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ORIGINAL PAPER

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Glycerol and arabitol production by an intergeneric hybrid, PB2, obtained by protoplast fusion between *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*

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Abstract An intergeneric osmotolerant hybrid yeast, PB2, was used together with the parental strains to study glycerol and arabitol production in batch culture. This fusion product was previously obtained by protoplast fusion between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*. Polyols and biomass production were determined in batch culture under aerobic conditions. Under the conditions tested, using PB2 hybrid and both parental strains, the best results were obtained with the hybrid. Arabitol reached a final concentration of 70 g/l and glycerol was increased to up to 50 g/l.

Introduction

Yeasts, like many other microorganisms, are able to produce polyhydroxy alcohols as an integral part of normal growth processes. Production and yields of polyols, however, can be influenced by growth conditions. Among the polyols produced by yeasts, glycerol, arabitol, erythritol, xylitol and mannitol are of the greatest potential interest. Most yeasts produce traces of one or more polyhydroxy alcohols. Higher yields have been obtained from species that show a relatively strong tolerance for high concentrations of salts or sugars (Deak and Beuchat 1993; Groleau et al. 1995; Loray et al. 1995; Rapin et al. 1994; Spencer and Spencer 1978).

Glycerol and arabitol are considered compatible solutes, which counterbalance the high osmotic pressure when yeast cells are exposed to osmotic stress (Blomerg and Adler 1992; Brown 1978). Glycerol and arabitol production by yeasts depends on environmental conditions. Controlling factors in osmotolerant yeasts, which are quite different from the corresponding factors in non-osmotolerant species, are still not known in detail. The metabolic pathways leading to the formation of glycerol, erythritol and arabitol are already known. Glycerol is formed via the Embden-Meyerhof pathway. Arabitol and erythritol are formed through the action of transketolase, which transfers a two-carbon unit to a glycerol-phosphate residue. In the case of erythritol, the resulting four-carbon residue is dephosphorylated to form erythritol (Spencer and Gorin 1960).

Polyols are usually used as additives in the food industry. Glycerol is a simple amphipathic three-carbon alcohol molecule with multiple commercial applications. The lack of color and odor, and its high viscosity, make glycerol suitable as an adjunct to ointments and cosmetics. Glycerol is part of many antifreezing agents, and it is used to stabilize enzyme solutions. Glycerol originating from yeast fermentation contributes to the consistency of beer, wine and bakery products, and the control of its level is of interest to these industries. Yeast stress tolerance in the yeast-producing industry is strictly related to the overproduction of glycerol (Prior and Hohmann 1997).

The aim of the present work was to study glycerol and arabitol production and the intracellular accumulation of each polyol by the intergeneric osmotolerant PB2 hybrid (Lucca et al. 1999). In order to compare polyol production between the hybrid and the parental strains, batch cultures using a production medium with high sugar concentration were performed.

Materials and methods

Yeast strains

The PB2 yeast hybrid was previously obtained by an intergeneric protoplast fusion between heat-treated protoplasts of *Torulaspora delbrueckii* CBS 813, osmotolerant, and viable protoplasts of *Saccharomyces cerevisiae* P-158, isolated from commercial grade baker's yeast, with some degree of glucose tolerance (Lucca et al. 1999). The PB2 yeast hybrid was deposited in the ARS Culture Collection, Peoria, USA. *T. delbrueckii* CBS 813 and *S. cerevisiae* P-158 were also used to allow comparison with the results obtained with the hybrid.

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Culture media

Complete high osmotic pressure medium (YEPD-600), containing in g/l: yeast extract, 10; peptone, 20; glucose, 600; agar, 20. Growth medium (YEPD), containing in g/l: yeast extract, 10; peptone, 20; glucose, 20. Osmotolerance assay media, containing in g/l: yeast extract, 10; peptone, 20 and glucose in the range from 100 to 800. Fermentation medium (YEPD-400), containing in g/l: yeast extract, 10; peptone, 20; glucose, 400.

Culture maintenance

S. cerevisiae and *T. delbrueckii* were maintained on YEPD agar slants. PB2 hybrid was maintained on YEPD-600. Yeast strains were subcultured at regular intervals of 15 days.

