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ORIGINAL PAPER

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Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process

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Abstract Several bottlenecks in the alcoholic fermentation process must be overcome to reach a very high and competitive performance of bioethanol production by the yeast *Saccharomyces cerevisiae*. In this paper, a nutritional strategy is described that allowed *S. cerevisiae* to produce a final ethanol titre of 19% (v/v) ethanol in 45 h in a fed-batch culture at 30°C. This performance was achieved by implementing exponential feeding of vitamins throughout the fermentation process. In comparison to an initial addition of a vitamin cocktail, an increase in the amount of vitamins and an exponential vitamin feeding strategy improved the final ethanol titre from 126 g l^{-1} to 135 g l^{-1} and 147 g l^{-1}, respectively. A maximum instantaneous productivity of 9.5 g l^{-1} h^{-1} was reached in the best fermentation. These performances resulted from improvements in growth, the specific ethanol production rate, and the concentration of viable cells in response to the nutritional strategy.

Introduction

Ethanol is an important industrial chemical with emerging potential as a biofuel to replace vanishing fossil fuels (Von Sivers et al. 1994; Wheals et al. 1999). The most commonly used ethanol producer is *Saccharomyces cerevisiae* (Zaldivar et al. 2001). Usually, sugar concentrations above 20% (w/v) are not used under industrial conditions because increasing concentration of ethanol delays the growth of the yeast, which eventually leads to stopping of the fermentation (Strehaiano et al. 1978; Novak et al. 1981; Kalmokoff and Ingledew 1985; Mota et al. 1987). The critical concentration of ethanol at which yeast ceases to grow is influenced by several factors, which have been comprehensively reviewed (Casey and Ingledew 1985; Van Uden 1985; D'Amore and Stewart 1987; Jones 1989). Several authors have observed that yeast extract (Casey et al. 1983; Thomas and Ingledew 1992; Thomas et al. 1993; Jones and Ingledew 1994; Bafrncova et al. 1999), ammonium (Leao and Van Uden 1983; Jones and Ingledew 1994; Niessen et al. 2000), magnesium (Dombek and Ingram 1986; Ciesarova et al. 1996; Birch and Walker 2000), or calcium (Nabais et al. 1988) have a protective effect either on growth, fermentation, or 'viability', which overall stimulates the rate of ethanol production. By modifying nutritional conditions, it is possible to increase ethanol production by traditional fed-batch processes with *S. cerevisiae* up to 11 °Gay Lussac (°GL) (85.8 g l^{-1}) in 125 h (Panchal and Stewart 1981). A high ethanol tolerant yeast (a yeast derivative from cross-breeding between *Saccharomyces diastaticus* and *Saccharomyces uvarum*) produced up to 13.3 °GL (103.7 g l^{-1}) in 66 h (Krishnan et al. 1995) and *Saccharomyces sake* produced up to 20.8 °GL (162.2 g l^{-1}) in 20 days (Hayashida and Ohta 1981). Mota et al. (1987) have described a fed-batch process in which substrate feeding was controlled by the CO_2 production rate. With this approach, the final ethanol concentration achieved was 124 g l^{-1} in 300 h with an ethanol yield of 0.47 g g^{-1}. Thomas et al. (Thomas and Ingledew 1992; Thomas et al. 1993) described an efficient process called VHG (Very High Gravity) that leads to an ethanol titre as high as 20.4 °GL (159 g l^{-1}) in 175 h at 20°C. This process is based on the bioconversion of hydrolysed wheat supplemented with yeast extract by growing cells of *S. cerevisiae* under aerobiosis.

