Journal of Industrial Microbiology, (1995) 14, 508–513

Hybrids obtained by protoplast fusion with a salt-tolerant yeast

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(Received 10 May 1994; accepted 18 November 1994)

Key words: Protoplast fusion; Hybrids; Osmotolerance; Polyols

SUMMARY

An industrial strain of *Saccharomyces cerevisiae* was fused with an osmotolerant yeast, *Debaryomyces hansenii*, to obtain hybrids having increased tolerance to elevated salt concentrations. The hybrids were intermediate to parent species in production of ethanol and polyols.

INTRODUCTION

The growth of microbial cells is often inhibited in environments where water activity is much reduced. However, both prokaryotic and eukaryotic microorganisms include species which can tolerate a wide range of salt concentrations and/or sugars in the culture medium during growth and are termed osmotolerant [6].

In the cells of most actively metabolizing organisms, the intracellular medium must remain relatively constant in ionic strength, pH and levels of metabolites. Thus, in media of low osmolality, there are homeostatic mechanisms which maintain these parameters within the required limits, especially in maintaining the intracellular osmolality [8]. After a transfer to a hyperosmotic medium, adaptation to the change is required. For most organisms, the adaptive response to this change is intracellular accumulation of organic compounds which act as compatible solutes which are not toxic to the cells, even at the high concentrations required for stabilization of the osmotic equilibrium. In yeasts, compatible solutes are polyhydroxy alcohols, such as glycerol, arabitol, mannitol, erythritol and xylitol [12,13,19].

For industrial yeasts, such as baker's, distiller's, brewer's and other beverage yeasts, osmotolerance is of varying importance. *Saccharomyces cerevisiae* has a certain degree of osmotolerance, but grows poorly on media of very high osmotic pressure, which limits its use in the fermentation of concentrated musts for production of fermented beverages. Legmann and Margalith [9] fused protoplasts of *Zygosaccharomyces mellis* with protoplasts of *Saccharomyces cerevisiae* to obtain strains which were more osmotolerant, and isolated hybrids which were able to ferment solutions containing up to 30% glucose, and produced good yields of ethanol. Spencer et al. [16] fused protoplasts of *Zygosaccharomyces rouxii* and *Saccharomyces diastaticus (S. cerevisiae)*, and obtained strains of baker's yeast with good performance in dough-raising tests in normal and sweet doughs and improved osmotic tolerance.

Protoplast fusion is now a recognized technique for the improvement of industrial yeasts for baking, brewing, ethanol production and other purposes [15]. Numerous strains have been constructed by intraspecific or interspecific fusions [11,14,16,17]. *Debaryomyces hansenii* is an osmotolerant yeast which shows a marked tolerance to high concentrations of salt or sugar in the medium [1]. Its osmotolerance makes it very important as a parental strain when using protoplast fusion as a technique for obtaining genetically modified industrial yeast strains having an increased tolerance to elevated concentrations of sugar in the musts.

In this work, we report the results of a fusion between a strain of *Saccharomyces cerevisiae* and a strain of *Debaryomyces hansenii*. We determined the growth rates, biomass yield, tolerance to elevated osmotic pressures and the production of polyhydroxy alcohols by hybrids obtained from this fusion.

MATERIALS AND METHODS

Yeast strains

Debaryomyces hansenii NRRL Y-7393 (osmotolerant) was a gift from Dr C. Kurtzman, Peoria, IL, USA; and *Saccharomyces cerevisiae* UCD 522, a wine yeast, from Dr R. Snow, University of California, Davis, CA, USA.

Media

Yeast cultures were maintained on complete medium, yeast extract 1%, peptone 2%, glucose 2% and agar 1.5% (YEPD). Regeneration medium was YEPD plus KCl 0.6 M and agar 3%. Selective medium was YEPD plus NaCl 12%.

