

Performance Fermentativa de uma Estirpe Recombinante de *Saccharomyces cerevisiae* consumidora de Lactose e Floculante

Fermentation Performance of a Recombinant Lactose-consuming Flocculating *Saccharomyces cerevisiae* Strain

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RESUMO

Recentemente, tem havido um interesse crescente em bioreactores que utilizam células imobilizadas ou floculantes em processos contínuos, para melhorar a produtividade dos bioprocessos. Uma das possíveis e promissoras aplicações de sistemas contínuos com células de levedura floculante é a bioremediação do soro do queijo, através da fermentação alcoólica da lactose.

O objectivo deste trabalho foi fazer uma análise cinética da fermentação alcoólica da lactose utilizando a estirpe NCYC869-A3/T1, uma estirpe recombinante de *S. cerevisiae* floculante que expressa os genes LAC4 (codifica a b-galactosidase) e LAC12 (permease da lactose) de *Kluyveromyces lactis*. As fermentações foram feitas numa coluna de bolhas de 600 mL, com diferentes concentrações iniciais de lactose.

A lactose foi consumida completamente em todas as fermentações. A taxa específica de crescimento máxima ampliou com o aumento da concentração inicial de lactose, atingindo o valor máximo para uma concentração inicial de lactose de 20 g L⁻¹ (tempo de duplicação de aproximadamente 2 h). Com concentrações iniciais de lactose mais elevadas, a taxa específica de crescimento diminuiu, indicando que o efeito da inibição pelo substrato se tornou significativo.

A concentração máxima de etanol produzido ampliou linearmente com o aumento da concentração inicial de lactose entre 5 e 200 g L⁻¹. Contudo, os rendimentos em etanol obtidos foram baixos (45-60% do valor teórico), provavelmente devido às elevadas taxas de arejamento utilizadas. Em fermentações realizadas em matrizes agitadas, em condições de micro-arejamento, a levedura foi incapaz de consumir completamente uma concentração inicial de lactose de 200 g L⁻¹, produzindo um máximo de 57 g L⁻¹ de etanol (aproximadamente a mesma concentração produzida no bioreactor a partir do consumo completo de 200 g L⁻¹ de lactose). Provavelmente, a levedura tem uma baixa tolerância ao etanol, e o etanol produzido inibiu a fermentação da lactose que restava.

A produtividade em etanol ampliou com o aumento da concentração inicial de lactose até 150 g L⁻¹ (1.23 g L⁻¹ h⁻¹). O aumento da concentração inicial de lactose para 200 g L⁻¹ conduziu a um ligeiro decréscimo da produtividade em etanol.

ABSTRACT

In recent years, there has been a growing interest in bioreactors utilizing immobilized or flocculating cells in continuous process in order to improve the bioprocess productivity. One of possible promising implementations of continuous flocculation yeast system is bioremediation of cheese whey by means of alcoholic fermentation of lactose.

The aim of this work was to carry out a kinetic analysis of alcoholic fermentation of lactose using strain NCYC869-A3/T1, a recombinant *Saccharomyces cerevisiae* flocculent strain expressing both the LAC4 (coding for b-galactosidase) and LAC12 (lactose permease) genes of *Kluyveromyces lactis*. Fermentations were performed in a 600 mL bubble column bioreactor, with different initial lactose concentrations.

The lactose was completely consumed in all the fermentations. The maximum specific growth rate was found to increase with initial lactose concentration, reaching its maximum at 20 g L⁻¹ initial lactose (doubling time of about 2 h). At higher initial lactose concentrations, specific growth rate decreased, indicating that the effect of substrate inhibition had become significant.

The maximum ethanol concentration produced increased linearly when the initial lactose concentration was increased between 5 and 200 g L⁻¹. However, the ethanol yields obtained were low (45-60% of the theoretical value), probably because of the high aeration rates used. In shake-flask fermentations, in conditions of micro aeration, the yeast was unable to completely consume 200 g L⁻¹ initial lactose, producing a maximum of 57 g L⁻¹ ethanol (which is about the same concentration produced in the bioreactor from complete consumption of 200 g L⁻¹ lactose). Probably, the yeast has low ethanol tolerance and the ethanol produced inhibits further lactose fermentation.