It is well known that yeast cells require vitamins such as meso-inositol (Vigie 1989; Winter et al. 1989), pantothenic acid (Taherzadeh et al. 1996) and biotin (Oura and Suomalainen 1978; Winter 1988; Pejin and Razmovski 1996) for growth. It has been reported that the assimilation and the storage of biotin conditioned the growth rate and the ethanol production of *S. cerevisiae* (Winter 1988). An initial concentration of 3–4 µg l^{-1} biotin was

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sufficient to obtain a maximum specific growth rate (Winter et al. 1989). The assimilation of biotin was effective at low ethanol concentrations and strongly inhibited at ethanol concentrations above 84 g l^{-1} (Winter 1988). It has been shown that the initial biotin added to the medium was totally assimilated within the first few hours of growth. The intracellular biotin was then distributed between mother and daughter cells by the division process. Thus, in order to maintain an optimal biotin concentration within the cells to reach high ethanol concentrations, it is preferable to carry out an exponential feeding of biotin based on the growth profile (Winter 1988). This procedure resulted in an improvement in ethanol production. In order to verify a similar effect of the other vitamins on ethanol production, the same nutritional strategy was considered by investigating the effect of (1) the amount and (2) the mode of feeding (exponential feeding vs an initial feeding) of other vitamins, such as pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, and para-aminobenzoic acid, on fed-batch *S. cerevisiae* cultures under aerobiosis.

Materials and methods

Microorganism, media and growth conditions

S. cerevisiae CBS8066 strain was supplied from the Centraal Bureau voor Schimmelcultures (The Netherlands). The strain was maintained on YPD [yeast extract 1% (w/v), bactopeptone 2% (w/v) and glucose 2% (w/v)] agar medium at 4°C. Pre-culture of yeast cells was carried out in a 5 ml tube of YPD rich medium containing NaCl 0.9% (w/v) at 30°C for 16 h on a rotary shaker (100 rpm). The culture was transferred to a 250 ml Erlenmeyer flask containing 50 ml mineral medium (pH 4) prepared as follows (all compounds are expressed in g l^{-1}): KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 3.0; $Na_2HPO_4 \cdot 12H_2O$, 3.0; sodium glutamate, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.04; $MnSO_4 \cdot H_2O$, 0.0038; $CoCl_2 \cdot 6H_2O$, 0.0005; $CuSO_4 \cdot 5H_2O$, 0.0009; $Na_2MoSO_4 \cdot 2H_2O$, 0.00006; $CaCl_2 \cdot 2H_2O$, 0.023; $(NH_4)_2Fe(SO_4)_6 \cdot 6H_2O$, 0.023; H_3BO_3, 0.003; pantothenate, 0.005; nicotinic acid, 0.005; meso-inositol, 0.125; thiamine, 0.005; pyridoxine, 0.005; para-aminobenzoic acid: 0.001, and biotin, 0.000012. Glucose was added at a final concentration of 40 g l^{-1}. After 10 h of growth at 30°C, the 50 ml culture was used to inoculate a 3 l Erlenmeyer flask containing 500 ml of mineral medium with vitamins as described above and incubated at 30°C for 10 h. Two flasks of the latter culture were used to inoculate 9 l of the same mineral medium in a 20 l bioreactor.

Fermentations

Three fed-batch experiments were performed in a 20-l fermentor using the Braun, Biostat E fermenting system. The temperature was regulated at 30°C, and the pH maintained at 4 by the addition of a 14% (v/v) NH_3 solution. The fermentor was connected to a computer. Software realiged the on-line acquisition of the controlled parameters (stirring rate, pH, temperature, partial pressure of dissolved oxygen, NH_3 and antifoam additions) and allowed the monitoring and the regulation of these parameters on-line. The pressure in the bioreactor was regulated at 0.2 bar (relative pressure). The fermentor was flushed continuously with air at a flow rate of 100 l h^{-1}, which corresponds to a maximum vvm of 0.2. The stirring rate was fixed at 400 rpm until the pO_2 reached 20%, then increased in order to avoid any oxygen limitation of the culture. The bioreactor was also equipped with a foam detection system and controlled addition of Struktol (Schill & Seilacher, Germany) antifoam. The maximum amount of Struktol added was 1.2 ml. The fermentor was supplied with a sterile feed using a peristaltic pump (Masterflex). Fermentation was started with a glucose concentration of 100 g l^{-1}. The glucose feed concentration was 700 g l^{-1}. The mass of glucose added in the fermentor was estimated instantly by weighing. The glucose concentration in the fermentor was automatically calculated by the software, based on all the inlet and outlet volumes (base addition, sampling, antifoam addition), the added mass, the density and the glucose concentration in the feeding solution measured by the DNS (3-5 dinitro-salicylic acid) method. The density of the feeding solution was measured by weighing a known volume. When the concentration of ethanol went above 90 g l^{-1}, glucose feeding of the fermentor was adjusted to bring the concentration up to 50 g l^{-1}.