Protoplast formation and fusion

These were done by methods previously described [18]. The fusogenic agent was polyethyleneglycol (PEG) MW 6000,

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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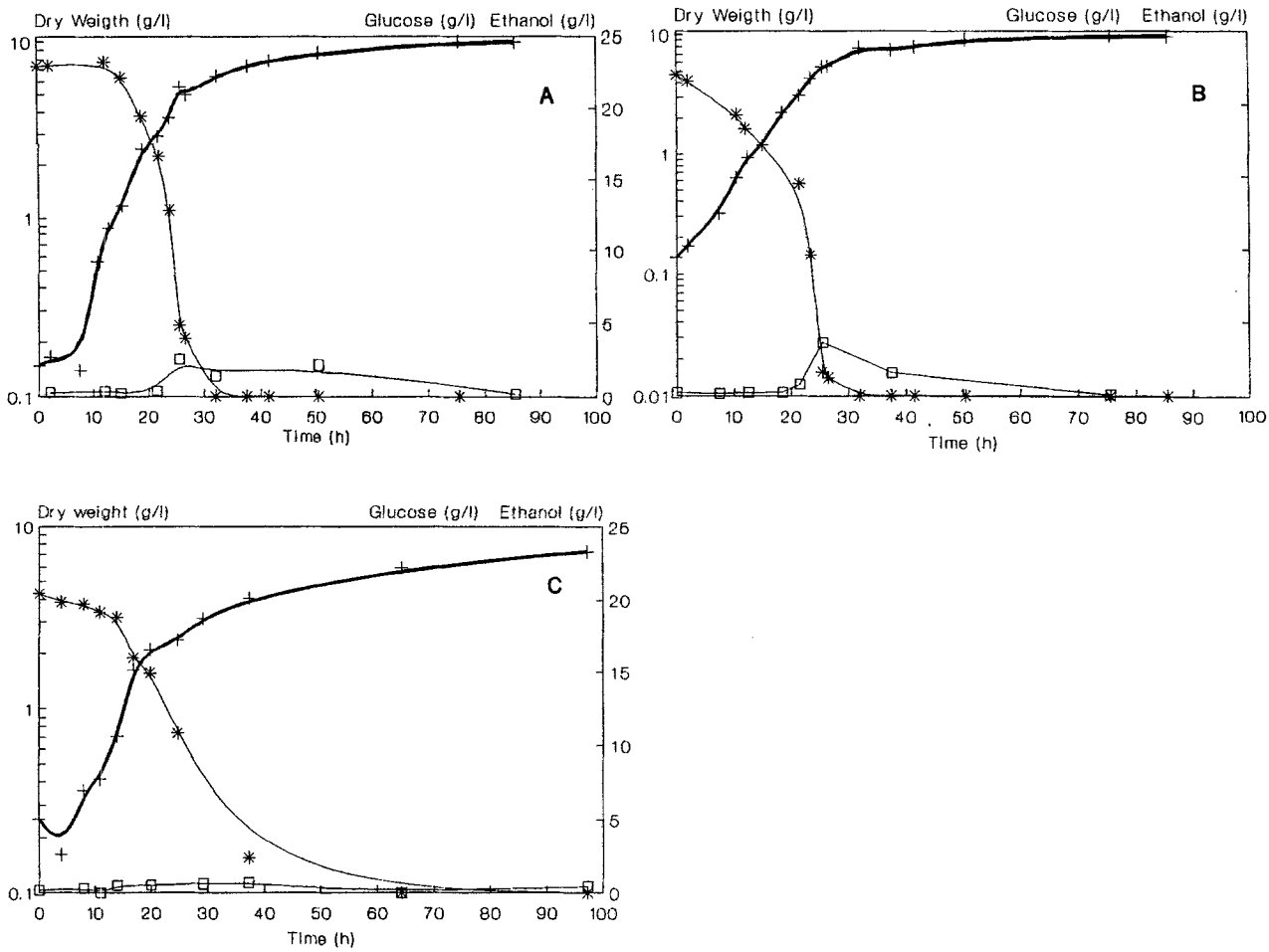

Fig. 1. Growth of the strains in YEPD with 12% NaCl. (A) Hybrid HM 92; (B) hybrid HM 93; (C) *Debaryomyces hansenii*. —+— Dry weight; —*— concentration of glucose; —□— production of ethanol.

