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PRODUCTION OF POLYOLS AND ETHANOL BY THE OSMOPHILIC YEAST *ZYGOSACCHAROMYCES ROUXII*

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SUMMARY

The yeast *Zygosaccharomyces rouxii* ATCC 12572 was selected for its ability to produce appreciable levels of ethanol and of various polyols from concentrated glucose media (20 %, w/v). *Z. rouxii* was shown to yield large quantities of glycerol and of the mixture arabitol + mannitol. Good agitation combined with appropriate aeration (1 vvm) allowed *Z. rouxii* to utilize glucose readily leading to high polyol production. Depending on the fermentation conditions used, *Z. rouxii* ATCC 12572 will give either ethanol or various polyols as main fermentation product(s).

INTRODUCTION

Osmophilic yeasts show interesting industrial potential. Under appropriate conditions, they can produce a liquid fuel (ethanol) and various polyols (glycerol, i-erythritol, arabitol, mannitol) which can be used in a number of industrial applications (examples: explosives, pharmaceuticals, plastics, food ingredients). The relatively high commercial value of some polyols could help to lower significantly the costs of producing ethanol as a biofuel, thus making the process more viable economically. In addition, because of their high tolerance for high osmotic pressures, osmophilic yeasts can easily ferment highly concentrated sugar solutions (20 %, w/v, and above). This ability leads to more concentrated fermentation liquors thus helping product recovery and allowing reduced capital and operation costs.

Interest in ethanol and polyol biosynthesis by osmophilic yeasts can be found in recent studies by Bellinger *et al.* (1990), O'Connor-Cox *et al.* (1991), Tokuoka *et al.* (1992) and Vijaikishore *et al.* (1986a,b). These reports followed excellent basic research performed many years ago by Onishi (1960;1963), Spencer (1968) and Peterson's group (Peterson *et al.*, 1958; Hajny *et al.*, 1960). Osmophilic yeasts accumulate various intracellular polyols during growth in media containing high concentrations of substrate in order to

counterbalance high extracellular osmotic pressure. Polyol accumulation slightly reduces water activity without affecting enzyme activity (Rapin *et al.*, 1994).

In this study, we report on the influence of yeast extract concentration and of aeration conditions on the metabolism of glucose by the osmophilic yeast *Zygosaccharomyces rouxii* and how this could be easily used to orient the metabolism of this yeast towards either polyol production or ethanol production.

MATERIALS AND METHODS

Microorganisms, media and culture conditions: *Zygosaccharomyces rouxii* (Boutroux) Yarrow, ATCC 12572 (formerly *Zygosaccharomyces bisporus* var. *mellis*, also *Saccharomyces rouxii*), obtained from the American Type Culture Collection (Rockville, MD), was chosen because it appeared to produce, in preliminary tests, appreciable quantities of ethanol and polyols (glycerol, arabitol and/or mannitol) from glucose among 37 species of osmophilic yeasts belonging to 8 genera (results not shown). Cultures were routinely maintained on Yeast Mold Agar slants (DIFCO Laboratories, Detroit, U.S.A.) enriched with 2 % (w/v) glucose; following growth at 28-30 °C for 2-3 days, the agar slants were floaded with sterile mineral oil and stored at 5 °C for up to 18 months before subculturing. The medium used in our studies, unless otherwise indicated, was that of Hajny *et al.* (1960). Its composition was (g/L): glucose, 200; yeast extract, 10; urea, 1. The medium was sterilized by autoclaving at 121 °C for various lengths of time depending on the volume. For media containing only 10 % glucose, the yeast extract concentration was lowered to 0.5 % as done by Hajny *et al.* (1960).

Fermentation protocols: The influence of yeast extract (Y.E.) concentration was evaluated using Hajny's medium (20 % glucose) containing various concentrations of Y.E. (0.25, 0.5, 1.0 and 1.5 %, w/v). The appropriate medium was distributed in 25 mL-aliquots into 150 mL-Erlenmeyer flasks, the flasks were inoculated at 4 % (v/v) using a 3 day-old culture (inoculum) and incubated at 35 °C and at an agitation rate of 250 rpm for up to 100 hours. For the agitation and aeration experiments, cultivation of the yeast was conducted in New Brunswich bioreactors (2 L-working volume, Multigen model F-2000, New Brunswick Sci. Co., U.S.A.). Hajny's medium (1.57 L) containg 20 % glucose was sterilized *in situ* at 121 °C for 20 minutes. Fermentations were carried out at 35 °C, at agitation rates ranging from 79 to 500 rpm, and with various levels of aeration (0, 0.33; 0.66; 1.0 vvm). Inoculation was 13 % in volume using a 3 day-old pre-culture. pH was monitored but not controlled. Finally, the fermentation process was tested in a 20 L Chemap bioreactor (model CF-11) using the following conditions: Hajny's medium (20 % glucose); working volume, 16 L; aeration, 1.0 vvm; agitation rate, 400 rpm; temperature, 35 °C. The bioreactor was inoculated at 8 % by volume using a 3 day-old pre-culture. pH was monitored but not controlled. Foaming was controlled using a mechanical device (Chemap's Fundafoam) but small quantities of a chemical antifoam were also added at time intervals (Antifoam A, Sigma Chemicals Co., St-Louis, U.S.A.).