Ethanol productivity increased with increasing initial lactose concentration up to 150 g L⁻¹ (1.23 g L⁻¹ h⁻¹). Further increase in initial lactose to 200 g L⁻¹ led to a slight decrease in ethanol productivity.

PALAVRAS-CHAVE KEY WORDS

Fermentação; lactose; etanol; *S. cerevisiae* recombinante; soro do queijo.
Fermentation; lactose; ethanol; recombinant *S. cerevisiae*; cheese whey.

1. INTRODUCTION

Cheese whey is a by-product of dairy industries, which presents rather high pollutant characteristics and is produced in high amounts. Since cheese whey has a high content of protein (1% w/v) and lactose (5% w/v), it is appropriate to consider it as a source of added value compounds and not just as an effluent.

Separation of whey protein content is typically achieved by ultrafiltration, producing whey protein concentrates (WPC). When preparing WPC, a lactose rich fraction, called permeate, is obtained. The permeate, which remains a major pollutant, can be used to produce ethanol by fermentation of the lactose.

Recently, there has been a growing interest in the development of continuously operating high-productivity systems with flocculating yeast cells for the production of ethanol, using lactose from cheese whey as fermentation substrate.

Some microorganisms are natural lactose consumers, like the bacteria *Escherichia coli* or yeasts from the *Kluyveromyces* genus. Conversely, *Saccharomyces cerevisiae* strains do not have the ability to consume lactose. Most frequently, *S. cerevisiae* is the microorganism of choice for alcoholic fermentation. Thus, construction of recombinant *S. cerevisiae* strains with the ability to metabolize lactose has been addressed by several authors (COMPAGNO et al., 1995; DOMINGUES et al., 1999b; PORRO et al., 1992; RAMAKRISHNAN and HARTLEY, 1993; RUBIO-TEIXEIRA et al., 1998; SREEKRISHNA and DICKSON, 1985).

Here, we describe a kinetic analysis of alcoholic fermentation of lactose, using NCYC869-A3/T1 yeast strain, at different initial lactose concentrations. Additionally, the effect of high initial lactose concentration on fermentative performance of the yeast was studied. This yeast is a recombinant *S. cerevisiae* flocculent strain with the ability to express both the LAC4 (coding for *b*-galactosidase) and LAC12 (lactose permease) genes of *Kluyveromyces lactis*, which makes it able to consume lactose. This was the first recombinant *S. cerevisiae* strain found to ferment lactose in a way comparable to that of the existing lactose-fermenting yeasts (DOMINGUES et al., 1999b).

2. MATERIAL AND METHODS

2.1 Microorganism

A recombinant *S. cerevisiae* flocculent strain with the ability to express both the LAC4 (coding for beta-galactosidase) and LAC12 (coding for lactose permease) genes of *K. lactis* was used. This yeast is named NCYC869-A3/T1 and its construction has been described (DOMINGUES et al., 1999b). The plasmid KR1B-Lac4-1 (ATCC N^o. 40186, U.S. Patent N^o. 5,047,340 dated September 10, 1991) harboring the LAC4 and LAC12 genes of *K. lactis*, was co-transformed with a 9 kb plasmid YAC4 (Sigma

V-0758, Lot. 103H6770) linear fragment into the flocculent host strain *S. cerevisiae* NCYC869-A3, which is a uracil-deficient strain (LIMA et al., 1995). Four clones expressing *b*-galactosidase activity were obtained, and only two kept a stable Lac⁺ phenotype. From these two, one showed better fermentation and growth parameters on lactose; it was taken for further studies and named T1 (DOMINGUES et al., 1999b).