Chemicals

Chemical products (glucose, salts, oligo-elements, orthophosphoric acid and NH_3) were obtained from Prolabo, vitamins were from Sigma and the sodium glutamate from Merck. All products were of the highest analytical grade available.

Feeding strategy

Vitamin feeding strategy

All fermentations were performed with an exponential feeding of biotin based on the growth profile (Fig. 1A). The total biotin concentration added was 32 µg l^{-1}. For the other vitamins, the following strategies were investigated: (1) an initial addition of a vitamin mixture at a concentration (α: concentration of vitamins except biotin) of 5 mg l^{-1} pantothenic acid, 5 mg l^{-1} nicotinic acid, 125 mg l^{-1} meso-inositol, 5 mg l^{-1} thiamine, 5 mg l^{-1} pyridoxine, and 1 mg l^{-1} para-aminobenzoic acid (fermentation A), (2) an initial addition at twice the former concentration (2.15α; fermentation B) and (3) a distribution of the vitamin mixture at a higher concentration (2.15α) via exponential feeding based on the growth profile (fermentation C) (Fig. 1B). The α value for the concentration was determined in order to avoid any growth limitation based on a final biomass concentration of about 20 g l^{-1} (Vigie 1989; Winter et al. 1989).

Glucose feeding strategy

Fermentation was started with a glucose concentration of 100 g l^{-1}. Whenever the residual glucose concentration was lower than 20 g l^{-1}, glucose feeding was carried out to restore a glucose concentration of 100 g l^{-1}. When the ethanol concentration was above 90 g l^{-1}, the glucose feeding brought the concentration back to 50 g l^{-1} (see Fig. 2). This set-point allowed minimisation of osmotic stress under conditions where the cells were already stressed by high ethanol concentrations.

Analytical methods

Yeast growth was evaluated by spectrophotometric measurements at 620 nm in a spectrophotometer (Hitachi U-1100) and calibrated against cell dry weight measurements. The spectrophotometric measurements enabled evaluation of doubling of the population during fermentation, and the generation time for the exponential addition of the vitamins during growth. Cells were harvested by filtration on 0.45-µm-pore-size polyamide membranes and dried to a constant weight at 60°C under a partial vacuum [200 mm Hg (ca. 26.7 kPa)]. A change of 1 U at OD_{620} was shown to be equivalent to 0.84 g of dry matter l^{-1}. The determination of glucose from fermentation supernatants was performed during fermentation with a glucose analyser (YSI model 27 A; Yellow Springs Instruments, Yellow Springs, Ohio). Concentrations of ethanol and acetic acid in the medium were determined by gas chromatography using a Pora-

