FED-BATCH BIOREACTOR PROCESS WITH RECOMBINANT *Saccharomyces cerevisiae* GROWING ON CHEESE WHEY

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Abstract - *Saccharomyces cerevisiae* strain W303 was transformed with two yeast integrative plasmids containing Kluyveromyces lactis LAC4 and LAC12 genes that codify β-galactosidase and lactose permease respectively. The BLR030 recombinant strain was selected due to its growth and β-galactosidase production capacity. Different culture media based on deproteinized cheese whey (DCW) were tested and the best composition (containing DCW, supplemented with yeast extract 1 %, and peptone 3 % (w/v)) was chosen for bioreactor experiments. Batch, and fed-batch cultures with linear ascending feeding for 25 (FB25), 35 (FB35), and 50 (FB50) hours, were performed. FB35 and FB50 produced the highest β-galactosidase specific activities (around 1,800 U/g cells), and also the best productivities (180 U/L.h). Results show the potential use of fed-batch cultures of recombinant *S. cerevisiae* on industrial applications using supplemented whey as substrate.
Keywords: Recombinant *Saccharomyces cerevisiae*; β-galactosidase; Cheese-whey; Fed-batch cultivation.

INTRODUCTION

Recombinant technology applied to micro-organisms, which are able to grow in large-scale bioreactors, can be used to obtain large quantities of protein, otherwise difficult or impossible to obtain. *Saccharomyces cerevisiae*, as a host micro-organism for the expression of heterologous proteins, has been particularly attractive for this purpose. This yeast is generally recognized as a safe micro-organism (GRAS), lacking endotoxins and lytic viruses, being able to perform many post-translational modifications, including glycosylation, acylation and folding of proteins (Alberghina et al., 1991). *S. cerevisiae* has been used as a host micro-organism to produce different heterologous proteins such as β-galactosidase (Cheng et al., 1997), glucoamylase

(Hardjito et al., 1993), α-2,6(N)-sialyltransferase (Borsig et al., 1995), antithrombotic hirudin (Kim et al., 2003), sporamin (Chen et al., 2003), Closterium sex pheromone (Sekimoto, 2002), cutinase (Calado et al., 2002), Rhizopus oryzae lipase (Ueda et al., 2002), capsid proteins of human polyomaviruses BK and JC (Hale et al., 2002), human interleukin-18 (Lim et al., 2002), α_1-antitrypsin (Tamer and Chisti, 2001), human XPA and XRCC1 DNA repair proteins (Pushnova et al., 2001), Aspergillus ficuum endoinulinase (Park et al., 2001).

Some studies for the production of recombinant protein by *S. cerevisiae* report the use of fed-batch cultivations to obtain higher productivity. Different feeding methodologies have been tried, such as constant feeding (Hardjito et al., 1993; Belem and Lee, 1999) or exponential feeding (Gombert and

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Kilikian, 1997; Shin et al., 1998) in order to obtain constant specific growth rates. Other studies rely on sophisticated systems, requiring fine instrumentation, such as on-line glucose and ethanol concentrations control (Alberghina et al., 1991; Horn et al., 1996). Other methodologies have been successfully developed based upon pH controlled feeding (Porro et al., 1991); dissolved oxygen controlled feeding for Kluyveromyces marxianus (Barberis and Segovia, 1997); off-line glucose measure (Patkar and Seo, 1992); and dissolved oxygen measure with the MSUR (maximum substrate uptake rate) for *S. cerevisiae* and *K. marxianus* (Oh et al., 1998, Nor et al., 2001).

Cheese whey, which is a by-product of cheese making, represents 85-95 % of the original milk volume and retains 55 % of the milk nutrients, mainly lactose (4.5-5 % (w/v)) and proteins (0.6-0.8 % (w/v)). However, since it contains low concentration of solids (6-7 % (w/v)), cheese whey usually is considered an effluent. Despite the fact that many technologies have been developed for cheese whey utilization, nearly half of worldwide cheese-whey production is still discarded as effluents (González-Siso, 1996). According to the USDA, the Brazilian cheese-whey production is increasing annually, reaching more than 550,000 tons in 2002 (USDA–NASS, 2002), generating approximately 4 millions tons of cheese-whey.

The fed-batch cultures of *S. cerevisiae* described so far by the literature, besides sophisticated controls, use defined or selective media, mainly with glucose as the carbon source. This work aimed to develop simple linear strategies of feeding for fed-batch cultures of recombinant *S. cerevisiae,* using an undefined culture medium based on supplemented cheese whey as carbon source.