Inoculum

Inoculum was prepared by incubating the strains in a 50 ml Erlenmeyer flask containing 10 ml of growth medium (YEPD) for 12 h at 30°C and 400 rpm. The culture was centrifuged 5 min at 5,000 g and the pellet was diluted to give a final concentration of 5×10^6 cells/ml in the different media used.

Osmotolerance assays

Osmotolerance assays were carried out in triplicate in 250 ml Erlenmeyer flasks containing 50 ml culture medium. Inoculated flasks were incubated at 30°C on a rotary shaker at 400 rpm and with aeration (1.0 vvm) until late exponential growth phase. The initial pH was 4.0. Samples were withdrawn at intervals of 12 h during 120 h.

Fermentations

Batch cultures were performed in a 2 l LH-502 (Inceltech) fermentor with a working volume of 1 l of fermentation medium, with automatic control of dissolved oxygen, pH, foam and temperature. The pH was maintained at 4.0 by the addition of either 0.1 N NaOH or 0.1 N H_2SO_4. The agitation speed was kept at 500 rpm and the temperature at 30°C. Dissolved oxygen was controlled at 60% saturation by supplying air automatically via a proportional integrative and derivative (PID) controller, the fluctuations being lower than 5%. Samples were withdrawn periodically until residual glucose in the medium was constant.

Analytical methods

Cell concentration was determined by drying washed biomass at 105°C to constant weight. Glucose and polyhydroxy alcohol (glycerol and arabitol) concentrations (g/l) were determined by HPLC, using Gilson (France) equipment with a 305 pump, a 2142 LKB (Sweden) differential refractometer and a CR 301 (Shimadzu, Japan) recorder/integrator chromatopac. The concentration of each substance was determined with a refractive index detector, under the following conditions: Rezex Organic Acid ROA (Phenomenex) column (300×7.8 mm); temperature: 55°C; eluent, 0.02 N sulphuric acid; flow rate, 0.6 ml/min; sample volume, 20 µl. Samples (0.7 ml) were mixed with an equal volume of 6% trichloroacetic acid, centrifuged and the supernatant was analyzed.

Polyhydroxy alcohols were extracted according to Adler et al. (1985), and the extracellular and intracellular concentrations of polyols were related to biomass and expressed as micromoles polyol per milligram dry weight (DW) biomass. The results reported are the mean values of three separate assays.

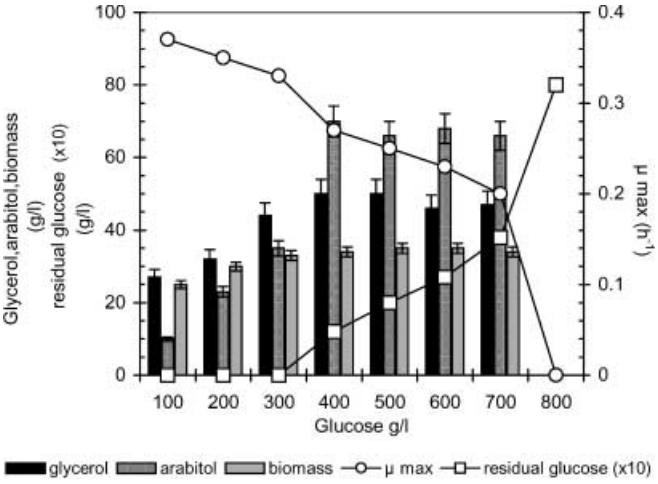

Fig. 1 Effect of glucose concentration on osmotolerance assays using strain PB2: maximum specific growth rate, biomass and residual glucose concentrations, and glycerol and arabitol production obtained

Results

An intergeneric osmotolerant hybrid yeast, PB2, was used together with the parental strains to study glycerol and arabitol production in batch culture. This fusion product was morphologically similar to the parental strain *S. cerevisiae* and could grow in medium with 700 g/l glucose, as could the other parent *T. delbrueckii* (Lucca et al. 1999). The fusant demonstrated its long-term stability, since it preserved all the original characteristics permanently.

