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ORIGINAL ARICLE

Continuous culture for the bioproduction of glycerol and ethanol by *Hansenula anomala* growing under salt stress conditions

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Abstract Continuous cultures of *Hansenula anomala* were carried out in media at low water activity, resulting from NaCl addition. Three dilution rates were compared and it was shown that a clear steady state was only recorded for the lowest dilution rate tested (0.03 h^{-1}), leading to a biomass concentration of 4.3 g L^{-1}. At 0.06 h^{-1} dilution rate, steady state was maintained for no more than 20 h and a clear wash-out was observed at 0.12 h^{-1}. The experimental ratio ethanol on glycerol produced at steady state (D=0.03 h^{-1}) was similar to that previously recorded in batch culture in similar conditions after 30 h of growth, 7.8 and 7.9, respectively. The comparison of continuous culture to repeated batch culture was clearly in favour of the continuous culture, which led to significantly higher glycerol and ethanol production rates, 4.5 and 4 times higher than those recorded in repeated batch culture, respectively, even if significant amounts of glucose remained in the bleeding of the bioreactor.

Keywords *Hansenula anomala* · Continuous culture · Repeated batch culture · Metabolite production

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Introduction

Osmotolerant yeasts are able to survive in media at low water activity induced by high salt or sugar concentrations. For this purpose, they produce polyols, like glycerol, the most abundant (Bellinger and Larher 1991; Van Eck et al. 1989). In addition to being a compatible solute for yeast, there is an increase in the industrial demand for glycerol, due to its numerous industrial applications (Palmarola-Adrados et al. 2005; Wang et al. 2001). Glycerol biosynthesis is mostly accompanied by ethanol biosynthesis, since the Embden-Meyerhof pathway is used for both glycerol and ethanol synthesis, as shown for *Saccharomyces cerevisiae* and *Torulopsis magnoliae* since 1956 (Spencer and Sallans 1956). Ethanol is also found in numerous industrial applications, especially as a substitute for fossil fuels (Palmarola-Adrados et al. 2005).

To improve rates of glycerol production, some authors have optimized yeast culture conditions. The main examined parameters were osmoticum, carbon and nitrogen substrates, as well as dissolved oxygen (Brown 1977; Djelal et al. 2005, 2006; Liu et al. 2003; Patil et al. 2002; Spencer and Sallans 1956).

Experiments are mainly carried out in batch culture, in shake flasks or in bioreactors (Taherzadeh et al. 2002), since they are easy to conduct and have a low risk of contamination. However, for a large number of processes, continuous cultures were proven to be more efficient than batch cultures. Indeed, ethanol production rates were found to be four times higher in continuous cultures than in batch cultures of *Saccharomyces bayanus* (Mota et al. 1987). The time needed to achieve maximum conversion yield of the carbon substrate into ethanol by a yeast *Pachysolen tannophilus* in continuous culture was half that recorded in batch culture (Ravinder and Kaur 1989). Production rate

is under control of the dilution rate: its increase results in increasing rates. However, the limit of the continuous process appears to be the relatively low cellular concentrations achieved, which may be overcome by cell recycling (Cheryan and Mehaia 1984; Mercier et al. 1998), cell immobilization (Kourkoutas et al. 2002; Lebeau et al. 1998), or by the continuous extraction of the inhibitory produced ethanol (Honorato da Silva et al. 1999).

In contrast, the literature dealing with continuous culture for polyols production is rather weak; some authors have examined polyols production in continuous mode with immobilized cells of *S. cerevisiae*, a relatively sensitive yeast to osmotic stress (Bisping et al. 1990; Hecker et al. 1990). A multistage cascade bioreactor was also proven to be efficient for the continuous production of glycerol by *Candida krusei* (Liu et al. 2002). Arabitol production in a two-stage continuous process has also been examined (Duflot et al. 2000).

In the available literature, there is therefore a lack of information concerning the simultaneous production of polyols and ethanol in continuous culture, which was the main purpose of our study. The continuous mode of culture was compared to the repeated batch process. Indeed, repeated batch culture has been proven to be efficient for ethanol production by flocculating yeast (Horiuchi et al. 2000), or immobilized yeast cells (Sakura et al. 2000), as well as for glycerol production by immobilized (Bisping et al. 1990) or free (Liu and Liu 2004) yeast cells. However, since immobilization limits oxygen transfer, while glycerol formation by osmophilic yeast is an oxygen-requiring process (Liu and Liu 2004), free cells were preferred to immobilized cells.