30%. For the osmotolerant yeast, the pretreatment solution contained β-mercaptoethanol, 0.1 M, and KCl, 1.2 M [3], and the enzyme used was Suc d'*Helix pomatia* at 10% (vol/vol) concentration in the protoplasting solution.

Regeneration of fused protoplasts and isolation of fusants (FP) was done using the overlay method on osmotically stabilized media [18].

Growth curves were determined using 10-ml cultures incubated in 125-ml Erlenmeyer flasks containing: a) YEPD or b) YEPD + NaCl 12%. In both assays the flasks were inoculated with 5×10^6 cells ml^{-1}, incubated at 30 °C on a rotary shaker at 200 r.p.m., and sampled periodically. Cell concentrations were determined according to dry weight. Glucose, ethanol and polyhydroxy alcohol concentrations were determined using HPLC. Polyhydroxy alcohols were extracted according to Adler et al. [1].

RESULTS

We carried out an intergeneric fusion between protoplasts of the wine yeast, *Saccharomyces cerevisiae*, and the salt-tolerant (halotolerant) species, *Debaryomyces hansenii*. The medium used for regeneration of the cell wall and isolation of the fusion products (FP) was YEPD + 12% NaCl, and the incubation temperature was 37 °C. *Saccharomyces cerevisiae* UCD 522 does not grow on media containing high concentrations of salt, and *Debaryomyces hansenii* NRRL Y-7393 does not grow at 37 °C, so only the hybrids survived. Their genomes therefore were presumed to be recombinants of the genomes of the parental strains. Two colonies of the 21 hybrids obtained were selected at random, and used in further experiments.

These hybrids and the parental strains were tested for their ability to grow in media containing high concentrations of salt. Growth curves, biomass, ethanol production and production of polyhydroxy alcohols were determined in YEPD medium + 12% salt and in the same medium without salt. The parental *Saccharomyces cerevisiae* did not grow in medium containing high salt, and the cells lysed.

Hybrids HM 92 and HM 93 (Figs 1 (A and B)) grew faster than the osmotolerant parental strain of *Debaryomyces hansenii* (Fig. 1 (C)). Under these conditions, the cultures of *D. hansenii* consumed all of the glucose and reached their highest cell density after 80 h of fermentation. The hybrids reached their maximum cell density and consumed all of the glucose between 10 and 30 h. Under these conditions, the parental osmotolerant strains produced practically no ethanol, while the hybrids produced it between 10 and 30 h of fermentation. It