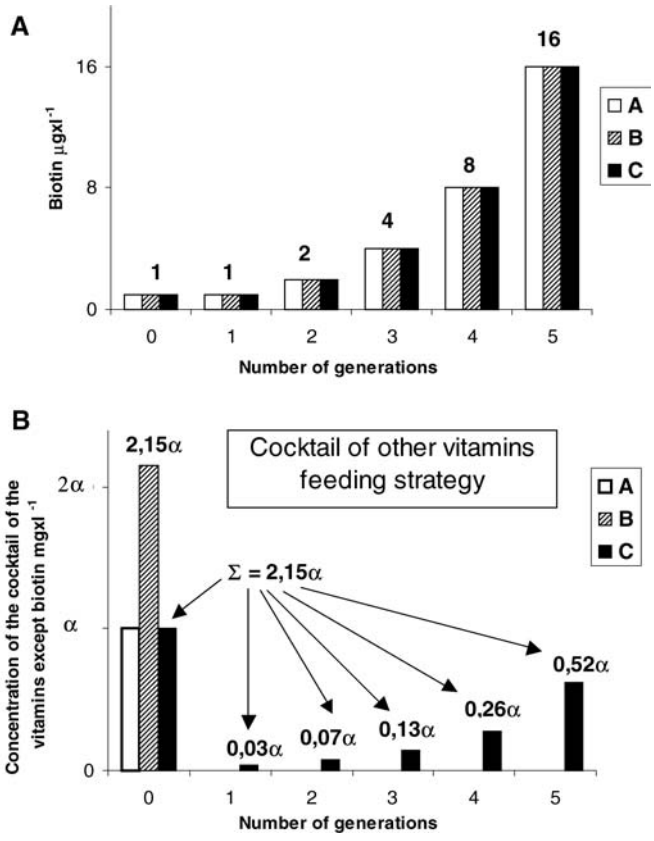

Fig. 1 Concentration of (**A**) biotin and (**B**) other vitamins except biotin added as a function of the number of generations for each of the three fed-batch fermentations *A*, *B* and *C*. The generation time was calculated by off-line measurements of optical density with a spectrophotometer to evaluate growth. α Concentration of vitamins except biotin (5 mg l^{-1} pantothenic acid, 5 mg l^{-1} nicotinic acid, 125 mg l^{-1} meso-inositol, 5 mg l^{-1} thiamine, 5 mg l^{-1} pyridoxine, 1 mg l^{-1} para-aminobenzoic acid)

plot Q column (25 m ×0.53 mm) with nitrogen as carrier gas and flame ionisation detection (Hewlett Packard, 5890 A) and the following conditions: an injection temperature of 250°C, initial oven temperature of 115°C to final temperature of 235°C at a rate of 12°C min^{-1} and an isotherm of 10 min, a flow rate of carrier gas of 20 ml min^{-1} and an injected volume of 50 nl. Determination of organic acids and glucose from fermentation supernatants was performed by HPLC using an Aminex HPX-87H+ column (300 mm ×7.8 mm) and the following conditions: a temperature of 50°C, with 5 mM H_2SO_4 as eluant (flow rate of 0.5 ml min^{-1}) and dual detection (refractometer and UV at 210 nm). The biomass formula was determined at ENSC (Toulouse, France) by elemental analysis of C, H, O, N and ashes. The biomass formula used to convert cell dry weights into molar carbon concentration was $C_1H_{1.79}O_{0.64}N_{0.16}$. The composition of the outlet gas (carbon dioxide, oxygen and nitrogen) was analysed by gas chromatography using a Porapak Q column followed by a molecular sieve of 5 Å maintained at 30°C with helium as carrier gas and catharometer detection (Intersmat, igc 120 mb). The calibration was done on air and on a standard gas composed of CO_2, O_2 and N_2. The true percentages of CO_2, O_2, N_2, the flow rates at the inlet and outlet of the fermentor, the number of moles of CO_2 formed, the number of moles of O_2 consumed, the percentage of saturation in water and the natural evaporation were calculated according to the measured percentages of CO_2, O_2, N_2, the existing temperature, humidity, volume of the outlet gas and the time of fermentation, using in-house developed software. Gas analysis was performed every 20 min during the fermentation.