Materials and methods

Microorganism

The osmotolerant yeast *Hansenula anomala* var. *anomala* (CBS 5759; Delft, Netherlands) was used. Stock cultures were maintained on a gelified medium (g L^{-1}): glucose, 20; peptone, 20; yeast extract, 10; and agar, 20; and were stored at 4°C.

Inoculum preparation

A given number of drops (10) of a yeast suspension in KCl 150 mmol L^{-1} were added to 25 mL of seed culture medium (g L^{-1}): glucose, 20; peptone, 20; and yeast extract, 10; contained in a 0.25-L Erlenmeyer flask. Cells were precultivated at 28°C and 180*g* until reaching a turbidity value of 1 unit at 610 nm. After centrifugation (4°C, 1,800 *g* and 5 min), cells were harvested, resuspended in 25 mL KCl 150 mmol L^{-1} and recentrifuged in similar conditions. The suspension obtained after harvesting cells and resuspending in 10 mL KCl 150 mmol L^{-1} was used to inoculate culture media.

Culture media

Vitamines were not required by *Hansenula anomala* for its growth (Burkholder et al. 1944). The synthetic medium used contained (mmol L^{-1}): NH_4Cl, 10; NaCl, 2.0×10^3 NaCl (added to ensure salt stress conditions); KH_2PO_4, 3.7; $MgSO_4{\cdot}7H_2O$, 4; as well as an EDTA mineral solution, derived from the Wikerham medium (Wickerham 1951) (mg L^{-1}): $CaCl_2{\cdot}6H_2O$, 150; $FeSO_4{\cdot}7H_2O$, 100; $ZnSO_4{\cdot}7H_2O$, 30; $CuSO_4{\cdot}5H_2O$, 0.79; H_3BO_3, 15; KI, 2; $NaMoO_4{\cdot}2H_2O$, 5; $MnSO_4{\cdot}H_2O$, 32; $CoCl_2{\cdot}6H_2O$, 5.6; EDTA, 100. Antifoam, 500 μL (silicone 426, aqueous emulsion of dimethyl-polysiloxanic), was added to the culture medium.

Glucose was also added at the required concentration during repeated batch culture (see next section) and at 0.4 mol L^{-1} during continuous cultures. KOH 1 mol L^{-1} was used to adjust the pH to 5.5.

Medium was sterilised by filtration on a 0.2-μm membrane (Sartorius, Goettingen, Germany).

Culture conditions

A 2-L bioreactor (Biolafitte, Saint Germain en Laye, France) was used. The working volume was 1.2 L and the airflow 100 L h^{-1}; the specific aeration rate was then 1.4 vvm, leading to an aeration number of 0.018, owing to the diameter of the stirring blade, 45 mm. Indeed, the aeration number N_a is given by:

$$N_a = \frac{Q}{r \cdot d^3} \quad (1)$$

where Q was the airflow, r the stirring rate and d the stirring blade diameter.

The pH (Mettler-Toledo, Wilmington, MA, USA) was maintained at 5.5 by automatic addition of 1 mol L^{-1} KOH, and the dissolved oxygen was monitored by means of a pO_2 probe (Mettler-Toledo).

The temperature was maintained at 28°C, the optimal value for *Hansenula anomala* growth (Burkholder et al. 1944). Growth was indirectly monitored by means of off-line turbidimetric measurements (Spectronic 20; Spectronic Analytical Instruments, Leeds, UK) at 610 nm, after calibration by dry cellular weight measurement. The experimental error of the dry weight was 0.2 g L^{-1}.

Repeated batch culture

Three experiments were successively carried out for 96 h at 140, 95 and 77 mmol L^{-1} glucose, respectively. After each experiment, agitation and aeration were stopped for 24 h to allow cell decantation. After harvesting 0.8 L of supernatant, sterile medium was added to the resting cells and a new run was carried out.

Mean production rates corresponded to the sum of the productions during each run for the considered metabolite on the total running time, namely, for each run, 96 h culture plus 24 h for cell decantation.

