitol supplemented medium (41% reduction) and only 1.1% w/v ethanol was produced in 30% mannitol supplemented medium (59% reduction).

The effect was more pronounced on strain 1384 as well (Fig. 5). Here it is seen that glucose uptake was not as rapid as with sorbitol supplemented medium and the comparative times needed to reach maximum ethanol concentrations were longer. After 30 hours of fermentation, while 3.75% w/v ethanol was produced in mannitol unsupplemented medium, 3.2% w/v ethanol was produced in 10% mannitol supplemented medium (15% reduction), 2.4% w/v ethanol was produced in 20% mannitol supplemented medium (36% reduction) and only 1.8% w/v ethanol was produced in 30% mannitol supplemented medium (52% reduction). Although the maximum ethanol levels reached in the mannitol supplemented media were higher than at 30 hours, the fermentation periods were much longer.

The effect of mannitol induced osmotic pressure on fermentation by the fusion product strain 1400 was not very dramatic and similar to the effect of sorbitol induced osmotic pressure (compare Figures 3 and 6). Thus glucose was very rapidly utilized in both mannitol unsupplemented and

supplemented media. After 25 hours of fermentation, 4.1% w/v ethanol was produced in mannitol unsupplemented medium, while 3.8% w/v ethanol was produced in both 10% and 20% mannitol supplemented media (7% reduction) and 3.6% w/v ethanol was produced in 30% mannitol supplemented medium (12% reduction).

The results thus demonstrate that the fusion product, strain 1400, is more tolerant to sorbitol and mannitol induced osmotic pressures than either of the fusion partners, strain 21 or strain 1384. While strain 1384 is more tolerant than strain 21, both partner strains are less efficient in mannitol supplemented media than in sorbitol supplemented media. The reasons for this effect are not clear but are probably due to the differences in physical properties of the two sugars. Mannitol has a higher melting point (166-168°C) than that of sorbitol (110-112°C, Merck Index, 1976) and is less soluble in water than sorbitol. Investigations are currently ongoing to elucidate the reasons for the different degrees of effects of sorbitol and mannitol upon the yeasts.

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