To determine the effect of osmotic pressure because of different sugar concentrations, the PB2 hybrid was grown on media containing different concentrations of glucose (osmotolerance assays). High glucose concentrations increase extracellular osmotic pressure and, as a consequence, osmotolerant yeasts accumulate polyols as compatible solutes. The initial glucose concentration was therefore varied in the range from 100 to 800 g/l and the effects examined. Maximum values obtained from glycerol, arabitol and biomass concentrations, and residual glucose are shown in Fig. 1. The strain was able to grow in the presence of up to 700 g/l of glucose in the medium and produced glycerol and arabitol. When the osmotic pressure of the medium increased, the specific growth rate decreased. Biomass concentrations were approximately the same for all glucose concentrations tested. Regarding polyol production, with ≤400 g/l glucose in the medium the hybrid produced more glycerol than arabitol, while from this glucose concentration up to 700 g/l, arabitol production was greater: this production was rather constant reaching 70 g/l arabitol. In these experiments, the carbon source was completely exhausted when the initial concentration of glucose was ≤300 g/l.

The PB2 hybrid was further studied, along with the parental strains *S. cerevisiae* and *T. delbrueckii*, for its ability to produce polyols. The time course of biomass,

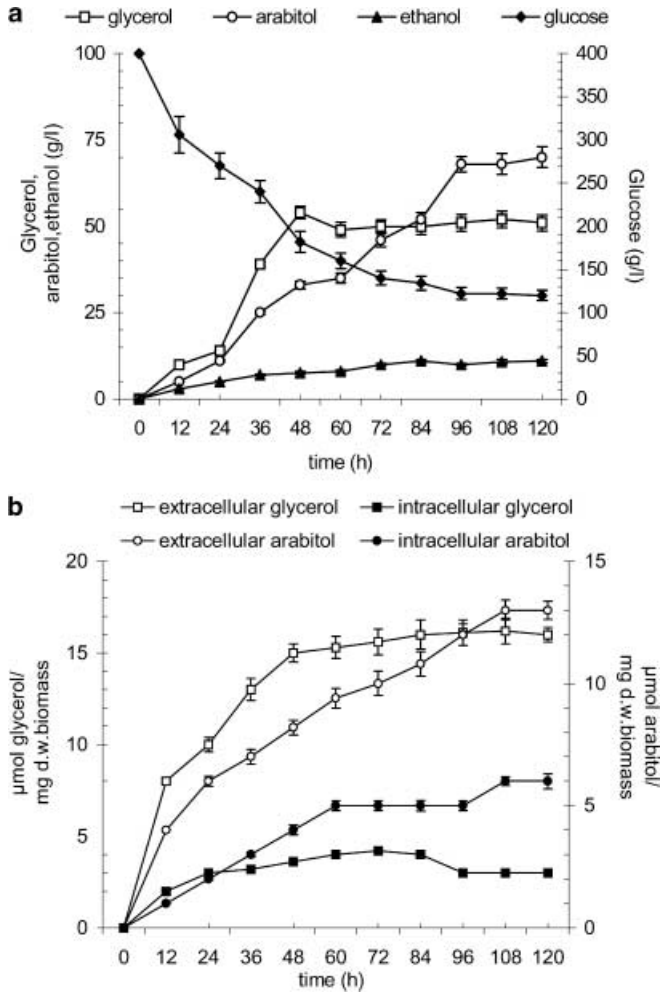

Fig. 2a, b Batch culture of PB2 hybrid in LH fermentor with YEPD-400 at 30°C, an initial pH of 4.0, with agitation (500 rpm) and aeration. **a** Glycerol, arabitol, ethanol and residual glucose (g/l); **b** extra and intracellular arabitol and glycerol, expressed as µmol polyol/mg dry weight (DW) biomass