MATERIALS AND METHODS

Chemicals

Except where otherwise indicated, all chemicals used were of analytical grade, bought from Sigma (St. Louis, USA) or Merck (Darmstadt, Germany).

Yeast Strain

S. cerevisiae W303 (Mata ade2-1 his3-11 leu2-3,112 ura3-1 trp1) was kindly provided by Prof.

JAP Henriques (Biotechnology Centre, UFRGS, Porto Alegre, Brazil) and maintained on agar-plates containing YPD medium (Saffi, et al., 2001).

Plasmids

Plasmids pMR4 and pMR11 were kindly provided by Dr. J Polaina (Instituto de Agroquímica y Tecnología de Alimentos, Valencia, Spain). These plasmids contain leu2-d gene, a rDNA locus sequence and the genes LAC4 and LAC12 under the control of a CYC-GAL promoter. The genes LAC4 and LAC12 code β-galactosidase and lactose-permease of Kluyveromyces lactis respectively (Rubio-Teixeira et al., 2000).

Yeast Transformation

Strain W303 was co-transformed with plasmids pMR4 and pMR11 by high efficiency lithium-acetate technique (Agatep et al., 1998) and incubated on lactose SD-agar plates at 30 $^{\circ}$C for 5 days.

Culture Media

Selective media were made up of lactose, glucose or maltose SD-agar plates supplemented with amino acids (lactose, glucose or galactose 20 g/L, yeast nitrogen base without amino acids 6.7 g/L, adenine 20 mg/L, histidine 20 mg/L, tryptophan 20 mg/L, uracil 20 mg/L. Complex media were YPD (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) or YPL (lactose 40 g/L, peptone 20 g/L, yeast extract 10 g/L).

To obtain deproteinized cheese whey (DCW), 70 g/L or 210 g/L (for concentrated feeding medium) of cheese whey powder (Elegê Laticínios S.A., Brazil) were dissolved in water and the pH adjusted to 4.5 with pure phosphoric acid. This solution was boiled during 10 min for protein precipitation and centrifuged at 15,000 g for 10 min. The supernatant was then filtered. The permeate, which is the DCW, contained about 50 g/L or 150g/L of lactose respectively. Supplementation studies were also carried out by the addition of 10 g/L of yeast extract and 10 (M1), 20 (M2) or 30 g/L (M3) of peptone. The pH of media were always adjusted to 6.0 with potassium hydroxide. For the bioreactor cultivations, 0.5 mL/L of antifoam polyoxyethylene-polyoxypropylene copolymers (Mazu DF 800 S, Mazer Chemicals Ltd., England) was added to the final culture medium.

Bioreactor Cultivations

Bioreactor experiments were performed in a 3 L stirred tank bioreactor (Biostat B, B.Braun Biotech International, Germany), filled with 1.5 L of culture medium. Culture conditions were 30 °C, 500 rpm, and aeration of 3 L air/min, previously described as the best growth conditions for the strain used in this work (Rech et al., 1999). Bioreactor was inoculated with 100 mL of a 30 hours pre-culture in YPL.

In the fed-batch cultures, feedings of 25, 35 or 50 hours started after 24 hours of batch stage. The feeding followed the equation F = at, where F is the feeding (mL/min), a is the feeding constant coefficient (mL/min²) and t is feeding time (min). The calculated coefficients for feedings of 25, 35 or 50 hours were, respectively, $a_{25h} = 8.889 \times 10^{-4}$ mL/min²; $a_{35h} = 4.535 \times 10^{-4}$ mL/min²; and $a_{50h} = 2.222 \times 10^{-4}$ mL/min². A total of 1000 mL of culture medium was fed.

Yields of biomass ($Y_{X/S}$), ethanol ($Y_{EtOH/S}$), and enzyme productivity (Q_P) were calculated as described previously (Sinclair and Cantero, 1990).

Enzyme Activity Assay

β-galactosidase assay was carried out in cell-free extracts, using ONPG (o-nitrophenol-β-D-galactopyranoside) as substrate, and performed as described by Rech et al., 1999. A unit of enzyme activity was defined as the amount of enzyme required to liberate one μmol of ONP (o-nitrophenol) in one minute at the assay conditions.

Biomass Concentration

Cell concentration was spectrophotometrically determined at λ = 600 nm (Hitachi U-1100 spectrophotometer, Hitachi, Japan), and co-relating the readings to biomass dry weight with a calibration curve. The cells were harvested at 16,000 g for 3 min and washed twice with cold distilled water.

Lactose Concentration

Lactose was measured as total sugars, determined by a phenol sulphuric acid method as previously described (Rech et al., 1999).