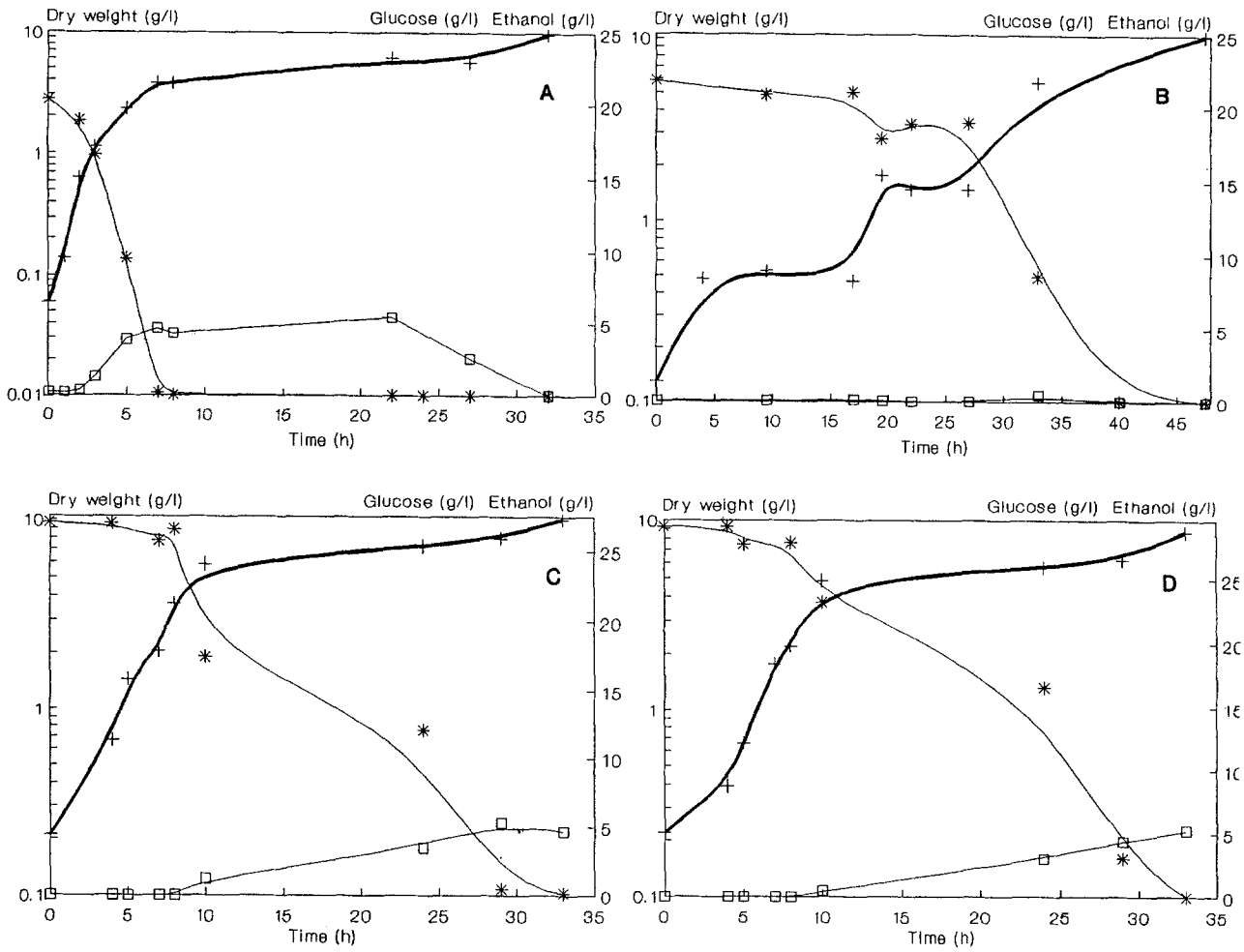

Fig. 2. Growth of the strains in YEPD basal medium. (A) *Saccharomyces cerevisiae*; (B) *Debaryomyces hansenii*; (C) hybrid HM 92; (D) hybrid HM 93. —+— Dry weight; —*— concentration of glucose; —□— production of ethanol.

may be assumed that this ability is conferred on the hybrids by the genetic information introduced into the genome from the other parental strain, *Saccharomyces cerevisiae*.

The same test was done in YEPD basal medium, to investigate the behavior of the strains in a medium of normal osmotic pressure. In the medium, the parental strain *Saccharomyces cerevisiae* used all of the glucose in 8 h of fermentation, reaching stationary phase and maximum ethanol production at this time (Fig. 2 (A)). The osmotolerant parental strain, *Debaryomyces hansenii*, metabolized all of the glucose (Fig. 2 (B)) in less time (45 h) than in the medium containing 12% NaCl, and produced practically no ethanol. The hybrids required about 30–35 h to utilize the glucose completely, and reached a maximum production of ethanol which was greater than that attained in the medium having an elevated osmotic pressure (Fig. 2 (C and D)).

