Production of β-galactosidase from recombinant *Saccharomyces cerevisiae* grown on lactose

Lucília Domingues, Carla Oliveira, Inês Castro, Nelson Lima and José A Teixeira*
Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Abstract: Improved productivity and costs reduction in fermentation processes may be attained by using flocculating cell cultures. The production of extracellular heterologous β-galactosidase by recombinant flocculating *Saccharomyces cerevisiae* cells, expressing the *lacA* gene (coding for β-galactosidase) of *Aspergillus niger* under the *ADHI* promotor and terminator in a bioreactor was studied. The effects of lactose concentration and yeast extract concentration on β-galactosidase production in a semi-synthetic medium were analysed. The extracellular β-galactosidase activity increased linearly with increasing initial lactose concentrations ($5-150\,\mathrm{g\,dm^{-3}}$). β-Galactosidase production also increased with increased yeast extract concentration. During the entire fermentation, no accumulation of the hydrolysed sugars, glucose and galactose, was observed. The catabolic repression of the recombinant strain when cultured in a medium containing equal amounts of glucose and galactose was confirmed. In complete anaerobiosis, the fermentation of lactose resulted in a very slow fermentation pattern with lower levels of β-galactosidase activity. The bioreactor operation together with optimisation of culture conditions (lactose and yeast extract concentration) led to a 21-fold increase in the extracellular β-galactosidase activity produced when compared with preliminary Erlenmeyer fermentations.
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Keywords: β-galactosidase production; flocculent yeast; extracellular protein production; high-cell-density fermentation

INTRODUCTION

β-Galactosidase (EC 3.2.1.23) is able to cleave β-linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose. β-Galactosidase preparations are widely used for hydrolysis of lactose in milk, milk products, and whey.[1–3]

Acidic β-galactosidase produced from moulds is very stable and does not require metal ion cofactors for its action.[4] Recovery costs for this enzyme are primarily at the level of production and purification stages. The selection of an inexpensive and easily available substrate together with a suitable producer microorganism, optimisation of culture conditions, and effective downstream processing are essential to reduce the cost of enzyme preparation.[5]

We have previously reported the construction of a flocculent yeast strain secreting high levels of *Aspergillus niger* β-galactosidase.[6] The recombinant strain is an attractive alternative to other fungal β-galactosidase production systems as the enzyme is produced in a rather pure form. Moreover, due to the phenonemon of cell flocculation, the downstream

processing is simplified as cells are easily separated from the fermentation broth (and hence from the enzyme) by stopping the agitation and aeration at the end of the fermentation. Also, we have previously shown that cheese whey permeate could be used as substrate for β-galactosidase production by this recombinant strain,[6] decreasing production costs. Nevertheless, the fermentation characteristics of this recombinant strain should be determined in order to further improve its production. In this work, the fermentation properties of *S cerevisiae* NCYC869 cells transformed by pVK1.1 grown in lactose are presented and β-galactosidase production optimised.

MATERIALS AND METHODS
Microorganism

A recombinant *S cerevisiae* NCYC869-A3/pVK1.1 flocculent strain expressing the *lacA* gene (coding for β-galactosidase) of *Aspergillus niger* under *ADHI* promotor and terminator was used. The construction of the recombinant strain has been previously described.[6]

* Correspondence to: José A Teixeira, Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
E-mail: jateixeira@deb.uminho.pt

Culture media

The recombinant yeast was maintained at 4 °C on slants or at −80 °C in a permanent culture of YNB selective medium having the following composition: 6.7 g dm^{-3} yeast nitrogen base (without amino acids), 20 g dm^{-3} lactose. For fermentation SSLactose medium of the following composition was used: KH$_2$PO$_4$ 5 g dm^{-3}; (NH$_4$)$_2$SO$_4$ 2 g dm^{-3}; MgSO$_4$.7H$_2$O 0.4 g dm^{-3}; yeast extract 2.5 or 10 g dm^{-3}; lactose, glucose or galactose at concentrations ranging from 5 g dm^{-3} to 150 g dm^{-3}.