Ethanol Assay

Ethanol concentration was determined by gas chromatography (CG-14B Shimadzu, Japan) properly set for this assay. To quantify the amount of ethanol, a calibration curve was constructed by adding a solution of cheese-whey (70 g/L), varying ethanol concentrations, and using a fixed amount of n-propanol as internal standard.

RESULTS AND DISCUSSION

Construction and Selection of a *S. cerevisiae* Strain that Produces β-Galactosidase

S. cerevisiae W303 was co-transformed with the SmaI linearized plasmids pMR4 and pMR11. After incubation at 30 °C for 5 days, 39 colonies were isolated from lactose SD-agar plates and sequentially denominated BLR001 to BLR039. These colonies were tested on SD-agar plates containing glucose, maltose or lactose as carbon source. Only 25 colonies maintained the capacity to grow on lactose as the sole carbon source (BLR007 and BLR008, BLR012 to BLR019, BLR021, BLR023 and BLR024, BLR026 to BLR031, BLR033 to BLR036, BLR038 and BLR039). These 25 positive transformants were grown on YPL and the production of β-galactosidase was quantified, using *S. cerevisiae* W303 (the parental strain) and K. marxianus CBS5665 as the negative and positive controls, respectively. Results represented in Figure 1a showed that strains BLR013, BLR015, BLR028, BLR035 and BLR036 presented poor growth, similar to the wild parental strain W303. The other strains had a similar growth profile, reaching around 10 OD units, compared to K. marxianus that has grown to 25 OD units. Results for β-galactosidase activity (Figure 1b) show that strains BLR014, BLR029, BLR030, BLR033, BLR038 and BLR039 presented very high enzyme production, reaching up to four times that for K. marxianus CBS6556. Strain BLR030 was finally chosen for bioreactor work.
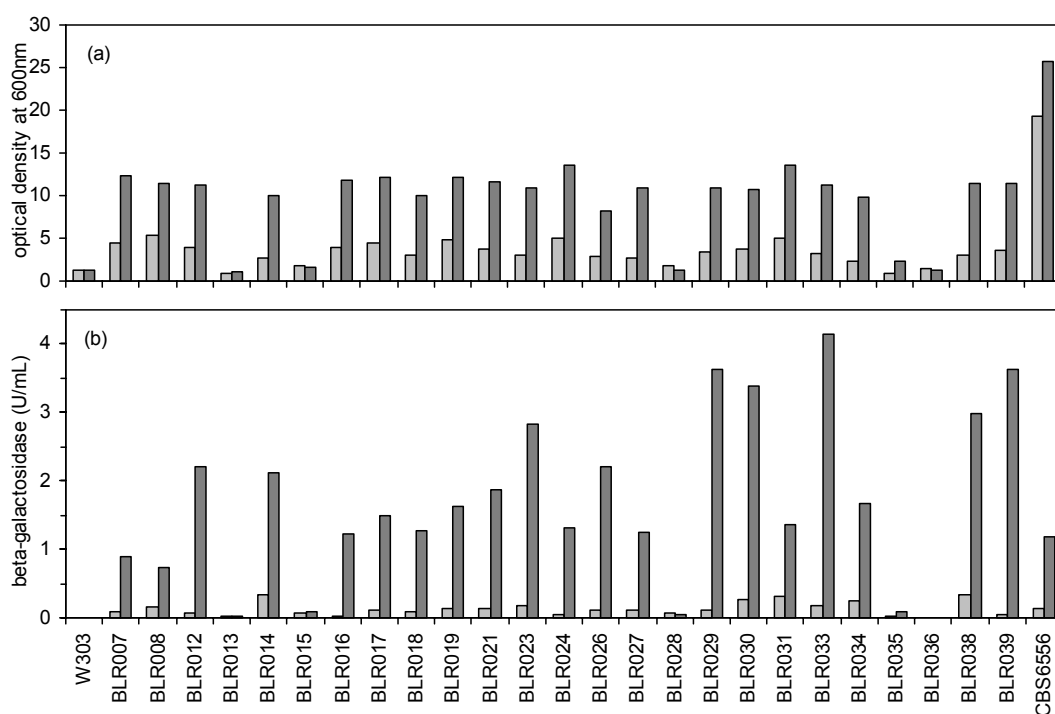
Figure 1: Growth (a) and β-galactosidase activity (b) of recombinant *S. cerevisiae* strains (BLR) compared with the wild strain (W303) and with K. marxianus CBS6556 in YPL at 24 hours (▣) and 48 hours (■) of cultivation.