In addition, both *Saccharomyces cerevisiae* and *Debaryomyces hansenii* produce the polyols, glycerol and arabitol, during growth [4,5] which are also produced by hybrids HM 92 and HM 93. However, when these strains were grown in a medium of normal osmotic pressure and one containing 12% NaCl, differences in production and accumulation of the two polyols were observed.

When the strains were grown in the basal YEPD medium, the levels of extracellular and intracellular glycerol decreased gradually to the stationary phase and did not change further (Fig. 3 (A,B,C,D)). However, when the cultures were grown in YEPD + 12% NaCl, both hybrids and the osmotolerant parent, *D. hansenii*, accumulated the greater part of the glycerol in the early logarithmic phase, and this was liberated into the medium later, in early stationary phase. In addition, the cells apparently began to re-utilize some of the glycerol produced, since the levels of extracellular glycerol decreased in this period (Fig. 4 (A,B,C)). The parent *S. cerevisiae* did not grow in this medium because of the high salt concentration.

S. cerevisiae produced but did not accumulate arabitol intracellularly when it was grown in the basal YEPD medium. Since the extracellular concentration of arabitol decreased with time, it was probably assimilated by the cells. However, *D. hansenii* excreted most of the arabitol produced in the early stage of growth. The fusion products, HM 92 and HM 93, accumulated arabitol during the late logarithmic phase, and later liberated it in the early stationary growth phase (Fig. 3 (A,B,C,D)).