Table 1 Effect of the amount of the vitamin mixture added and the mode of vitamin feeding (see Fig. 1) on biomass, ethanol production and by-product formation of *Saccharomyces cerevisiae* CBS 8066 during the fed-batch fermentations *A*, *B* and *C* at 30°C. μ_{max} Maximum specific growth rate, X_{max} maximum concentration of biomass, $Y_{X/S}$ yield factor of biomass on substrate, $Y_{P/S}$ yield factor of ethanol on substrate, *°GL* °Gay Lussac

Fermentation Feeding mode	A Initial	B Initial	C Exponential
Vitamin concentration	α	2.15α	2.15α
Fermentation time (h)	45	45	45
Final volume (l)	18	19.2	18.9
μ_{max} (h^{-1})	0.39	0.44	0.43
X_{max} (g l^{-1})	13	16	15
$Y_{X/S}$ (g g^{-1})	0.041	0.057	0.047
$Y_{P/S}$ (g g^{-1})	0.38	0.38	0.42
[Ethanol] final (g l^{-1})	126	132	147
[Ethanol] final (°GL)	16.2	16.9	18.9
[Glycerol] final (g l^{-1})	5	9	4
Average ethanol productivity (g $l^{-1}h^{-1}$)	2.8	3.0	3.3

Determination of cell 'viability'

The cellular 'viability' was determined by the methylene blue technique (Postgate 1967). A 200 µl sterile solution of methylene blue (0.3 mM in 68 mM Na_3 citrate) was mixed with 200 µl of a yeast suspension diluted to reach an $OD_{620\,nm}$ of 0.4–0.7. The mixture was shaken and, after 5 min incubation, placed in a Thomas's counting chamber. The number of stained (non-active cells) or unstained (active cells) and the number of buds were counted in five different fields with a total of at least 200–300 cells. The percentage of 'viable' cells (%MB 'viability') was the number of unstained cells (live cells) divided by the total number of cells (stained and unstained). Under the conditions used, the mean (m) viability was estimated with an accuracy of 10% (Nielsen et al. 1991), i.e. the interval m ±0.1m contains the true value of viability with a probability of 95%.

Results

Fed-batch fermentations

The mass of glucose consumed, and ethanol and biomass produced during the different fed-batch processes are shown in Fig. 2. It can be observed that all fermentations occurred in two phases: a first phase characterized by a concomitant growth and ethanol production and a second phase where growth stopped but cells kept producing ethanol.

Growth and metabolite production parameters are summarized for the three fed-batch processes in Table 1. Note that the final volumes are similar for the different fermentations, allowing direct comparison of metabolite concentrations. Thus, the dilution factor does not affect interpretation of the variation of the different parameters.

The major by-product detected in the broth was glycerol. Glycerol production occurred only during the growth phase and followed a linear relationship with the biomass produced (data not shown).

It can be observed that the initial addition of twice the concentration of vitamins (2.15α) led to a slight increase