Bioreactor operation

Batch culture experiments were performed using a 2 dm^3 bioreactor (Braun, Biostat M) fitted with agitation and aeration control, temperature measurement and control, and pH measurement and control. The temperature was maintained at 30 °C, the pH at 4.0 by automatic addition of an ammonia solution, agitation speed was set at 150 rpm and the bioreactor was aerated with filtered air at a flow rate varying from 0.5 to 3 vvm (volume of air per volume of reactor per minute). Anaerobic conditions were attained by sparging with oxygen-free nitrogen at the rate of 0.5 vvm. The fermenter vessel and tubing were autoclaved for 30 min at 121 °C.

A 10 dm^3 bioreactor filled with 7 dm^3 SSlactose medium with 150 g dm^{-3} lactose and 15 g dm^{-3} yeast extract was also used. In these fermentations a set-point of 20% was fixed for the oxygen level. This value was kept constant automatically, throughout the fermentation, by changing the aeration rate from 1.4 to 2.9 vvm and the agitation speed from 150 rpm to 825 rpm.

Inocula, with a volume corresponding to 10% of fermentation broth used in batch and fed-batch fermentations, were prepared in Erlenmeyer flasks and incubated at 30 °C and 150 rpm.

β-Galactosidase activity measurements

The β-galactosidase activity in the culture medium was measured as the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG) as previously described.[7] Samples were incubated with 1.7 mmol dm^{-3} substrate in 0.075 mol dm^{-3} Na-acetate buffer, pH 4.5, for 10 min at 65 °C. The pH was raised to 10 with 1 mol dm^{-3} Na$_2$CO$_3$ and the activity was measured spectrophotometrically at 405 nm on a scanning multiwell spectrophotometer (SLT Spectra).[8] One unit of activity was defined as the amount of enzyme that hydrolysed 1 nmol *p*NPG min^{-1} at 65 °C.

Protein determination

The protein concentration was measured with a Coomassie Protein assay reagent (Pierce, Rockford, IL, USA), a method based on the dye-binding technique.[9]

Biomass determination

Biomass concentrations were measured as dry-weight (DW) and/or using absorbance methods. The DW was determined by filtering the sample through 0.2-μm filter-paper and then drying at 105 °C for 24 h. The absorbance was measured at 600 nm on a scanning multiwell spectrophotometer (SLT Spectra) and compared with a previously constructed standard curve for absorbance versus DW. Samples were treated with deflocculation solution (NaCl 1.5% (w/v), pH 3.0) before the absorbance was read.

Lactose, glucose, galactose and ethanol measurements

Total reducing sugar concentration was determined by the dinitrosalicylic acid method.[10] Lactose, glucose, galactose and ethanol concentrations were determined by HPLC (PL Hi-Plex Pb Column). The solvent used was ultrapure water, at a flow rate of 0.6 cm^3 min^{-1}, while detection was effected with a refractive index detector. Temperature was maintained at 80 °C.

Determination of β-galactosidase content in the fermentation broth

The fermentation broth was purified by ion-exchange chromatography. The FPLC Smart system from Pharmacia was used. The column Mono Q^R HR 5/5 prepacked with Mono Q (quaternary amino ethyl), a strong anion exchanger based on a beaded hydrophilic polymer, was equilibrated with 25 mmol dm^{-3} Tris−HCl buffer, pH 7.8. Proteins were eluted with a linear gradient from 0 to 1 mol dm^{-3} NaCl in 24 min at a flow rate of 1 cm^3 min^{-1} and 2 cm^3 fractions were collected.

RESULTS AND DISCUSSION

Lactose fermentation characterisation

Some common features were observed for all the fermentation conditions tested (different lactose and yeast extract concentrations). A lag phase of around 10 h was usually observed, after which lactose was rapidly metabolised, without significant accumulation of the hydrolysed sugars. The extracellular β-galactosidase concentration increased during the exponential and stationary phase, increasing further during the ethanol-consumption phase. In Fig 1 a typical fermentation time course for 100 g dm^{-3} initial lactose concentration is represented.