The plasmid KR1B-Lac4-1 remains autonomous in the recombinant strain. Under positive selective pressure conditions (lactose as sole carbon source), the plasmid stability is very high, since cells have to keep the plasmid in order to retain viability (DOMINGUES et al., 1999a).

2.2 Culture Media and Cultivation Conditions

Yeast was maintained at 4 °C on plates of a SSLactose medium containing (per litre) 5 g KH₂PO₄, 2 g (NH₄)₂SO₄, 0.4 g MgSO₄.7H₂O, 1 g yeast extract, 20 g lactose.

Fermentations were performed in yeast defined mineral medium (VERDUYN et al., 1992) containing (per litre) 5 g (NH₄)₂SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄.7H₂O. Trace elements were added using 1000 times concentrated solution. Final trace elements concentrations per litre were: EDTA, 15 mg; ZnSO₄.7H₂O, 4.5 mg; CoCl₂.6H₂O, 0.3 mg; MnCl₂.2H₂O, 0.84 mg; CuSO₄.5H₂O, 0.3 mg; CaCl₂.2H₂O, 4.5 mg; FeSO₄.7H₂O, 3 mg; Na₂MoO₄.2H₂O, 0.4 mg; H₃BO₃, 1 mg; KI, 0.1 mg. Filter-sterilized vitamins were added after heat sterilization of the medium, using 1000 times concentrated solution. Final vitamin concentrations per litre were: biotin, 0.05 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; inositol, 25 mg; thiamine HCl, 1 mg; pyridoxine HCl, 1 mg; para-aminobenzoic acid, 0.2 mg. Lactose was autoclaved separately and added after heat sterilization of the medium. Lactose concentrations (in g L⁻¹) were: 5, 10, 20, 50, 100, 150 and 200. For lactose concentrations above 10 g L⁻¹, the concentrations of trace elements and vitamins were doubled.

Fermentations were made in a 600 mL bubble column bioreactor with H/D ratio of 2.0. Fermentors were filled with 400 mL of defined mineral medium. The temperature and pH were maintained at 30 °C and 4.0, respectively. A high air flow rate of 1.0 vvm (adjusted by mass flow control) was applied using a sintered porous plate located at the bioreactor bottom, in order to obtain sufficient aeration and bulk mixing in the bioreactor. A magnetic stirrer was used to help air flowing to keep the entire reactor volume well-mixed.

For start-up the fermentations, cells were grown in a 100 mL Erlenmeyer flask filled with 40 mL of culture medium. After cultivation at 30 °C with 150 rpm agitation for 20-30 h, the cell suspension was aseptically pumped to the fermentor. Therefore, the initial volume of medium in the fermentor was 440 mL.

2.3 Analytical Methods

Cell growth was monitored by following the optical density at 600 nm (OD₆₀₀). A standard curve of optical density against cellular dry weight (DW) was previously constructed, and used to estimate the biomass concentration. Cellular dry

weight was determined by filtering 10 mL of yeast culture through a preweighed 0.45 mm filter and washing with 20 mL of water. The filter was dried overnight at 104 °C, cooled in a desiccator, and weighed.

Lactose and ethanol were quantified by HPLC analysis, using a Chrompack Organic Acids column. The column was eluted at 60 °C with 0.005 M H₂SO₄ at a flow rate of 0.6 mL min⁻¹. Detection was by means of a Jasco 830-RI refractive-index detector.

2.4 Gas Analysis

Exhaust gas was cooled in a condenser (4 °C), and O₂ and CO₂ concentrations were determined with a TanDem dual gas analyzer (Adaptive Biosystems, U.K.).

2.5 Determination of Physiological Parameters

Maximum specific growth rates (μ) were calculated from numerically smoothed experimental biomass concentration data (Boltzmann fit function), using the following definition:

$$\frac{1}{X} \frac{dX}{dt}$$

Microcal™ Origin® software (Northampton, U.S.A.) was used for numerical and differential calculation.