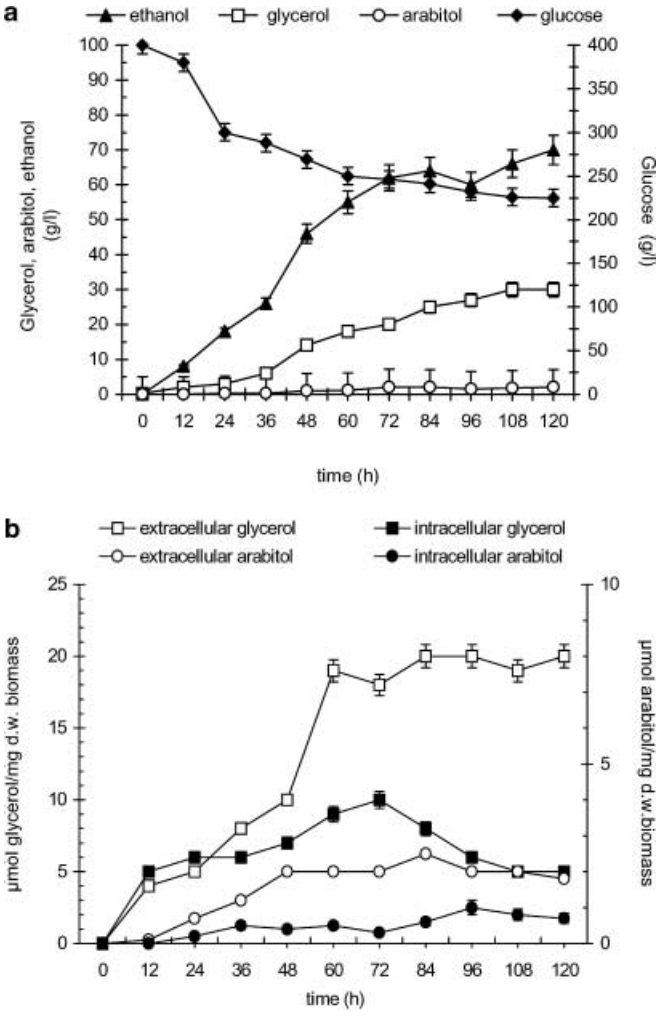

Fig. 3a, b Batch culture of *Saccharomyces cerevisiae* in LH fermentor with YEPD-400 at 30°C, an initial pH of 4.0, with agitation (500 rpm) and aeration. **a** glycerol, arabitol, ethanol and residual glucose (g/l); **b** Extra and intracellular arabitol and glycerol, expressed as µmol polyol/mg DW biomass

ethanol, glycerol and arabitol production as well as sugar uptake were determined.

Fermentation assays using YEPD-400 were performed in batch culture under controlled growth conditions, dissolved oxygen was kept at 60% saturation. The results depicted in Fig. 2a show that the PB2 hybrid produced glycerol (50 g/l) mainly in the exponential growth phase while arabitol concentration increased up to the end of the fermentation (70 g/l). Ethanol concentration in the culture supernatant was significantly lower (12 g/l) than that obtained (75 g/l) when the fermentation assay was performed under non-controlled growth conditions (Lucca et al. 1999). As can be observed, higher oxygen availability increased the cell mass and glycerol concentration, and reduced the concentration of ethanol.

The total concentration of polyols (intra and extracellular) produced by the PB2 hybrid is shown in Fig. 2b. The strain reached the highest specific intracellular arabitol production (6 µmol/mg DW biomass) during the stationary growth phase, while the highest specific intracellular glycerol production (3 µmol/mg DW biomass) was reached after 36 h, before remaining constant. Both specific extracellular glycerol and specific arabitol production increased up to 15 and 13 µmol/mg DW biomass respectively, after 120 h of fermentation. These results are in accordance with those reported by Blomerg and Adler (1992), for *S. cerevisiae* grown in the presence of high external osmolarity. They also observed that the production and accumulation of glycerol were enhanced.

Batch cultures using both parental strains, *S. cerevisiae* (Fig. 3a, b) and *T. delbrueckii* (Fig. 4a, b) were performed under the same culture conditions as the corresponding PB2 hybrid cultures.