Using a stronger inoculum can reduce the lag phase, and significantly reduce the fermentation time. However, the yeast culture will always need sufficient time for β-galactosidase secretion and to initiate lactose hydrolysis. The inoculum is a determinant factor in the fermentation time. If an inoculum comprising yeast cells and culture supernatant is used, the enzyme present in the inoculum supernatant will initiate lactose hydrolysis, so reducing the lag phase. This effect will be more marked with greater β-galactosidase activity in the inoculum supernatant.
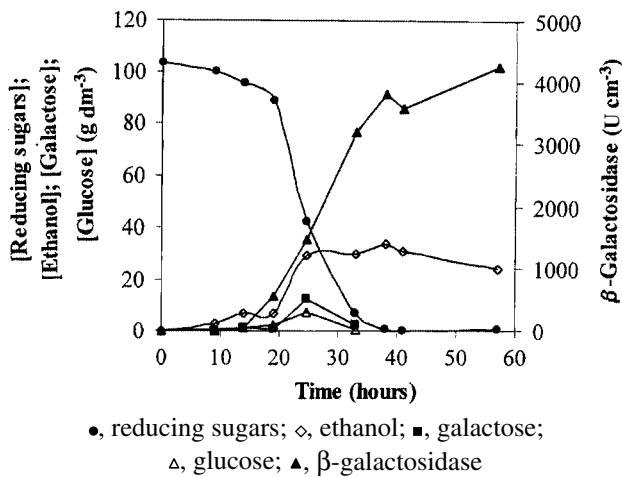
Figure 1. Fermentation time course for 100 g dm⁻³ initial lactose concentration.

•, reducing sugars; ◇, ethanol; ■, galactose; △, glucose; ▲, β-galactosidase

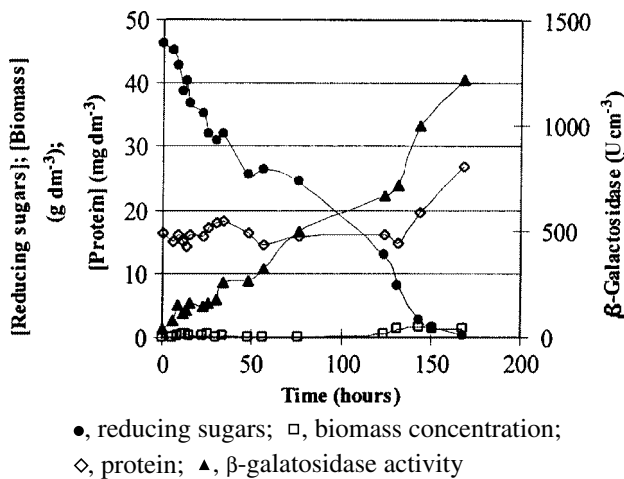•, reducing sugars; □, biomass concentration; ◇, protein; ▲, β-galatosidase activity

Figure 2. Anaerobic fermentation time profile for the recombinant strain *S cerevisiae* NCYC869-A3/pVK1.1 using lactose as carbon source.

Figure 3. Sugar and ethanol profile for the anaerobic fermentation.

•, lactose; △, glucose; ■, galactose; ◇, ethanol

△, glucose; o, galactose; ▲, β-galactosidase activity

Figure 4. Aerobic fermentation time profile for the recombinant strain *S cerevisiae* NCYC869-A3/pVK1.1 using glucose and galactose as carbon sources.

The promotor used may also explain the observed 10 h lag phase[11] as well as the increase in β-galactosidase activity during the ethanol-consumption phase.[12,13]