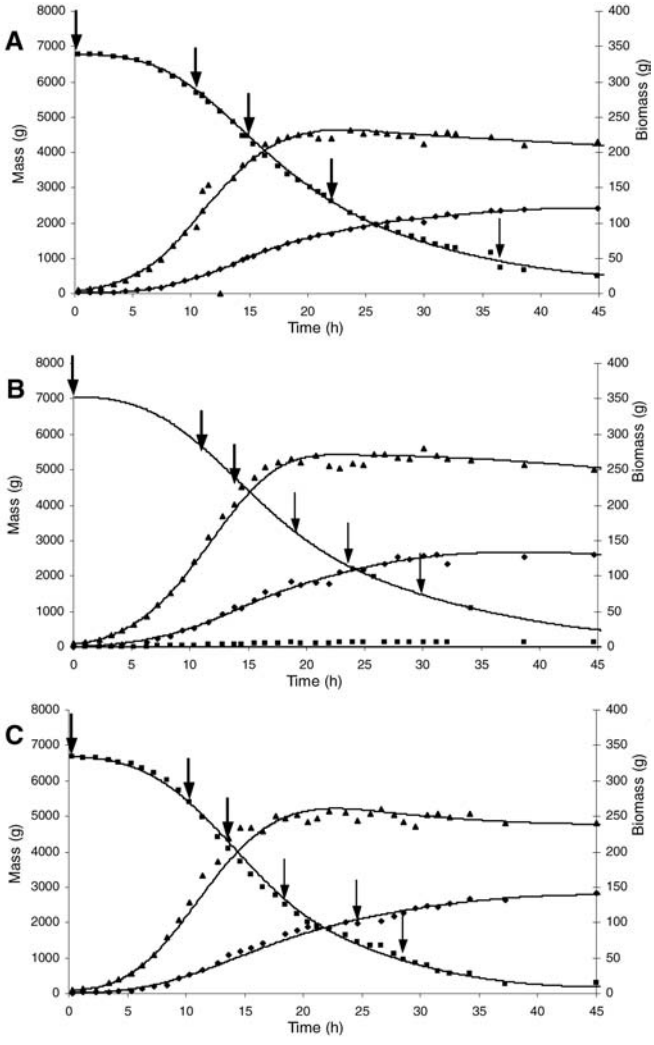

Fig. 2A–C Change in the mass of glucose (*squares*), ethanol (*diamonds*) and biomass (*triangles*) in fermentations *A*, *B* and *C*. *Thick arrows* represent the feeding of the fermentor with glucose to a final concentration of 100 g l^{-1} and *thin arrows* the feeding of the fermentor with glucose to a final concentration of 50 g l^{-1}

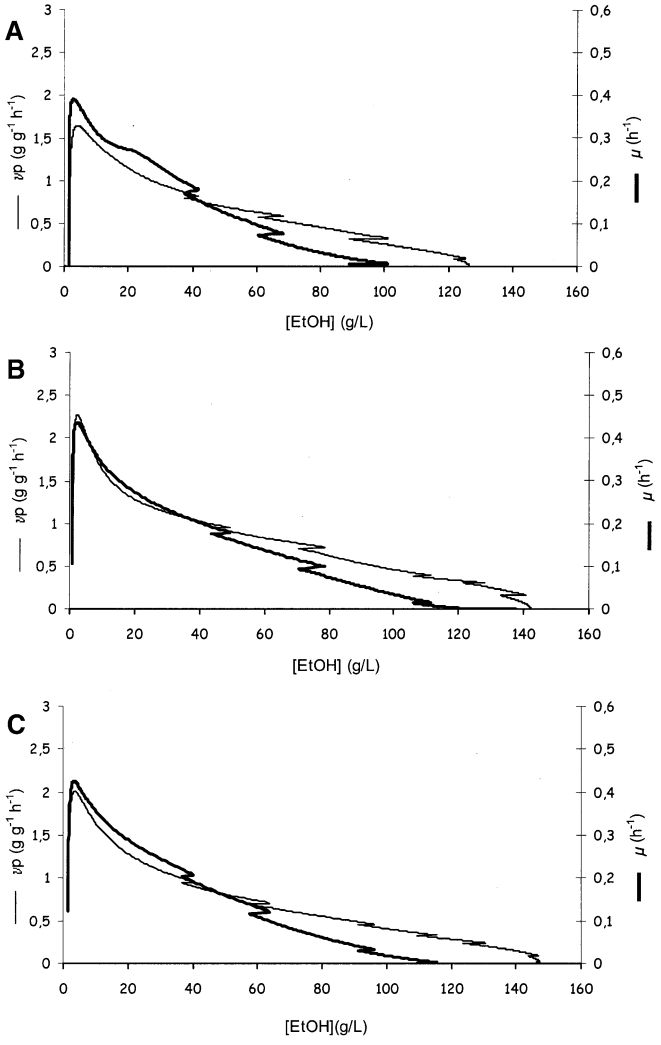

Fig. 3A–C Specific growth rate, μ, (*thick line*) and specific ethanol production rate (ν_p) (*thin line*) vs ethanol concentration for fermentations *A*, *B* and *C*

in the growth rate, a higher final biomass concentration and a higher glycerol concentration (cf. fermentations A and B), but an identical yield of ethanol production on glucose. The exponential feeding of vitamins improved the final ethanol titre (132 vs 147 g l^{-1}) and the yield of ethanol production on glucose ($Y_{P/S}$) reaching a value of 0.42 g g^{-1} (cf. fermentations B and C). Carbon and redox balances were calculated for each fermentation. All fermentations showed a small carbon balance deficit that ranged from 7.8% in fermentation A, 8.5% in fermentation B, to 6.9% in fermentation C. The redox balances were also similar, with deficits of 9.6% in fermentation A, 10% in fermentation B and 6.5% in fermentation C.