Selection of Culture Medium for Fed-Batch Bioreactor Cultures

These experiments were aimed to determine an efficient culture medium, based on DCW, for the growth of the recombinant *S. cerevisiae* BLR030 strain, and the production of β-galactosidase. Batch cultures with DCW containing about 50 g/L of lactose were supplemented with 10 g/L of yeast extract and 10 (M1), 20 (M2) or 30 g/L (M3) of peptone. Biomass and β-galactosidase activity were measured at 24 and 48 hours, as presented in Table 1. This set of experiments was necessary to establish the amount of nitrogen source needed to support growth and enzyme production. Results have shown that medium M3 is the best composition for biomass and β-galactosidase production, thus being chosen for bioreactor experiments.

Table 1: Effect of medium composition on biomass formation and volumetric β-galactosidase activity of *S. cerevisiae* BLR030 growing on shake flasks. Data represent the mean of two experiments.

	Biomass (g/L)			β-gal (U/mL)		
time	M1	M2	M3	M1	M2	M3
24h	1.12	1.23	1.31	1.17	1.53	1.87
48h	2.47	3.40	4.17	2.36	2.86	8.34

Bioreactor Cultures

In order to study the influence of the feeding time on fed-batch cultivations of *S. cerevisiae* BLR030, fed-batch cultures with linear ascending feeding profile of 25 (FB25), 35 (FB35), and 50 (FB50) hours were performed and the results were compared to the batch cultivation. Culture and feeding media were based on DCW enriched with yeast extract and peptone. Results are presented in Figures 2 (a), (b) and (c). Table 2 compares the values obtained for biomass and product yields of all cultures. Low biomass yields ($Y_{X/S}$) were obtained for batch and FB25. However, for 30 and 50 hours feedings, good

biomass formation was obtained. Similar results were observed by Ramakrishnan and Hartley (1993) who grew aerobically *S. cerevisiae* GRF167 in lactose. Domingues et al. (1999) obtained small $Y_{X/S}$ values in cultures of recombinant *S. cerevisiae* T1 growing in lactose minimal medium, suggesting that this medium composition does not make for a suitable medium to obtain high cell density cultures. Whenever achieved, high cell density cultures of *S. cerevisiae* always used complex defined media, with glucose or glucose and galactose combinations as carbon sources and some kind of sophisticated strategy to control substrate feeding according to the variations of cells metabolism during the fed-batch phase (Alberghina et al., 1991, Porro et al., 1991, Oh et al., 1998), however, these systems are based on expensive hardware and costly media.

Surprisingly, despite the strong aeration during all cultivations (oxygen concentration never felt below 50 % saturation), a small amount of ethanol was always formed, about 10 g/L (results not shown), indicating that, even under slow growth control, *S. cerevisiae* BLR030 was showing catabolic repression by lactose, a fermentative sugar that will trigger the Crabtree effect.

β-galactosidase specific activity, shown in Figure 2 (b), increased during the first hours after the feeding starts for all feeding strategies, between 30 and 50 hours of run, while cell growth was nearly absent (Figure 2 (a)), showing that the feeding with lactose induced β-galactosidase activity of cells. Similar results were obtained for K. marxianus CBS6556 in