The results of anaerobic fermentation with the recombinant strain *S cerevisiae* NCYC869-A3/pVK1.1 are presented in Figs 2 and 3. In fermentation using nitrogen sparging, less β-galactosidase was produced than in aerated cultures, also the growth rate was less. In the absence of oxygen, the lactose metabolism (50 g dm⁻³) continued for 150 h, whereas in the presence of oxygen only 24 h were needed for total lactose consumption. Also, the accumulation of galactose was more pronounced in the absence of oxygen. The biomass concentration was much lower in the anaerobic fermentation, leading to the observed results. Ramakrishnan and Hartley[14] also observed slow growth in anaerobic conditions for the industrial strains transformed with pVK1.1. The yield of β-galactosidase per mass unit of biomass was, overall, slightly higher for the anaerobic fermentation.
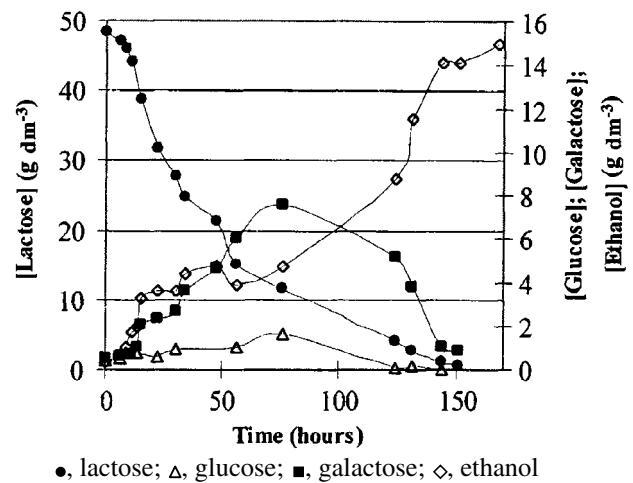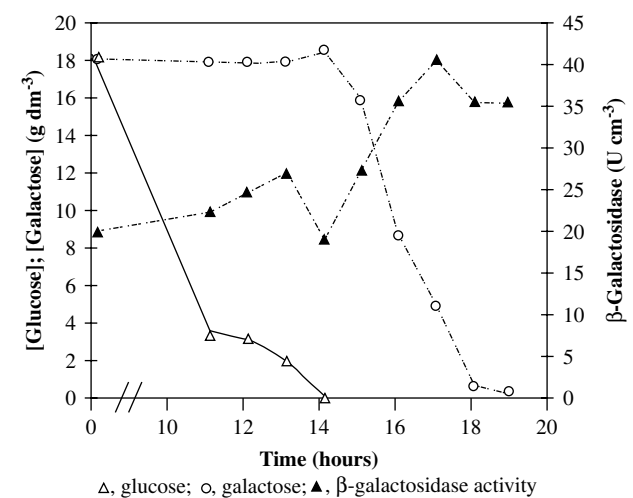
Fermentation on medium containing equal amounts of glucose and galactose

Independently of the experimental conditions, there was simultaneous metabolisation of the two hydrolysed sugars, glucose and galactose, in the Lac⁺ transformants secreting β-galactosidase. To confirm that the host yeast strain was not resistant to catabolic repression, an assay was conducted with the recombinant yeast strain *S cerevisiae* NCYC869-A3/pVK1.1 in semi-synthetic medium with glucose and galactose both at 20 g dm⁻³. As observed in Fig 4, the glucose was metabolised first whereas galactose was only metabolised after exhaustion of the glucose, confirming the diauxic behaviour of the host strain. However, pVK1.1 transformants grown on lactose did not exhibit any sign of catabolic repression. Ramakrishnan and Hartley[14] have determined the enzymatic activity of galactokinase in an industrial strain transformed with pVK1.1 and observed that the induction of this enzyme was 83% in a medium containing 10% lactose, against 100% for the induction observed with 2% galactose. The absence of catabolic repression

can be due to the low amount of glucose available in the culture medium.[14] Although the level of expression of the galactose pathway enzymes varies from one strain to another, for complete repression of galactose utilisation a minimum amount of glucose is required.[15] Below this threshold the galactose metabolic pathway operates to varying degrees depending upon the strain and the availability of galactose.[15] Our data corroborate the data obtained by Ramakrishnan and Hartley.[14] For the joint glucose and galactose fermentation, the lag phase for sugar metabolisation was not observed as the secreted enzyme was not needed for glucose metabolisation. However, in respect of the β-galactosidase secretion the 10 h lag phase was again observed, confirming the results obtained for lactose fermentation on the promotor characteristics described by Vainio.[11] Also, the amounts of β-galactosidase produced were considerably less than those obtained with lactose, despite the final biomass concentration being similar to that obtained with 50 g dm^{-3} lactose.

Effect of lactose concentration on β-galactosidase production

Different lactose concentrations (5, 10, 20, 50, 100, 150 g dm^{-3}) were tested as substrates for β-galactosidase production. In Fig 5 the effect of lactose concentration on extracellular β-galactosidase production is shown. β-Galactosidase production increased linearly ($R^2 = 0.996$) with increase in lactose concentration. However, at low lactose concentrations (5, 10 and 20 g dm^{-3}) the β-galactosidase produced was similar for the three lactose concentrations. As a consequence, the substrate conversion yield on β-galactosidase was maximum at 5 g dm^{-3} initial lactose concentration, decreased for 10 and 20 g dm^{-3} and stabilised at higher lactose concentrations.