Impact of the vitamin feeding strategy on kinetic parameters

A comparison of the fed-batch fermentations was performed in terms of growth rate and ethanol production rate throughout the process. To this end, the specific growth rate (μ) and the specific ethanol production rate (ν_p, in g g^{-1} h^{-1}) were plotted versus ethanol concentration throughout fermentation. They both decreased gradually, probably due to inhibition by the ethanol accumulated during the process. This representation convincingly demonstrated that doubling the amount of vitamins improved both parameters, and affected the uncoupling between growth and ethanol production that is triggered at very high ethanol concentrations (cf. fermentations A and B in Fig. 3). No significant effect on either the growth rate or the uncoupling between growth

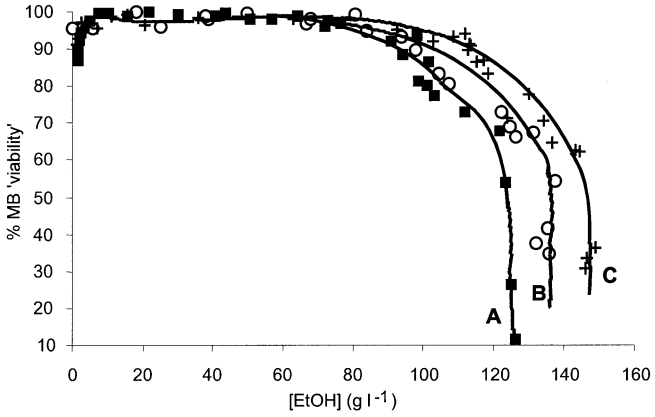

Fig. 4 Comparison of the percentage of methylene blue-stained cells (%MB 'viability') as a function of ethanol concentration in fermentations *A* (*squares*), *B* (*circles*) and *C* (*crosses*)

and ethanol production was seen when the same amount of vitamins was supplied in an exponential way as compared to an initial feeding (cf. fermentations B and C in Fig. 3). The specific production rate (ν_p) reached a maximum above 2 g g^{-1} h^{-1} and varied identically in both fermentations.

Differences were observed in the average ethanol productivity, which increased from 2.8 g l^{-1} h^{-1} (fermentation A) to 3.0 g l^{-1} h^{-1} (fermentation B) and 3.3 g l^{-1} h^{-1} (fermentation C) (Table 1). A maximum instantaneous ethanol productivity of about 9.5 g l^{-1} h^{-1} (1.21 °GL h^{-1}) was attained in fermentation C.

Impact of the vitamin feeding strategy on cell 'viability'

Cell viability was measured throughout the fermentations and the percentage of viable cells was plotted versus ethanol concentration (Fig. 4). Over 90% of viable cells was measured until the ethanol concentration reached a threshold value. Above this value the cell viability dropped to reach 10–30% of viable cells at the end of the fermentation. The threshold value of ethanol concentration was 90 g l^{-1} in fermentation A, 100 g l^{-1} in fermentation B and 115 g l^{-1} in fermentation C.

A dramatic drop in the viable population – from 90% to 10% – was observed in the reference fermentation A while the ethanol concentration increased from 90 g l^{-1} to 126 g l^{-1}. The effect of doubling the initial amount of vitamins avoided such a drastic decline in cell viability. As shown in Fig. 4, the percentage of viable cells remained above 50% for a concentration of ethanol up to 135 g l^{-1} (fermentation B). The exponential feeding of vitamins further improved the percentage of viable cells during the fermentation, since 70% of the population were still viable at 135 g l^{-1} ethanol (Fig. 4 fermentation C).