Analyses: Glucose utilization and product accumulation were monitored using a Waters HPLC unit equiped with an Aminex HPX-87P column (Bio-Rad Labs) heated to 75 °C. Degassed and filtered distilled water served as the mobile phase (flow rate: 0.5 mL/min). Detection was done using a refractive index detector (Waters model R401). An external calibration was performed at least once a day, using a mixture of ethanol, sugars and polyols. Our method was unable to separate arabitol from mannitol; consequently, results are reported as the mixture arabitol + mannitol. Samples consisted of supernatant fluids from fermentation broths after centrifugation at 7,200 rpm at room temperature (International Centrifuge, model Centra 4); when required, samples were diluted with distilled water before chromatography.

Influence of yeast extract. Shake flask investigations were undertaken in order to measure more precisely the influence of Y.E. on various parameters of this fermentation (results not shown). In regard to glucose utilization, the results indicated the following: (a) glucose utilization was almost directly proportional to Y.E. concentration in the range 0.5 to 1.0 % and (b) glucose was not readily consumed at Y.E. concentrations below 0.5 % whereas almost 100 % consumption was obtained with 1.0 % Y.E. As for ethanol production, ethanol accumulation was obtained only in media containing more than 0.5 % Y.E. and was almost directly proportional to Y.E. concentration up to 1.5 %. Ethanol concentration appeared to decrease very appreciably during prolonged incubation (over 96 hours) probably due to consumption by the yeast cells and to evaporation. Finally, in regard to polyol accumulation, the results indicated that: (a) the production of glycerol and of the mixture arabitol + mannitol was directly proportional to Y.E. concentration up to 1.0 % and (b) a Y.E. concentration of 1.5 % led to polyol concentrations lower than expected; this was probably due to consumption by the yeast cells of a fraction of the polyols following glucose depletion. Some strains of *Z. rouxii* are known to grow on several polyols including mannitol (Barnett *et al.*, 1990). It is therefore reasoned here than excess Y.E. stimulated growth favoring first glucose utilization then polyol consumption.

At least two important lessons were derived from these results: (a) a relatively high concentration of Y.E., around 1 %, is critical for maximal glucose consumption, and for ethanol and polyol accumulation; (b) prolonged fermentations will lead to significant consumption of ethanol and of some polyols following glucose exhaustion.

Bioreactor experiments: influence of agitation alone. Bioreactors experiments were first run in order to evaluate ethanol and polyol production by *Z. rouxii* under low aeration conditions; here, aeration was provided solely by agitation. These experiments showed conclusively (Table 1) that agitation alone led to incomplete glucose consumption with concomitant low polyol accumulation; the highest glucose consumption was about 35 % and was obtained with an agitation rate of 500 rpm. However, ethanol accumulation was quite significant and reached close to 30 g/L at an agitation rate of 500 rpm. An ethanol yield of 0.42 from glucose (g/g) or 83 % of theoretical yield was calculated.

Table 1. Influence of agitation alone on glucose utilization and product accumulation by *Z. rouxii* ATCC 12572. Experiments performed in 2 L-bioreactors without pH control. Results at 108 h. Initial glucose concentration was 200 g/L.

Agitation rate (rpm)	Glucose consumed (g)	Concentration (g/L) of:		
		Ethanol	Glycerol	arabitol + mannitol
79	41.2	19.7	6.5	7
200	53.7	23	6.5	8
400	56.3	22	6.5	8
500	70.7	30	8.5	11.5

Bioreactor experiments: influence of agitation coupled with aeration. Since agitation alone did not lead to high polyol accumulation, it became necessary to supply additional oxygen (air) to the cultures. In order to determine the influence of additional aeration (*via* air sparging), three 2L-bioreactors containing 1.57L of medium were inoculated and the fermentations run under the following conditions: (1) the aeration rate was either 0.33, 0.66 or 1.0 vvm; (2) the agitation rate was fixed at 300 rpm following initial trials.

Agitation coupled with aeration greatly improved glucose utilization (Figure 1A). Glucose consumption reached 100 % at around 80 h at aeration rates of 0.66 and 1.0 vvm. Glycerol accumulation was maximal at an aeration rate of 1.0 vvm (Figure 1C) as the accumulation of the mixture arabitol + mannitol (Figure 1D). Ethanol accumulation was highest with an aeration rate of 0.66 vvm (Figure 1B). Figure 1 shows conclusively that ethanol disappeared relatively quickly following glucose consumption, especially at the higher aeration levels, due to the combined effect of ethanol consumption by the yeast cells and evaporation. The beneficial effect of aeration on glucose utilization and polyol production has also been observed by other investigators (Peterson *et al.* 1958; Ramachandran and Sulebele 1979). On the other hand, the negative influence of aeration on ethanol production is a well-known phenomenon with osmophilic yeasts according to Spencer (1968); where ethanol is found in a fermentation aeration is inadequate for maximal polyol yields.