Continuous culture

Experiments were carried out at 3 dilution rates, 0.03, 0.06 and 0.12 h^{-1}. A level probe was used to maintain the volume constant in the bioreactor. Culture was carried out in batch mode for 30 h, before sterile medium was fed into the reactor.

Mean production rates corresponded to the product of the concentration at steady state by the dilution rate.

Analysis

The time courses of polyol, (e.g. glycerol and arabitol), disaccharides (e.g. trehalose), alcohols (e.g. ethanol), and organic acids (e.g. acetic acid), were deduced from off-line sampling (6×10^{-3} L). Metabolites identification and determination were carried out by HPLC involving an ions exclusion column HPX-87H (300×7.8 mm,;Bio-Rad, Hercules, CA, USA), maintained at 45°C (Oven Croco-Cil_{TM}; Cluzeau–Info–Labo, Ste Foy La Grande, France). The elution was performed at a flow rate of 0.6×10^{-3} L min^{-1} (Waters pump, Milford, MA, USA) using sulphuric acid 5 mmol L^{-1}. An ERC 7512 refractometer (Saitama, Japan) was used for the detection of the different compounds. The standard solution contained 5 mmol L^{-1} each of glycerol, arabitol, trehalose, ethanol and acetic acid. The experimental error was 2%, namely 0.1 mmol L^{-1}.

Results

Figure 1 shows that the amounts of glycerol produced remained nearly similar for all runs, between 19 and 23 mmol L^{-1}, while the production of arabitol increased from 4.3 to 13.1 mmol L^{-1}. Contrarily, ethanol and acetic acid productions decreased from 241 to 126 mmol L^{-1} and from 7.1 to 0 mmol L^{-1}, respectively. Low amounts of trehalose were also produced, between 0.1 and 0.2 mmol L^{-1}. An amount of 3.6 g L^{-1} of biomass was formed during the first run, while similar amounts of biomass were formed during the second and the third runs, 1.5 and 1.7 g L^{-1} respectively.

Figure 2a shows that, during continuous culture, no steady state can be achieved for 0.12 h^{-1} dilution rate; biomass concentration increased to 3.1 g L^{-1} after 30 h of culture before it decreased almost continuously until cessation of culture (0.58 g L^{-1} after 84 h). For a lower dilution rate (0.06 h^{-1}), a steady state was maintained for less than 20 h, since biomass concentration was constant (4 g L^{-1}), and then decreased after 55 h of culture. A clear

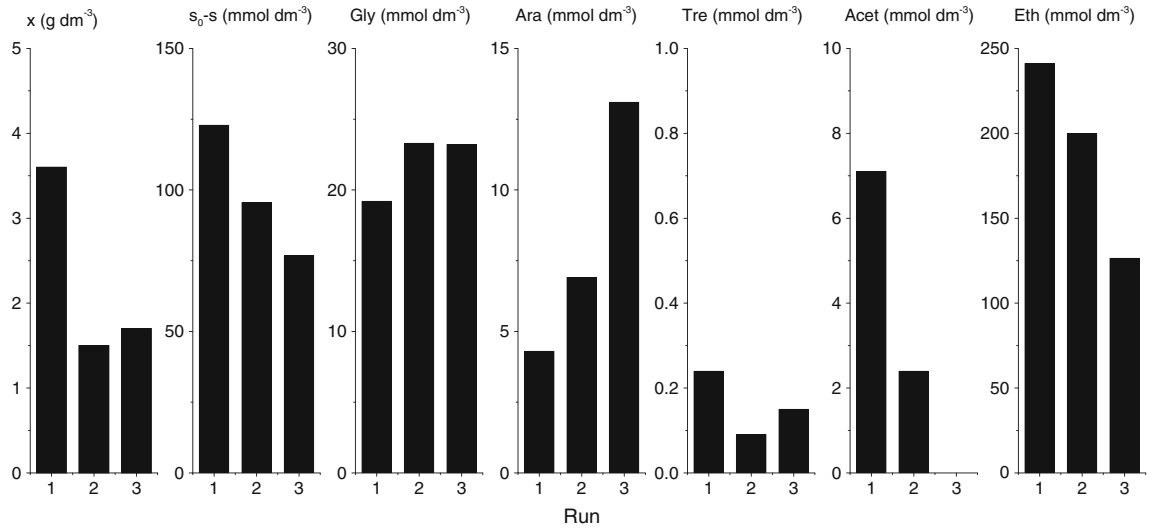

Fig. 1 Final biomass production (*x*), glucose consumption (s_0–s), production of glycerol (*Gly*), arabitol (*Ara*), trehalose (*Tre*), acetic acid (*Acet*) and ethanol (*Eth*) at the end of each run of the repeated batch process of *Hansenula anomala* growing on 2 mol L^{-1} NaCl, 10 mmol L^{-1} ammonium and 140 (run 1), 95 (run 2) and 77 (run 3) mmol L^{-1} glucose