Effect of yeast extract on extracellular β-galactosidase production

For the higher lactose concentrations (50, 100, 150 g dm^{-3}) three concentrations of yeast extract were tested (2, 5, 10 g dm^{-3}). Overall, β-galactosidase
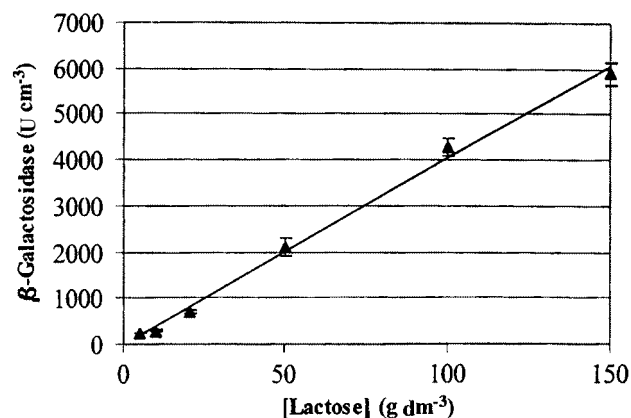
activity increased (from 1.7 to 2 times) with increase in yeast extract concentration. The increase in yeast extract concentration allowed for an increase in biomass concentration (1.1–1.8 times) due to more pronounced ethanol consumption. However, the increase in β-galactosidase activity was not proportional to the increase in biomass concentration. As the promotor used was the short *ADHI*, the ethanol-consumption phase also had a stimulatory effect on the promotor.[11,12] In conclusion, the increase in the yeast extract concentration gave higher extracellular β-galactosidase production. This was mainly due to the existence of a more pronounced ethanol-consumption phase, which in turn led to higher biomass concentration and to greater activation of the promotor. The stimulatory effect of yeast extract on biomass enzyme production has been observed by several authors for different organisms and enzymes, namely for the production of xylanases by *Aspergillus awamori*,[16] on growth of *Monascus purpureus*,[17] on luciferase activity by *Saccharomyces cerevisiae*[18] and β-galactosidase activity by *Streptococcus thermophilus*.[19] Table 1 shows that up to the point of sugar exhaustion the extracellular β-galactosidase activity profiles were very similar for the different yeast extract concentrations.

Effect of oxygen limitation on extracellular β-galactosidase production

In all the conditions tested, we observed low values of dissolved oxygen in the culture medium as fermentation proceeded (less than 10% of the saturation value). To ensure that this was not limiting the β-galactosidase production, fermentation was planned such that the dissolved oxygen was set to be always greater than 20% of the saturation value. These fermentation experiments were conducted in a 10 dm^3 bioreactor and to keep the dissolved oxygen above 20% of the saturation value, the aeration rate reached the maximum value of 2.9 vvm and the agitation speed 825 rpm. The main fermentation results are represented in Fig 6. When considering the sugar exhaustion point, it can be

Figure 5. Dependence of extracellular β-galactosidase activity on lactose concentration for aerobic fermentations.

Table 1. Effect of yeast extract concentration on extracellular β-galactosidase activity $\pm$ interval with 95% confidence, at the sugar exhaustion point

Lactose (g dm^{-3})	Yeast extract (g dm^{-3})	β-Galactosidase (U cm^{-3})
50	2	2108 $\pm$ 197
50	5	2206 $\pm$ 234
100	2	4269 $\pm$ 188
100	5	4875 $\pm$ 242
100	10	3979 $\pm$ 225
150	2	5894 $\pm$ 265
150	5	5617 $\pm$ 263
150	10	5867 $\pm$ 289
150	15	5652 $\pm$ 325

●, reducing sugars; ◇, ethanol; ▲, β-galactosidase activity
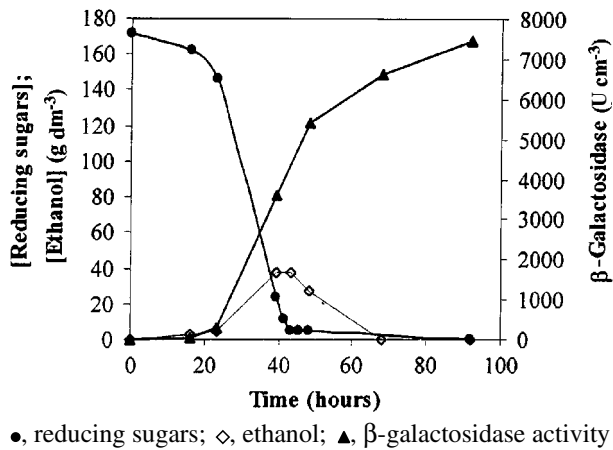
Figure 6. Reducing sugars, ethanol, and β-galactosidase activity profiles for batch operation with SSlactose medium, $150\,\mathrm{g\,dm^{-3}}$ lactose and $15\,\mathrm{g\,dm^{-3}}$ yeast extract; dissolved oxygen was kept above 20% of the saturation value.