our previous work (Rech et al., 1999), suggesting that the mechanisms for enzyme production was similar for the recombinant strains of S. cerevisiae. The maximum specific activities were obtained at 49 hours of cultivation in FB35 and FB50 runs, with 1,778U/g cell and 1,934U/g cell, respectively. This specific activity is 2.8 times higher than that obtained in fed-batch cultures of K. marxianus CBS6556 using cheese-whey as culture medium and twice as high as obtained by Rubio-Teixeira et al. (1998) with *S. cerevisiae* MRY276, an haploid recombinant strain, but when they crossed MRY276 with S288C, a wild strain of S. cerevisiae, the specific β-galactosidase activity of the diploid strain raised 4 folds. These experiments were carried out in batch cultures using defined complex medium containing lactose as the carbon source instead of cheese-whey, as is the case in our work. β-galactosidase volumetric activities shown in Figure 2 (c), and the productivity values Q_P (Table 2) confirms that there are no important differences between feeding for 35 or 50 hours: 180 and 185U/L.h, respectively. Although volumetric activities were similar to those obtained for K. marxianus growing under the same feeding strategies and medium, the productivities are about 35 % lower (Rech et al., 1999). This fact can be explained by the lower growth rates of the recombinant *S. cerevisiae* obtained in this work, consequently imposing longer cultivation times. However, it is interesting to note that *S. cerevisiae* and K. marxianus batch cultivations have similar productivities: 120 and 118U/L.h, respectively.
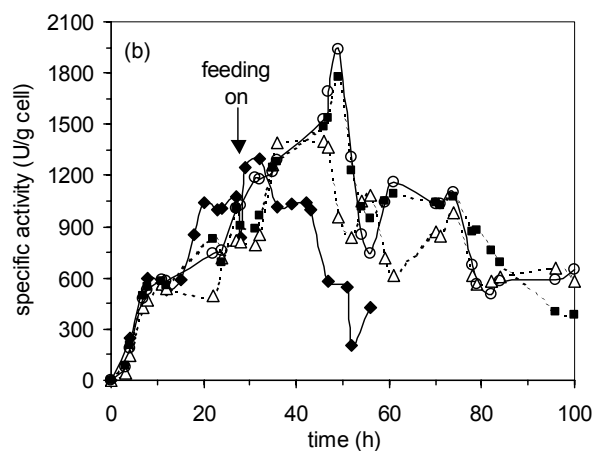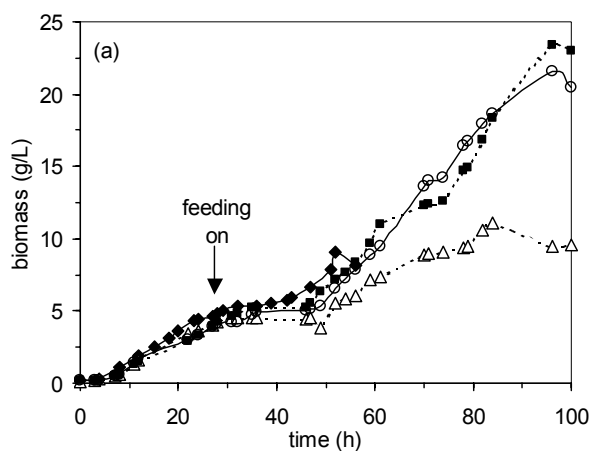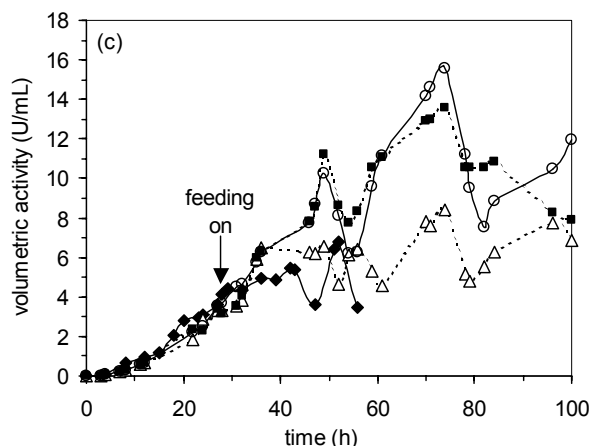
Figure 2: Time course for batch (—◆—) and fed-batch bioreactor cultivation of recombinant *S. cerevisiae*
BLR030 with linear ascending feeding profile for 25 hours (··△··), 35 hours (—○—) and 50 hours (··■··).
(a) biomass; (b) β-galactosidase specific activity; (c) β-galactosidase volumetric activity.
(b) Experiments are the mean of three cultivations.

**Table 2: Yields and productivities for batch and fed batch cultivations of *S. cerevisiae* BLR030 growing on
shake flasks. Data represent the mean of two experiments.**

	Batch	**FB25**	**FB35**	**FB50**
$Y_{X/S}$	0.18	0.16	0.23	0.26
$Y_{EtOH/S}$		0.05	0.05	0.06
Q_P (U/(L.h))	120	125	180	185

CONCLUSIONS

The construction of recombinant strains of *S. cerevisiae* as host for heterologous protein expression has been widely used in research and starts now being used in industrial processes. The insertion of LAC4 and LAC12 into the genome of S. cerevisiae, under the control of a strong promoter such as the one used in this work (CYC-GAL) is a promising strategy to allow the use of this well known and safe yeast in cultures based on lactose, a sugar that is not normally metabolised by S. cerevisiae. In this way, large amounts of cheese whey, a by-product of dairy industry, can then be used as an inexpensive carbon source for cell cultivation in order to reduce costs of enzyme productions, although some supplementation with yeast extracts and peptone might be necessary. Moreover, we developed a simple feeding strategy, not relying on sophisticated control systems. The results showed promising perspectives for further developments, if we consider that higher volumetric and specific enzyme activities were obtained when compared to cultures of K. marxianus growing under the same conditions and medium.

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