Biomass conversion yield (Y_{XS}) was calculated as the ratio between the final biomass concentration (X_f) and the initial lactose concentration (S_0). Ethanol conversion yield (Y_{PS}) was calculated as the ratio between the final ethanol concentration (P_f) and the initial lactose concentration. Lactose was completely consumed in all the fermentations; X_f and P_f were determined at "end time", defined as the time when lactose concentration dropped to zero, i. e., before ethanol respiration.

The % of theoretical ethanol conversion yield (η) was calculated as follows (considering that 0.538 g of ethanol are produced from fermentation of 1 g of lactose):

$$(\%) \frac{P_f}{S_0 \cdot 0.538} \cdot 100$$

The ethanol productivity (QP) was defined as the ratio between the final ethanol concentration (P_f) and the time taken to complete lactose consumption. It is worth noticing that the calculated productivity does not represent process productivity, since the time spent between consecutive batches is not taken into account.

3. RESULTS

Figure 1 shows the time course of lactose concentration in the fermentations. Lactose was completely consumed in all the fermentations. The time taken to complete

Figure 1 Evolution of lactose concentration (S) during batch fermentations in bubble column bioreactor with the following initial lactose concentrations, in g L⁻¹: 5 (■; A), 10 (●; B), 20 (▲; C), 50 (◇; D), 100 (□; E), 150 (○; F) and 200 (△; G).

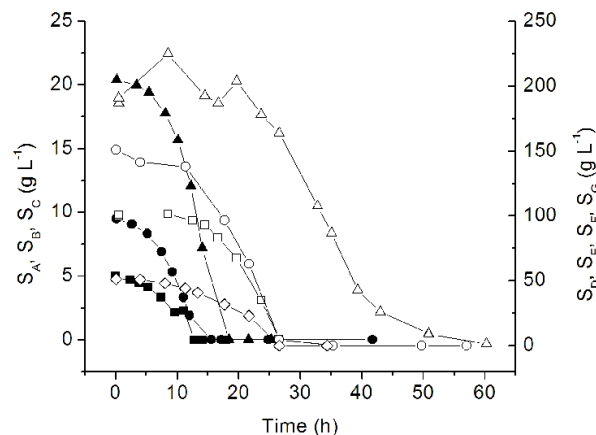
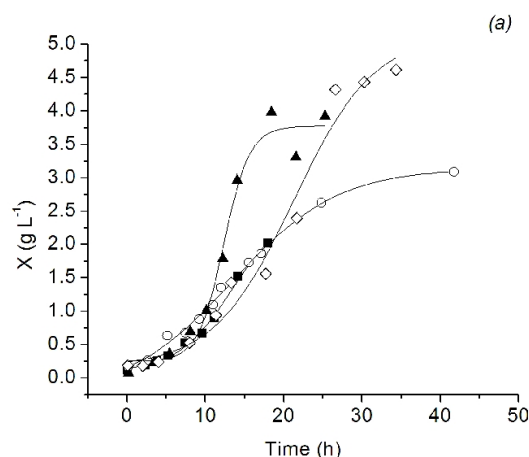


Figure 2 Evolution of biomass concentration (X) during batch fermentations in bubble column bioreactor. Initial lactose concentrations, in g L⁻¹, were: (a) 5 (■; A), 10 (○; B), 20 (▲; C), 50 (◇; D) and (b) 100 (□; E), 150 (●; F), 200 (△; G).



lactose consumption increased with the initial lactose concentration. The higher increase was observed between 150 g L⁻¹ (30 h, which was about the same time taken to consume 100 g L⁻¹) and 200 g L⁻¹ (60 h) initial lactose.

The final biomass concentration increased with the initial lactose concentration (figure 2). The higher biomass conversion yield (0.31 g g⁻¹) was obtained with 5 g L⁻¹ initial lactose. Biomass yield markedly decreased for 10 and 20 g L⁻¹ initial lactose (0.16 and 0.19 g g⁻¹, respectively). Further strong decrease (to about 0.08 g g⁻¹) was observed for initial lactose concentrations of 50 and 100 g L⁻¹, whereas there was a slight increase for initial lactose concentrations of 150 and 200 g L⁻¹ (table 1).

