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Utilization of protein-hydrolyzed cheese whey for production of β-galactosidase by *Kluyveromyces marxianus*

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We studied the utilization of protein-hydrolyzed sweet cheese whey as a medium for the production of β-galactosidase by the yeasts *Kluyveromyces marxianus* CBS 712 and CBS 6556. The conditions for growth were determined in shake cultures. The best growth occurred at pH 5.5 and 37°C. Strain CBS 6556 grew in cheese whey *in natura*, while strain CBS 712 needed cheese whey supplemented with yeast extract. Each yeast was grown in a bioreactor under these conditions. The strains produced equivalent amounts of β-galactosidase. To optimize the process, strain CBS 6556 was grown in concentrated cheese whey, resulting in a higher β-galactosidase production. The β-galactosidase produced by strain CBS 6556 produced maximum activity at 37°C, and had low stability at room temperature (30°C) as well as at a storage temperature of 4°C. At −4°C and −18°C, the enzyme maintained its activity for over 9 weeks.

Keywords: cheese whey; β-galactosidase; *Kluyveromyces marxianus*

Introduction

By the year 2000, chemical, agricultural, and food products obtained by biosynthesis will have risen from the 1990 market of US$275 million to around US$17 billion. Both microorganisms and mammalian cells are used to produce a variety of products such as insulin, many antibiotics, and polymers. It is expected that, in the future, living cells will produce a number of organic chemicals currently derived from petroleum. The advantages of bioconversion are, among others, the mild reaction conditions (temperature and pressure), and high yields [4].

The cheesemaking industry generates large quantities of cheese whey (about 83% by volume of total milk utilized). This product contains about 50% of the total solids of original milk [15]. Due to its high level of organic substances, mostly lactose (70% of the total solids), cheese whey imposes a high level of biochemical oxygen demand (BOD) at effluent treatment plants, about 30 000–60 000 mg L^{-1}, depending on the cheesemaking process utilized. In comparison, 5000 liters of cheese whey are equivalent to the treated effluent of 2000 persons [13]. It is estimated that cheese whey worldwide production would be about 4.0×10^7 tons per year [17], of which 3.15×10^6 tons would be generated in Brazil [13].

In this work we investigated the use of protein-hydrolyzed sweet cheese whey as a culture medium to produce β-galactosidase (lactase), the enzyme used in reduced lactose milk production, an outstanding industrial product used by a large lactose-intolerant population [9,12].

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Materials and methods

All chemicals were of analytical grade.

Yeast strains

Two strains of *Kluyveromyces marxianus*, CBS 712 and CBS 6556, obtained from Centraalbüreau vor Schimmel-Cultures and provided by The Center for Biotechnology Development, Joinville, Brazil were used. The strains were maintained on agar slants at 4°C, as previously described [5].

Media composition

The basic medium used was made up with either 70 g L^{-1} or 210 g L^{-1} of reconstituted sweet cheese whey powder (composition: 71% lactose, 11.1% soluble protein, 0.7% fat, 3% humidity, 7.2% ashes; Elegê Laticínios SA, Lageado, RS, Brazil); yeast extract to give a final concentration of 10 g L^{-1} of soluble protein (produced in our laboratory as described below); and urea (10 g L^{-1}, Synth, Rio de Janeiro, Brazil). For growth in a bench bioreactor, 1 ml L^{-1} of antifoam polyoxyethylene-polyoxypropylene co-polymers (Mazu DF 800 S, Mazer Chemicals Ltd, Manchester, UK) was used.

To avoid protein precipitation during the sterilization process (121°C, 15 min), cheese whey proteins were hydrolyzed with a commercial protease (Alcalase 2.4 L, 1 ml L^{-1}, Novo Nordisk A/S, Copenhagen, Denmark), at pH 8.5 and at 55°C for 3 h.

Preparation of yeast extract

The yeast extract used in this work was obtained as follows: 100 g L^{-1} of dry *Saccharomyces cerevisiae* (Fleischmann Royal, Rio de Janeiro, Brazil) was suspended in phosphate buffer, pH 7.0, and the suspension was held at 55°C for 24 h. The suspension was centrifuged for 20 min at $3400 \times g$ and the supernatant constituted the yeast extract,

which was characterized by soluble protein analysis, carried out as described by Révillion and co-workers [14].

Pre-inoculum

Isolated yeast colonies were aseptically transferred to a 300-ml shake flask containing 50 ml of culture medium, and incubated on an orbital shaker at 200 rpm for 15–20 h. The culture medium and growth conditions used for the pre-inoculum were the same as described below.

Growth in orbital shaker

The experiments carried out on an orbital shaker were run to establish the best growth conditions (pH, temperature and culture medium) for both strains. Cultures were grown in 300-ml flasks containing 50 ml of medium. The flasks were inoculated with the necessary pre-inoculum volume to give an initial cell concentration between 0.05 and 0.1 g L^{-1}. The cultures were incubated at 200 rpm for 24 h. After 5, 10 and 24 h of cultivation, pH, cell concentration (g L^{-1}), and total sugar concentration (g L^{-1}) were measured. All experiments were done in duplicate.