Discussion

In order to verify whether the amount of vitamins and the mode of vitamin feeding influenced the alcoholic performance of *S. cerevisiae*, as reported in the literature for biotin, experiments were carried out in fed-batch fermentation with various amounts of a vitamin mixture and two feeding strategies. The results presented here showed that both an increase in the amount of vitamins and an exponential feeding strategy improved the performance of the process (in terms of final ethanol titre, μ and ν_p) and the ethanol tolerance of the micro-organism.

In this study, the initial addition of a double dose of vitamins and the exponential feeding of these vitamins increased the final ethanol titre by 7% and 17%, respectively, compared to the reference fermentation (A). A final ethanol titre of 19% (v/v) was reached in 45 h at 30°C by fed-batch fermentation using an exponential vitamin feeding strategy on a synthetic defined medium. Our process is therefore highly competitive as regard to ethanolic performances reported in the literature. To the best of our knowledge, a comparable performance [18.2% (v/v) i.e. 142 g l^{-1}] was reached in 192 h at 30°C using VHG technology (Thomas et al. 1993). Also, 16.8% ethanol (v/v) in 100 h has been obtained with sake yeast (Hayashida and Ohta 1981) and 16.73% (v/v) in a multi-stage continuous fermentation system (Bayrock and Ingledew 2001). In sake brewing, the accumulation of ethanol can reach 20.8% (v/v) in 20 days at 20°C by supplementing the fermentation broth with proteolipid extracted from *Aspergillus oryzae* (Hayashida and Ohta 1981). An ethanol titre as high as 20.4% (v/v) has been obtained in 175 h at 20°C using VHG technology (Thomas and Ingledew 1992).

Our results showed an essential role of the vitamins in improving the kinetics of ethanol production of *S. cerevisiae*. Doubling the amount of vitamins, whatever the strategy used, increased the maximum specific rate of ethanol production by 34% (ν_{pmax} =2.2 vs 1.64 g g^{-1} h^{-1}) and the average ethanol productivity by 7%. An improvement of 18% on the productivity was reached with the exponential feeding strategy.

The specific rate variations related to the ethanol concentration pointed out the existence of an uncoupling between growth rate and ethanol production rate, and revealed the ability of non-growing yeast cells to sustain ethanol formation. This phenomenon occurred for higher ethanol concentrations when the amount of vitamins was doubled, no matter which feeding strategy was used.

It is known that loss of viability is a function of ethanol concentration (Casey and Ingledew 1985). It has already been observed that cell viability determined by methylene-blue staining decreased as the concentration of ethanol increased during *S. cerevisiae* fermentation (Thomas et al. 1993). In this study, it was clearly shown that the cell viability during ethanol fermentation is also affected by the vitamin feeding strategy. Both an increase in the amount of vitamins and exponential feeding

of the vitamins slowed down the decline in cell viability at high ethanol concentrations. As a result, a higher viable population was detected at higher ethanol concentrations: 10% at 126 g l^{-1} in fermentation A, 30% at 135 g l^{-1} in fermentation B, and 70% and 30% at 135 and 147 g l^{-1}, respectively, in fermentation C. This shift most likely accounted for the increased ethanol productivity and final titre that eventually reached 147 g l^{-1} (18.9 °GL) in 45 h.

To summarise, the vitamin feeding strategy implemented improved the ethanol performance of *S. cerevisiae* in terms of final titre and average productivity. By definition, the productivity is a function of the specific ethanol production rate and the concentration of viable cells. Doubling the amount of vitamins was shown to increased both parameters, while the exponential feeding of vitamins further improved the concentration of viable cells. As a result, a combination of both strategies led to a higher average productivity and final titre. The determinism of very high ethanol tolerance and adaptation of *S. cerevisiae* is under the control of bioavailability of these vitamins.

Further investigation of ethanol tolerance during fermentation processes will require the molecular elucidation of mechanisms that render yeast cells fed with vitamins less sensitive to high ethanol concentration, and capable of elevated ethanol productivity under non-growth conditions.

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