Larger-scale bioreactor experiment. In order to validate some earlier observations and also to carry out initial scale-up studies, the fermentation was also performed at the 16 L scale (Figure 2). The experimental conditions were selected to favour polyol accumulation; aeration was consequently set at 1 vvm. Glucose was completely used

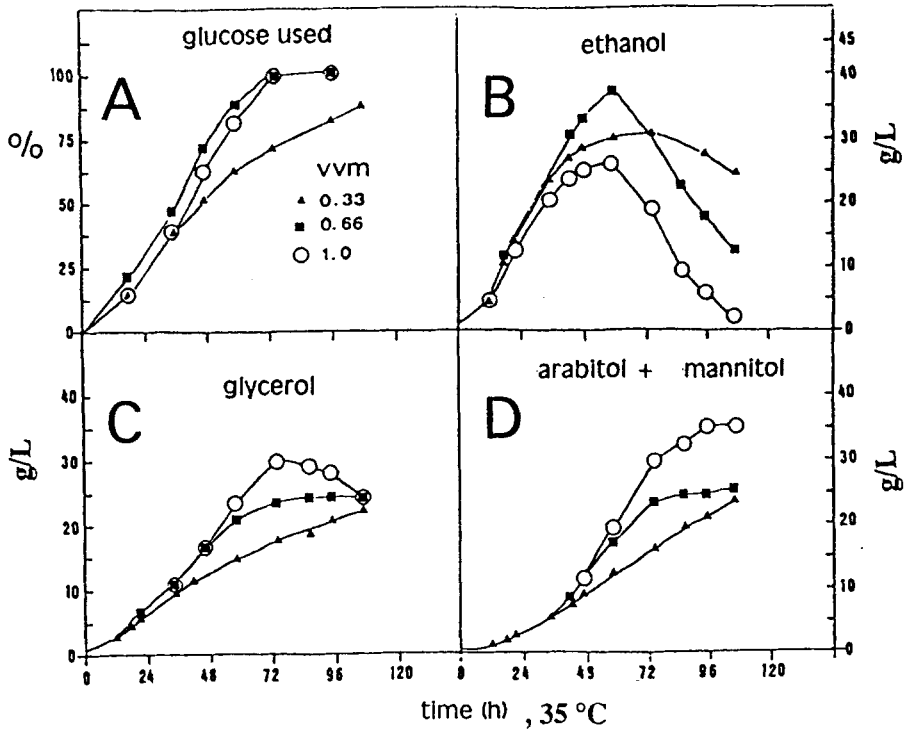

Figure 1: Influence of aeration coupled with agitation (300 rpm) on glucose utilization and on ethanol and polyol accumulation in small (2 L) bioreactors.

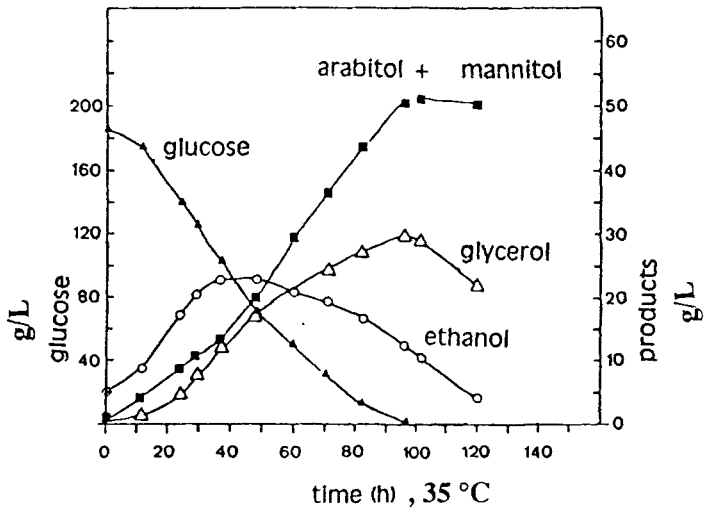

Figure 2: Fermentation of glucose by *Z. rouxii* at the 20 L-scale under partially optimized conditions.

within 100 h, similarly to our earlier smaller scale experiments. Ethanol accumulated in the medium up to about 48 h, its concentration reached 23 g/L then decreased regularly to 4 g/L at 120 h. Polyol accumulation, however, continuously increased until glucose exhaustion (around 96 h). Following glucose exhaustion, the concentration of glycerol decreased significantly while that of the mixture arabitol + mannitol remained stable.

Conclusion. This fermentation with *Z. rouxii* is a good example of a biomass conversion process able to yield chemicals (polyols) and a fuel (ethanol). By simple manipulation of some fermentation conditions, one can favour either ethanol accumulation or polyol accumulation. This fermentation process has been only partially optimized but was however able to give industrially attractive final product concentrations (values at 100 h, Figure 2). Later experiments indicated most of the arabitol + mannitol mixture consisted of mannitol (results not shown). Increased process optimization should lead to even higher product concentrations in the final fermentation broth in the future. As suggested by de Troostenbert *et al.* (1984), the polyols present in the cell-free fermentation liquor might be used as such, without refining, for certain applications in the polymer industry.

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