The lines correspond to the numerically smoothed experimental biomass concentration data (Boltzmann fit function). In some cases, the later points, which may correspond to growth in ethanol at the end of fermentation,

were not used in the numerical adjustment.

The maximum specific growth rate was found to increase with initial lactose concentration reaching its maximum at 20 g/L initial lactose (doubling time of about 2 h). At higher initial lactose concentrations, specific growth rate decreased (table 1), indicating that the effect of substrate inhibition had become significant.

The ethanol concentration produced increased with increasing initial lactose concentration (figure 3). Moreover, the maximum ethanol concentration obtained increased linearly with increasing initial lactose concentration (table 1, figure 4). The highest ethanol bioconversion yield about 60% of theoretical was obtained with initial lactose between 20 and 100 g L⁻¹, decreasing to 53 % for higher initial lactose concentrations (table 1).

Figure 3 Evolution of ethanol concentration (P) during batch fermentations in bubble column bioreactor with the following initial lactose concentrations, in g L⁻¹: 5 (■ ; A), 10 (● ; B), 20 (▲ ; C), 50 (◇ ; D), 100 (□ ; E), 150 (○ ; F) and 200 (△ ; G).

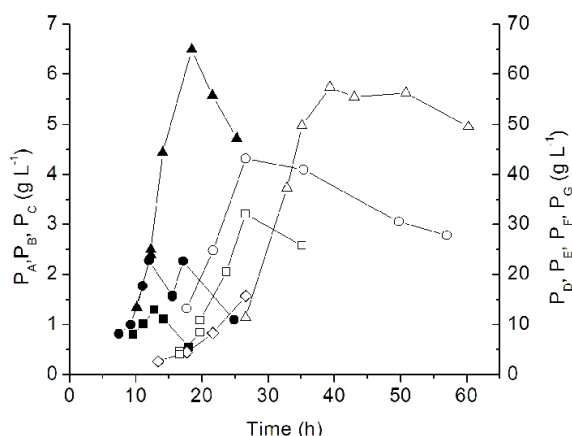


Figure 4 Variation of the maximum ethanol concentration (P_f) obtained in batch fermentations in bubble column bioreactor according to the initial lactose concentration.

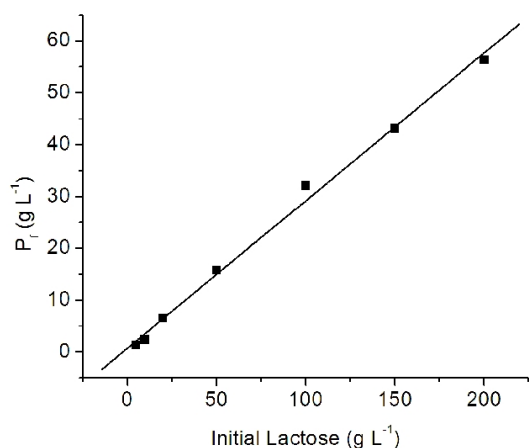


Table 1 Variation of fermentation parameters with the initial lactose concentration; 5 g L⁻¹ (A); 10 g L⁻¹ (B); 20 g L⁻¹ (C); 50 g L⁻¹ (D); 100 g L⁻¹ (E); 150 g L⁻¹ (F); 200 g L⁻¹ (G).