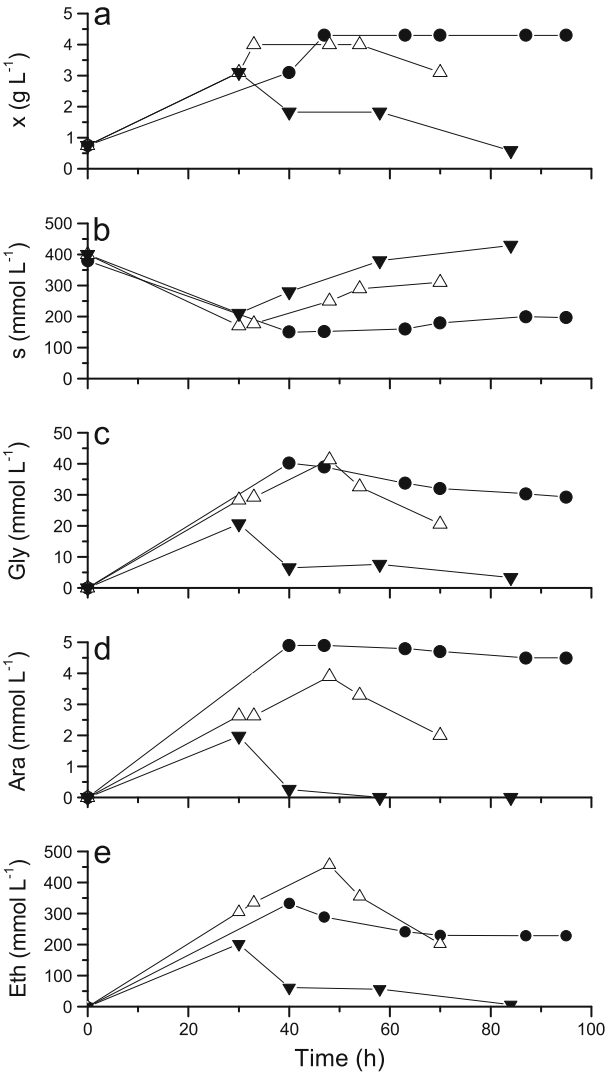

Fig. 2 Time-courses of biomass (**a**), glucose (**b**), extracellular glycerol (**c**), extracellular arabitol (**d**) and ethanol (**e**) concentrations during continuous culture of *Hansenula anomala* growing on 2 mol L^{-1} NaCl, 10 mmol L^{-1} ammonium and 40 mmol L^{-1} glucose at dilution rates of 0.03 (●), 0.06 (Δ) and 0.12 (▼) h^{-1}

steady state, from 47 h and until the end of culture (95 h), was only recorded for the lowest dilution rate (0.03 h^{-1}), also leading to the highest biomass concentration (4.3 g L^{-1}).

In close relationship with biomass concentrations, the highest concentrations of non-assimilated glucose were recorded for the highest dilution rate (0.12 h^{-1}; Fig. 2b), until the initial glucose was recorded at the end of culture, showing a washout of the bioreactor. For D=0.06 h^{-1}, the time-course of glucose concentration also displayed a peak after 30 h of culture and then increased to a final concentration of 0.31 mol L^{-1}. Glucose concentrations remained nearly constant only during the steady state recorded for the lowest dilution rate (0.03 h^{-1}; Fig. 2b).