D. hansenii and both hybrids exhibited similar behavior in media having elevated osmotic pressure. During exponential

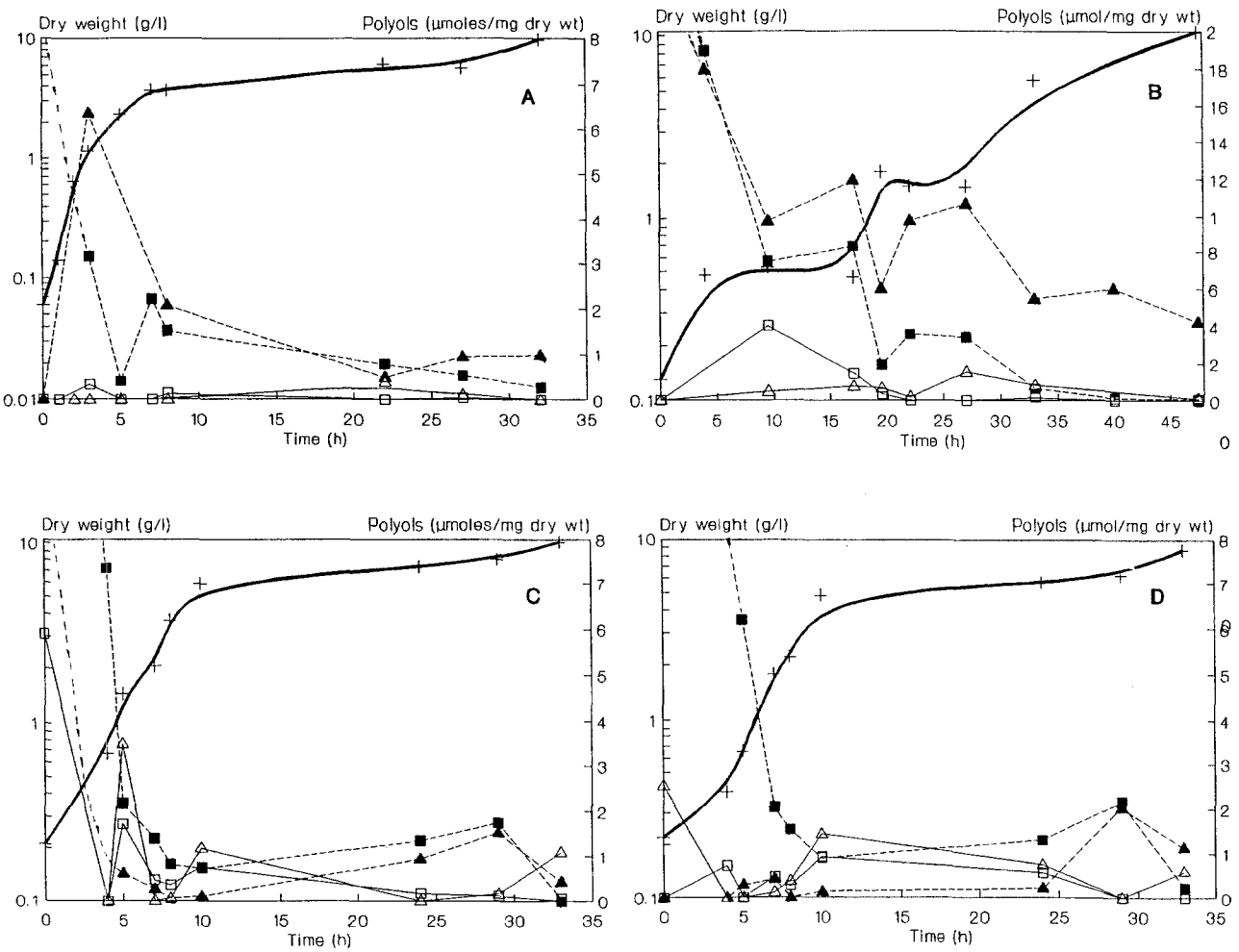

Fig. 3. Production of polyols during growth in YEPD basal medium. (A) *Saccharomyces cerevisiae*; (B) *Debaryomyces hansenii*; (C) hybrid HM 92; (D) hybrid HM 93. —+— Dry weight; —□— intracellular glycerol; --■-- extracellular glycerol; —△— intracellular arabitol; --▲-- extracellular arabitol.

growth the intracellular levels of glycerol remained high, arabitol occurred extracellularly. Later, when the intracellular level of glycerol decreased, arabitol began to accumulate within the cell (Fig. 4 (A,B,C,D)). *D. hansenii* and both of the hybrids responded similarly to osmotic shock in the growth medium. The levels of production of polyols were increased, and the intracellular levels were maintained. However, the proportions of the two polyols varied according to the stage of growth. This behavior resembles that observed in *D. hansenii* by other investigators, under similar cultural conditions [2,7].

DISCUSSION

The results obtained by Onishi and Shiromaru [10] using *Zygosaccharomyces rouxii* were similar to those reported here for *Debaryomyces hansenii*. These data also showed that when *Z. rouxii* cells were transferred from a medium containing a high salt concentration (12%) to one having the same salt concentration and differing little in sugar concentration, there was little change in the polyol concentration. These conditions are similar in the natural habitat of these yeasts, where they are not subject to osmotic shock. In nature, osmotolerant yeasts are found in fermenting honey and confectionery, spoiled salt meats, salty foods and other habitats having high osmotic pressures, and this aspect of their osmotolerance has not been investigated.

Compatible solutes protect the cell by stabilizing the structure of enzymes, probably in the same way as increased concentrations of KCl protects temperature-sensitive, osmotic-remedial mutants of *Saccharomyces cerevisiae* (L.H. Hartwell, pers. comm.). Conversion of glucose to glycerol and other polyhydroxy alcohols probably reduces the intracellular sugar concentration, reducing the osmotic tension and relieving the osmotic stress in the intracellular environment. In addition, conversion of glucose to compatible solutes is accompanied by ethanol formation and synthesis of ATP, so that energy is available to the cell from the process.

The method of protoplast fusion can be used to obtain hybrids having improved performance over the parental strains. The hybrids had more rapid growth rates in saline media and produced ethanol in the same medium. The hybrids obtained in the present work will be used for further investigations on the fundamental basis for osmotolerance and its practical applications.