observed (also from Table 1) that the extracellular β-galactosidase activity did not differ significantly from the other fermentations with the same initial lactose concentration. As expected the ethanol produced was less and after the ethanol-consumption phase an increase in β-galactosidase production was observed. The oxygen concentration did not significantly affect β-galactosidase production. The oxygen concentration should be sufficient to allow yeast growth but oxygen limitation had no effect on the final amount of β-galactosidase produced. If the oxygen was in excess, the same effect as that observed with yeast extract concentration was seen. These latter conditions allow for an ethanol-consumption phase, increasing further the β-galactosidase produced after the complete utilisation of sugar.

Plasmid stability

At the end of fermentation the proportion of cells secreting β-galactosidase was determined by counting the blue colonies on SSlactose-Xgal plates. For aerobic fermentations the percentage of β-galactosidase-secreting cells at the end of fermentation was greater than 70% while for anaerobic fermentations the value was slightly less. It was observed that the use of SSlactose inoculum slightly decreased plasmid stability, so inocula were made with YNB selective medium ($20\,\mathrm{g\,dm^{-3}}$ lactose). Plasmid stability data are presented in Table 2.

Table 2. Percentage of cells secreting β-galactosidase at the end of fermentation time

| Fermentation type		% Cells secreting β-galactosidase[a]
Aerobic	YNB selective medium inoculum	78 ± 8
	SSlact medium inoculum	68 ± 6
	Anaerobic	68 ± 2

[a] Mean value ± standard deviation.

Characterisation of fermentation broth

FPLC analysis of the fermentation broth indicated that β-galactosidase represents as much as 30% of total protein.

Fed-batch lactose fermentations

The fed-batch fermentations were undertaken to better characterise β-galactosidase production by using three different feed profiles with SSlactose medium containing in total $100\,\mathrm{g\,dm^{-3}}$ lactose and $10\,\mathrm{g\,dm^{-3}}$ yeast extract. Also, it was observed that the effect of substrate concentration on β-galactosidase yield was maximum at the lowest initial lactose concentration, which may indicate inhibition at higher substrate concentrations. The fed-batch operation mode would be advantageous in this case.

In Fig 7 the main fermentation results obtained with fed-batch operation with constant feed profile are represented. The bioreactor was fed at $0.2\,\mathrm{dm^3\,h^{-1}}$ with SSlactose medium containing $118\,\mathrm{g\,dm^{-3}}$ lactose and $11.8\,\mathrm{g\,dm^{-3}}$ yeast extract. The fermentation experiment was initiated as a batch operation in $300\,\mathrm{cm^3}$ containing $5\,\mathrm{g\,dm^{-3}}$ lactose. After sugar utilisation, the feed was initiated until a total volume of $1.7\,\mathrm{dm^3}$ fermentation broth was attained, followed by a further batch culture. The concentration of reducing sugars increased during the continuous operation phase, reaching a value of $50\,\mathrm{g\,dm^{-3}}$. Thereafter the concentration decreased in the continuous phase and the reducing sugars were totally consumed in the discontinuous phase. The extracellular β-galactosidase activity increased during the continuous phase, decreased during the discontinuous phase and increased again after total sugar utilisation. The β-galactosidase activity detected in the supernatant was $1900\,\mathrm{U\,cm^{-3}}$ at the end of the continuous phase, decreased to $1570\,\mathrm{U\,cm^{-3}}$, and reached $5050\,\mathrm{U\,cm^{-3}}$ $20\,\mathrm{h}$ after sugar exhaustion, which corresponds to the ethanol-consumption phase. In the batch fermentation, the β-galactosidase activity increased continuously until sugar exhaustion, reaching a value 2.5 times greater than that obtained with this feed
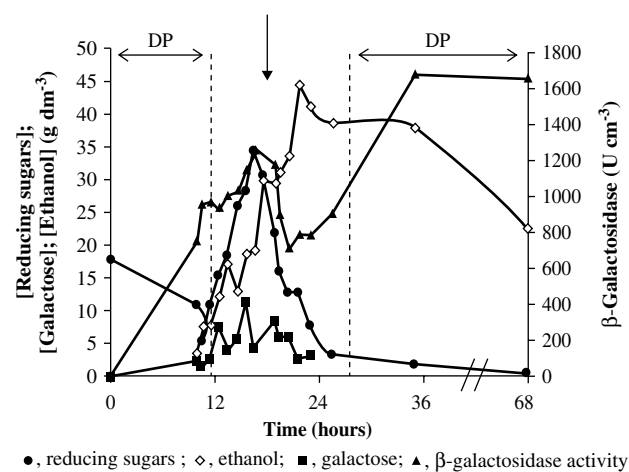