The main compatible solutes, glycerol and arabitol, as well as the main metabolite produced, ethanol, displayed similar behaviour (Fig. 2c–e). Indeed, for D=0.12 h^{-1}, the maxima for extracellular productions were recorded after 30 h of culture, namely concomitantly to the maximum for biomass concentration (Fig. 2a); then their concentrations decreased to negligible values at the end of culture. For D=0.06 h^{-1}, extracellular productions were at a maximum after about 50 h of culture, namely at the end of the short steady state, and then decreased. For D=0.03 h^{-1}, from the beginning of the steady state, extracellular productions decreased slightly and then remained constant or nearly constant, as was the case for glycerol production, until the end of culture.

Discussion

Figure 1 demonstrates the feasibility of the repeated batch process. Indeed, after total glucose depletion from the medium, cell decantation can be easily carried out and a new run can proceed after the addition of sterile medium. Glycerol concentration increased throughout the three runs to a final concentration of 42.5 mmol L^{-1}, since at the end of each batch, after harvesting of the supernatant, a significant part of the broth remained in the bioreactor (0.4 L). However, similar amounts of glycerol, the main compatible solute (Brown 1978), were produced during each run, and had to be related to the constant NaCl concentration (2 mol L^{-1}). The increase observed for the arabitol production after each repeated batch had to be related to the later accumulation of arabitol in the cell (unpublished results), if compared to the intracellular glycerol accumulation, which displayed a maximum at the end of growth (Djelal et al. 2005). Indeed, in addition to be a compatible solute resulting from osmotic adjustment (Bellinger and Larher 1991; Van Eck et al. 1989), arabitol is also often considered as a stationary state marker (Adler et al. 1985; Nobre and Da Costa 1985; Witter and Anderson 1985). The weak amounts of trehalose produced during each run (0.4% of carbon from the consumed glucose was converted into trehalose at best) can be related to the low implication of trehalose in the osmoregulation (Brown et al. 1986). Indeed, high salinity of culture media inhibited trehalose biosynthesis (Brown et al. 1986; El Mokadem et al. 1986).

Counterbalancing the increase observed in the production of arabitol, the production of metabolites, ethanol and acetic acid, which are not involved in the osmoregulation, decreased after each run (Fig. 1).

It should be noted that the behaviour recorded during the first run was in agreement with previous results recorded in similar culture conditions and at the same salinity but for 0.56 mol L^{-1} glucose (Djelal et al. 2005).

However, even if the feasibility of the repeated batch process has been demonstrated, its comparison to continuous

cultures, which were carried out in similar conditions, was in favour of the continuous culture. Indeed, the rates recorded at steady state for the lowest dilution rate (0.03 h^{-1}; Fig. 2) were significantly higher than those recorded in batch culture (Fig. 1): growth rate was 0.13 g L^{-1} h^{-1}, while only 0.04 g L^{-1} h^{-1} was recorded at best during the first batch of the repeated runs; extracellular production rates at steady state were 0.9, 0.14 and 6.8 mmol L^{-1} h^{-1} for glycerol, arabitol and ethanol, but only 0.2, 0.07 and 1.7 mmol L^{-1} h^{-1}, respectively, during repeated batch culture. In favour of the repeated batch mode, the almost total glucose depletion from the medium should, however, be noted, while the residual glucose concentration at steady state was close to 0.2 mol L^{-1}, namely half of the initial concentration (Fig. 2b). Lower residual glucose concentrations can most likely be obtained, but at the expense of a reduction of the dilution rate, leading to lower production rates.

It should be noted that the experimental ratio ethanol on glycerol produced at steady state for D=0.03 h^{-1}, namely 33 h mean residence time, was 7.8 (Fig. 2c, e), namely close to the ratio recorded after about 30 h of batch culture in similar conditions (7.9) (Djelal et al. 2005).

The relatively low cellular density can be considered as the limit of the continuous mode of culture, due to an inhibition of the "microbial biosynthesis" by the osmotic potential of the culture medium. Indeed, during continuous cultures of *Zygosaccharomyces rouxii* at dilution rates in the range 0.05–0.2 h^{-1}, the maximum biomass concentration at steady state, reached for the lowest dilution rate, was only 1.6 g L^{-1}, obtained in media at low water activity (0.96) induced by NaCl addition (Van Zyl and Prior 1990). Accounting for the low cellular density recorded during culture in media at low water activity, an important part of the assimilated glucose is used for polyols production and for energy supply, needed to maintain the required amount of intracellular polyols, which allows osmotic adjustment to be ensured (Djelal et al. 2005).

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