According to the results obtained, *S. cerevisiae* (Fig. 3a) produced more ethanol than either the osmotolerant parental strain *T. delbrueckii* (Fig. 4a) or the fusant. Production of polyols differed according to the strain. For example, *S. cerevisiae* produced a very low arabitol

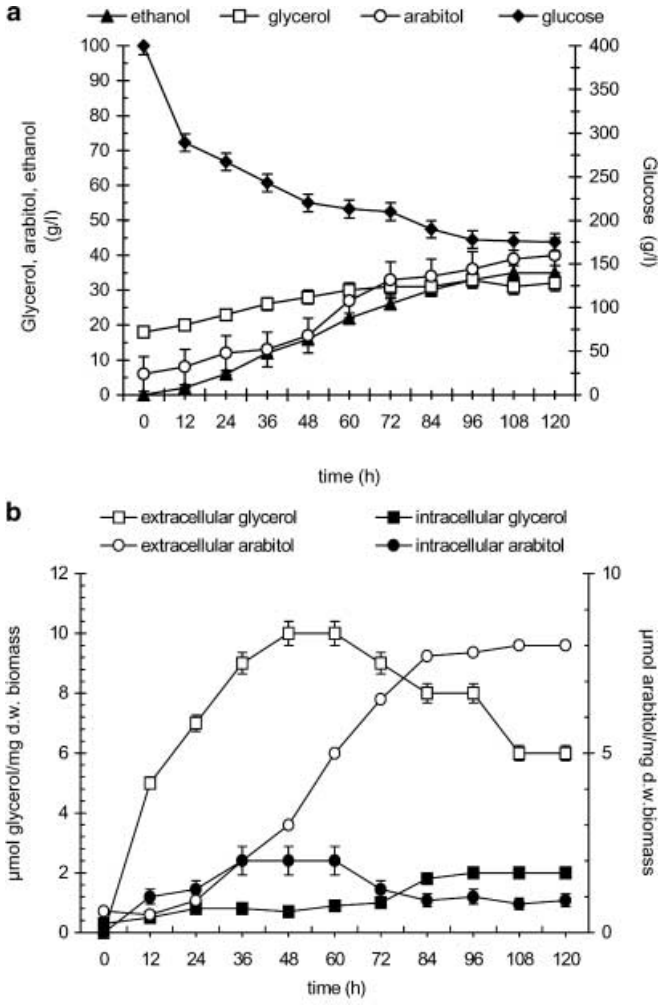

Fig. 4a, b Batch culture of *Torulaspora delbrueckii* in LH fermentor with YEPD-400 at 30°C, an initial pH of 4.0, with agitation (500 rpm) and aeration. **a** Glycerol, arabitol, ethanol and residual glucose (g/l); **b** extra and intracellular arabitol and glycerol, expressed as µmol polyol/mg DW biomass