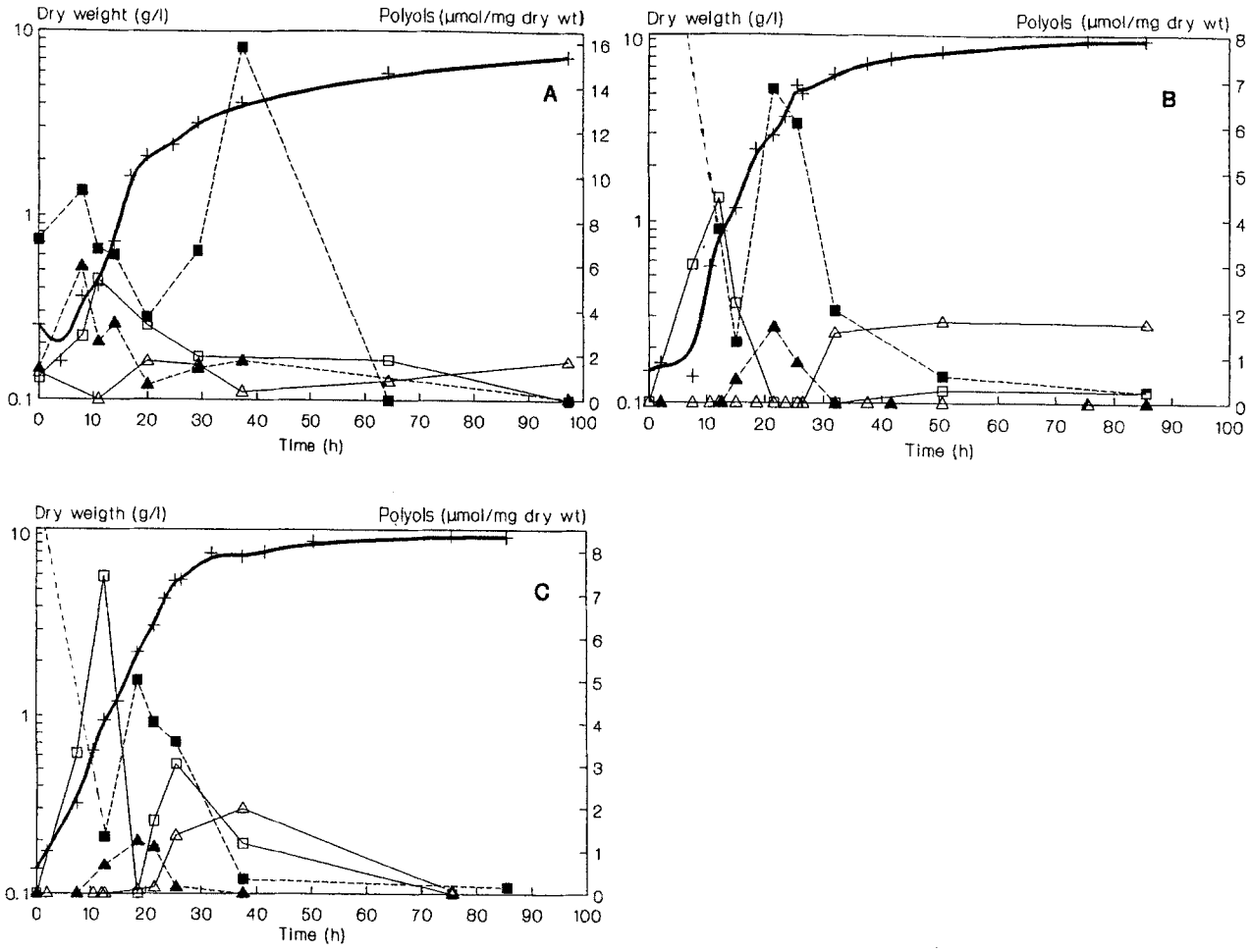

Fig. 4. Production of polyols during growth in YEPD with 12% NaCl. (A) *Debaryomyces hanesenii*; (B) hybrid HM 92; (C) hybrid HM 93. —+— Dry weight; —□— intracellular glycerol; --■-- extracellular glycerol; —△— intracellular arabitol; --▲-- extracellular arabitol.

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