	A	B	C	D	E	F	G
Maximum specific growth rate (h ⁻¹)	0.20	0.34	0.36	0.14	0.19	0.18	0.16
Biomass conversion yield Y _{xs}	0.31	0.16	0.19	0.08	0.07	0.10	0.10
Final ethanol concentration P _f (g L ⁻¹)	1.3	2.3	6.5	15.7	32.1	43.2	56.3
Ethanol conversion yield Y _{ps}	0.26	0.24	0.32	0.31	0.32	0.29	0.28
Ethanol conversion yield n (%)	48	44	59	57	59	53	53
Ethanol productivity Q _p (g L ⁻¹ h ⁻¹)	0.10	0.14	0.36	0.58	0.97	1.23	0.94

4. DISCUSSION

The yeast used in this work was the first recombinant *S. cerevisiae* strain found to ferment lactose in a way comparable to that of the existing lactose-fermenting yeasts (DOMINGUES et al., 1999b). The efficiency of this strain was already tested in pilot-scale (1000 m³) continuous high cell density system feed with cheese whey permeate. In that system, the yeast consumed lactose completely with an ethanol bioconversion yield of about 80% of the theoretical value (KLEIN; unpublished results).

Here, we describe a kinetic analysis of alcoholic fermentation of lactose using NCYC869-A3/T1 yeast at different initial lactose concentrations (ranging from 5 to 200 g L⁻¹). This strain has never been tested in fermentations with initial lactose concentrations higher than 100 g L⁻¹.

When the initial lactose concentration is increased between 5 and 100 g L⁻¹, an increase in the ethanol bioconversion yield and a decrease in the biomass yield are observed. This is in agreement with the respiratory bottleneck model for *S. cerevisiae* (SONNLEITNER and KÄPPEL, 1986). This model was described for glucose degradation under conditions of aerobic ethanol formation. Although, in the present case, the substrate is lactose, the bottleneck model should hold, because lactose is hydrolysed intracellularly into glucose and galactose. The validity of the bottleneck model for this strain was already reported by DOMINGUES et al. (1999b), for the same initial lactose concentration range (5 - 100 g L⁻¹). However, when initial lactose was increased to 150 and 200 g L⁻¹, biomass conversion yield slightly increased and conversely ethanol yield decreased. This may indicate that ethanol formation is subject to product (ethanol) inhibition at high concentrations.

The low ethanol bioconversion yield obtained (45-60% of theoretical) may be related with the high aeration rate used in the fermentations. In fact, the dissolved oxygen in liquid phase never dropped to zero during the fermentations (data not shown). In continuous ethanol fermentation of lactose using the same strain, ethanol conversion yields as high as 80%

have been obtained (DOMINGUES et al., 1999a; DOMINGUES et al. 2001). Moreover, batch fermentation of two times concentrated cheese whey permeate (about 100 g L⁻¹ lactose) using NCYC869-A3/T1 resulted in a product with 53 g L⁻¹ ethanol, which means a bioconversion yield close to the theoretical value. In that fermentation an aeration rate of 0.1 vvm was used, which was 10 times lower than used in this work (DOMINGUES et al., 2001).

We attempted to do shake-flask fermentations in conditions of micro aeration: cultures were performed with 150 rpm agitation in 250-mL Erlenmeyer flasks filled with 100 mL of medium (for details on cultivation conditions see table 2). Potassium hydrogen phthalate was added to the medium at 100 mM final concentration, in order to avoid acidification (BLANK and SAUER, 2004). The initial pH was adjusted to 5.0 and the final pH was 4.2 ± 0.1. In these conditions, the yeast produced 48 g L⁻¹ ethanol from 114 g L⁻¹ initial lactose (ethanol yield was 78% of theoretical). Thus, low aeration led to a higher ethanol yield, as expected. In another shake-flask fermentation, with 217 g L⁻¹ initial lactose, the yeast only consumed about 140 g L⁻¹ lactose. After 48 h of cultivation, the ethanol concentration was 57 g L⁻¹ and the lactose residual was still 87 g L⁻¹ (ethanol yield at this point was 81% of theoretical). From this point on, ethanol concentration started to decrease, due either to evaporation or ethanol respiration, although there was still lactose in high concentration (table 2).