●, reducing sugars ; ◇, ethanol; ■, galactose; ▲, β-galactosidase activity
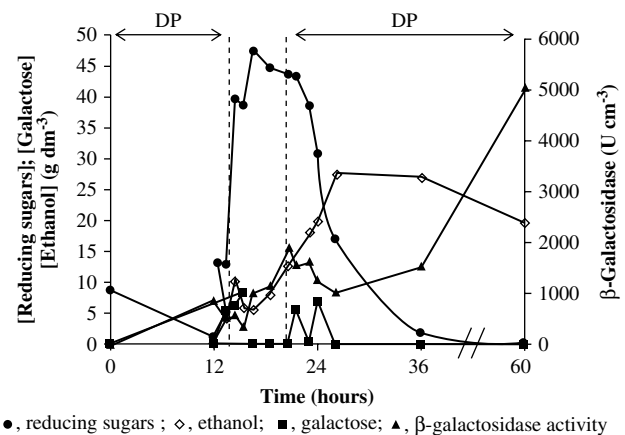
Figure 7. Reducing sugars, ethanol, galactose, and β-galactosidase activity profiles for fed-batch operation with constant feed rate; DP — discontinuous phase.

profile at the point of sugar exhaustion. For the fed-batch fermentation a slight increase in final cell concentration was observed ($30.4\,g\,dm^{-3}$ dry weight for fed-batch and $29.4\,g\,dm^{-3}$ dry weight for batch fermentation).
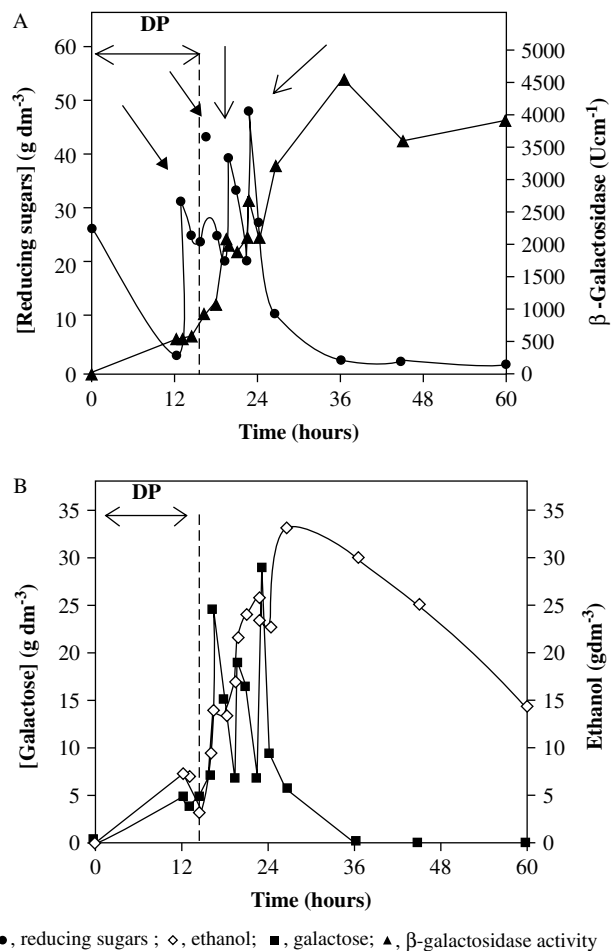
These data allow the conclusion that the fed-batch operation with constant feed rate is not advantageous for β-galactosidase production under the tested conditions.