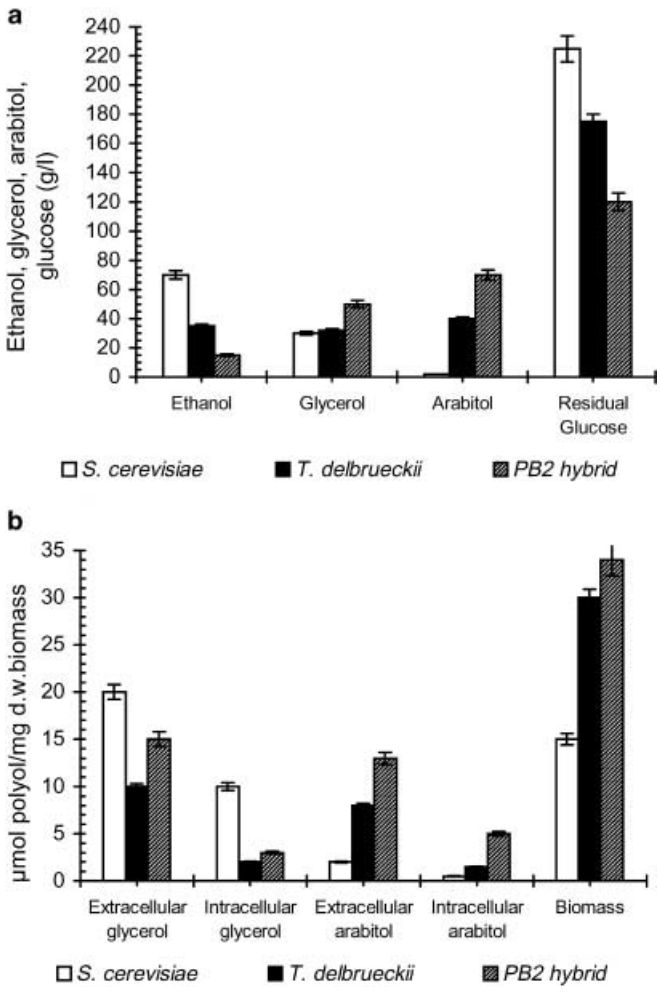

Fig. 5a, b Batch cultures of PB2 hybrid and the parental strains in LH fermentor with YEPD-400 at 30°C, an initial pH of 4.0, with agitation (500 rpm) and aeration. **a** Maximum glycerol, arabitol, ethanol and residual glucose concentrations obtained, expressed as g/l; **b** maximum intra and extracellular polyol concentrations obtained, expressed as µmol polyol/mg DW biomass

concentration, while glycerol reached 30 g/l in the medium. The behavior of *T. delbrueckii* during the fermentation was similar to that of *S. cerevisiae* with respect to glycerol production; however, the concentration of arabitol reached 40 g/l. The PB2 hybrid produced more glycerol and arabitol (Fig. 2a) than either parental strain and also showed the highest sugar uptake.

The distribution of glycerol and arabitol inside and outside the cells was noticeably different between the strains (Figs. 2b, 3b, 4b). All three strains synthesized glycerol and arabitol as a response to the high osmotic pressure due to the high glucose concentration. It seems that for the osmotolerant hybrid, the ability to grow in the presence of high sugar concentrations in aerobic cultures and the improvement of biomass yield were related to the synthesis of both polyols, i.e., glycerol and arabitol. The PB2 hybrid was able to grow in high glucose concentration media. Glucose uptake and growth rate were increased under fully aerobic conditions. Kim et al. (1997) also observed an improvement in polyol production in aerobic cultures of the yeast *Trigonopsis variabilis*.

Figure5a, b summarizes and compares the maximum values obtained with the PB2 hybrid and its parental strains, *S. cerevisiae* and *T. delbrueckii* in the batch cultures performed. During the assays, the highest biomass yield was obtained with PB2 hybrid (34 g/l), while yield values for parental strains *T. delbrueckii* and *S. cerevisiae* were 30 and 15 g/l, respectively.

Discussion

The results obtained in this paper show that aerobic conditions improved polyol and biomass production by the osmotolerant hybrid (Lucca et al. 1999). Although in the osmotolerance assays performed, this strain consumed

all the available carbon source when the initial concentration of glucose was ≤300 g/l, subsequent studies were done with 400 g/l of initial glucose. Under these high osmotic pressure conditions, both polyols reached their maximum concentrations in the culture supernatant. The increase of initial glucose improved mainly arabitol production. Optimal fermentation conditions should be adjusted to between 300 and 400 g/l glucose in order to achieve a complete exhaustion of the substrate.

Glycerol and arabitol yields obtained were 0.19 g glycerol/g glucose consumed and 0.27 g arabitol/g glucose consumed. These results are similar to those reported by Yun and Song (1994) using *Aureobasidium pullulans*. Wang et al. (2001) described a yield of 0.25 g glycerol/g glucose consumed and a volumetric productivity of 15 g glycerol l^{-1} day^{-1} for *S. cerevisiae*. They also described a yield of 0.43 g polyol/g glucose consumed and a volumetric productivity of 20 g polyol l^{-1} day^{-1} for *Candida magnoliae* (osmotolerant). The volumetric productivities achieved with the fusant PB2 for both polyols were 25 g glycerol l^{-1} day^{-1} and 17.5 g arabitol l^{-1} day^{-1}.

Under the experimental conditions used in the present study, a significant increase in glycerol and arabitol production by the PB2 hybrid with respect to the parental yeasts, *S. cerevisiae* and *T. delbrueckii*, was achieved.

Recent studies carried out with the PB2 hybrid showed that polyol production was improved by using sugarcane-molasses as a carbon source (Lucca and de Figueroa 2000).

Even if the results obtained in this work do not reach the level needed to establish cost effective industrial fermentation, it is worth considering the possibilities of a fully optimized process in order to improve its performance.

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