Table 2 Variation of lactose and ethanol concentrations during cultivation in shake-flasks with 100 g L⁻¹ (A) and 200 g L⁻¹ (B) initial lactose. Cultures were carried out at 30 °C by rotary shaker with 150 rpm agitation, in 100 mL of culture medium in 250 mL Erlenmeyer flasks. The flasks were inoculated with 1/10 volume of preinocula grown overnight (30 °C, 150 rpm) in the respective medium. Potassium hydrogen phthalate was added to the medium at 100 mM final concentration, in order to avoid acidification. The concentrations of trace elements and vitamins in the medium were triplicated. Moreover, 300 mL of 1000 times concentrated trace elements solution and 300 mL of 1000 times concentrated vitamins solution were added aseptically to cultivation B after 24 and 56 h.

	A			B					
Cultivation time (h)	0	24	26	0	24	48	56	78	150
Lactose (g L ⁻¹)	114	<0.5	<0.5	217	109	87	83	81	75
Ethanol (g L ⁻¹)	-	48	45	-	52	57	55	55	51

In shake flask micro aerated fermentations the yeast produced more ethanol (80% of theoretical yield). Additionally, in these conditions, the yeast was unable to consume 200 g L⁻¹ lactose, producing a maximum of 57 g L⁻¹ ethanol, which is about the same concentration that was produced in the bubble column bioreactor after complete consumption of 200 g L⁻¹ lactose. These observations lead us to believe that this is a critical ethanol concentration for this yeast strain. The strain may not tolerate higher ethanol concentrations.

In order to be able to produce higher ethanol concentrations with this strain we may need to improve its ethanol tolerance. This would involve the selection of mutants of this strain with higher ethanol tolerance, and that retain the

ability to ferment lactose at high rates.

The productivity increased with increasing initial lactose concentration until 150 g L⁻¹. Further increase in initial lactose to 200 g L⁻¹ led to a decrease in productivity, because the time taken to complete fermentation strongly increased. At 200 g L⁻¹ initial lactose there was a longer lag phase, before the yeast start growing exponentially and fermenting lactose.

5. CONCLUSIONS

In the batch fermentations in bubble column described here, the recombinant *S. cerevisiae* strain NCYC869-A3/T1 was able to completely consume as much as 200 g L⁻¹ of initial lactose concentration. The final biomass concentration increased with increasing initial lactose concentration. The maximum specific growth rate was found to increase with initial lactose concentration reaching its maximum at 20 g/L initial lactose (doubling time of about 2 h). At higher initial lactose concentrations, specific growth rate decreased, indicating that the effect of substrate inhibition had become significant.

The maximum ethanol concentration produced increased linearly when the initial lactose concentration was increased between 5 and 200 g L⁻¹. However, the ethanol yields obtained were low (45–60% of the theoretical value), probably because of the high aeration rates used. In shake-flask fermentations using buffered medium, in conditions of micro aeration, the yeast was unable to completely consume 200 g L⁻¹ initial lactose. In these conditions, the yeast produced a maximum of 57 g L⁻¹ ethanol (which is about the same concentration that was produced in the bubble column bioreactor from 200 g L⁻¹ lactose) in 48 h, after which the ethanol concentration started to decrease, although there was still a lactose residual higher than 80 g L⁻¹. Probably, the ethanol produced inhibits further lactose fermentation.

The highest ethanol productivity (1.23 g L⁻¹ h⁻¹) was obtained with 150 g L⁻¹ initial lactose. This productivity is higher than previously reported for batch fermentation of lactose based on metabolically engineered *S. cerevisiae* cells: 0.1–0.2 g L⁻¹ h⁻¹ (PORRO et al., 1992), 0.139–0.4 g L⁻¹ h⁻¹ (RAMAKRISHNAN and HARTLEY, 1993) and 1 g L⁻¹ h⁻¹ (COMPAGNO et al., 1995).

In order to be able to produce higher ethanol concentrations with this strain we may need to improve its ethanol tolerance.

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