The main fermentation data for fed-batch operation with exponential feed profile followed by a constant feed rate are given in Fig 8. In this fermentation, the discontinuous phase was initiated with $500\,cm^3$ culture volume with SSlactose medium with $20\,g\,dm^{-3}$ lactose and $2\,g\,dm^{-3}$ yeast extract. The exponential feed phase was initiated after sugar exhaustion ($t = 10.5\,h$). The exponential feed phase was maintained for 6 h after which a $139\,cm^3\,h^{-1}$ constant feed rate was used until a total volume of $1.7\,dm^3$ fermentation broth was attained (total fermentation time $\sim24\,h$). The reducing sugar concentration increased continuously during the exponential feed phase, reaching $35\,g\,dm^{-3}$. During the constant feed rate phase, the reducing sugars concentration decreased continuously, the reducing sugar concentration being $5\,g\,dm^{-3}$ when the feed stopped. The β-galactosidase activity increased during the exponential phase, reaching $1250\,U\,cm^{-3}$ and decreased during the constant feed rate. After the continuous phase, which coincides with sugar exhaustion and ethanol consumption, the β-galactosidase activity increased again, reaching $1675\,U\,cm^{-3}$. The final yeast cell concentration was similar to that obtained in batch fermentation, though slightly less ($28.7\,g\,dm^{-3}$ dry weight). The β-galactosidase production in the fermentation with an exponential feed rate was less than with a constant feed rate and also less than with batch fermentation.

As for batch fermentations, it was observed that the more pronounced β-galactosidase production

●, reducing sugars ; ◇, ethanol; ■, galactose; ▲, β-galactosidase activity

Figure 9. Reducing sugars and β-galactosidase activity (A); ethanol and galactose (B) profiles for fed-batch operation.
DP — Discontinuous phase; the arrow points indicate the addition of culture medium; black full arrows — addition of $200\,cm^3$; black line arrows — addition of $400\,cm^3$.

●, reducing sugars ; ◇, ethanol; ■, galactose; ▲, β-galactosidase activity

Figure 8. Reducing sugars, ethanol, galactose and β-galactosidase activity profiles for fed-batch operation with exponential feed rate followed by constant feed rate. DP — Discontinuous phase; the arrow point indicates the change from exponential to constant feed rate.

phase occurred during the phase of rapid reducing sugar metabolisation, a pulse feed profile was tested. The reason for adding fresh medium was to guarantee that β-galactosidase production would always be at its highest level. The instant of addition (~3-h intervals) was confirmed by evaluating the reducing sugars concentration. In Fig 9 the main fermentation results for fed-batch fermentation with pulse feed profile are represented. The fermentation experiment was initiated as a batch phase with SSlactose medium with $25\,g\,dm^{-3}$ ($12\,h$) after which $200\,cm^3$ of SSlactose medium containing $150\,g\,dm^{-3}$ lactose were added. After $3.5\,h$ a further $200\,cm^3$ were added, after a further $3.25\,h$ period $400\,cm^3$ were added, with $400\,cm^3$ added $3.1\,h$ after (the medium addition points are represented as arrows on Fig 9). The reducing sugar concentration showed an oscillatory profile, exhibiting a maximum value when medium was added and decreasing afterwards until each new addition of medium. The β-galactosidase activity increased continuously until sugar exhaustion, stabilising thereafter. The maximum β-galactosidase activity obtained in this experiment was $4515\,U\,cm^{-3}$ and the cell

concentration was slightly greater than for other experiments (32.7 g dm^{-3} dry weight). Considering the fermentation time to give sugar exhaustion, this latter feed profile gave β-galactosidase production comparable to the batch fermentation.

CONCLUSION

The results reported show the possibility of optimisation of the medium composition and growth conditions to achieve increased production and secretion of β-galactosidase activity by recombinant *S cerevisiae* cells. A 21-fold increase in extracellular β-galactosidase activity could be attained by bioreactor operation together with changing culture conditions (lactose and yeast extract concentration) compared with Erlenmeyer flask fermentations.[6]

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