Bioreactor cultivation

Growth in a bioreactor was followed to analyze growth kinetics for each strain under the conditions previously selected, the specific growth rate (μ), the doubling time (t_d) and the yields ($Y_{P/X}$,$Y_{P/S}$,$Y_{X/S}$). The experiments were performed in a 2-L stirred tank bioreactor (New Brunswick Scientific Co, New Brunswick, NJ, USA), filled with 1.5 L of culture medium, agitation speed set at 500 rpm and an airflow rate of 3 L min^{-1}.

Cell concentration

The cell concentration was estimated by measuring absorbancy in a spectrophotometer at 620 nm, and relating the readings to biomass dry weight with a calibration curve. The cells were harvested at $16000 \times g$ for 3 min and washed twice with phosphate buffer.

Lactose concentration

The lactose concentration was determined by the phenol-sulfuric acid method described by Barale [2].

Enzyme activity assay

The assays were carried out at 30°C, utilizing ONPG (*o*-nitrophenol-β-d-galactopyranoside) as substrate, and performed as described by Lederberg [10].

Ethanol assay

The ethanol concentration was determined by gas chromatography (Varian Star 3400 CX, USA) with a 50-m length and 0.25-mm internal diameter capillary column LM-100 (L&M, São Carlos, Brazil). The chromatograph was programmed as follows: injector at 250°C; detector at 250°C; column at 50°C for 2 min, followed by heating to 200°C at a rate of 5°C min^{-1}, completing analysis in 32 min. To quantify the amount of ethanol, a calibration curve was constructed by adding a solution of cheese whey (7% w/v), varying ethanol concentration, and a fixed amount of *n*-propanol as internal standard.

Results and discussion

Growth optimization of Kluyveromyces marxianus in cheese whey medium

Initial pH of culture medium: Growth of the *Kluyveromyces marxianus* strains CBS 712 and CBS 6556 were compared in reconstituted cheese whey with an initial pH 4.5 or 5.5. This acid pH range was chosen since ideal yeast growth pH is around 5.0–6.0. The growth curves illustrated in Figure 1 show that initial pH 5.5 resulted in a greater initial growth rate for the tested strains. These results are in agreement with those of Furlan *et al* [5] who found that several *K. marxianus* strains showed best growth at pH of 5.5.

Growth temperature: In order to determine the best growth temperature, the strains were grown in cheese whey at 30 and 37°C. These temperatures were chosen because they are the conventional temperatures used by most fermentation industries. As shown in Figure 2, there was no appreciable difference in growth between these temperatures. These results suggest that the two *Kluyveromyces*

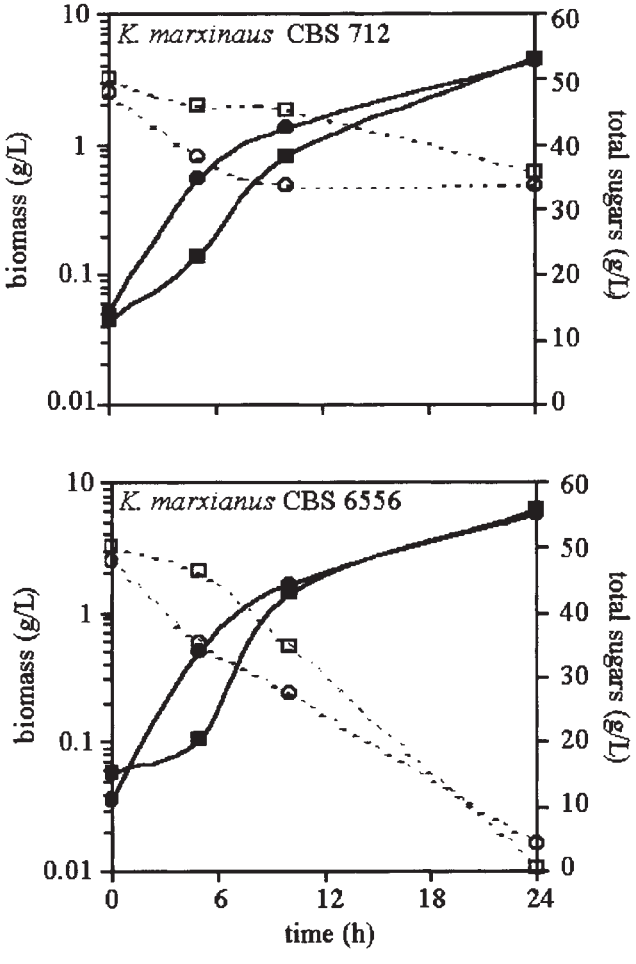

Figure 1 Growth of *Kluyveromyces marxianus* strains CBS 712 and CBS 6556 at pH 4.5 and pH 5.5 on an orbital shaker. Culture medium and conditions: cheese whey 70 g L^{-1}, temperature: 30°C, 200 rpm. ■ Biomass at pH 4.5; □ total sugars at pH 4.5; ● biomass at pH 5.5; ○ total sugars at pH 5.5.

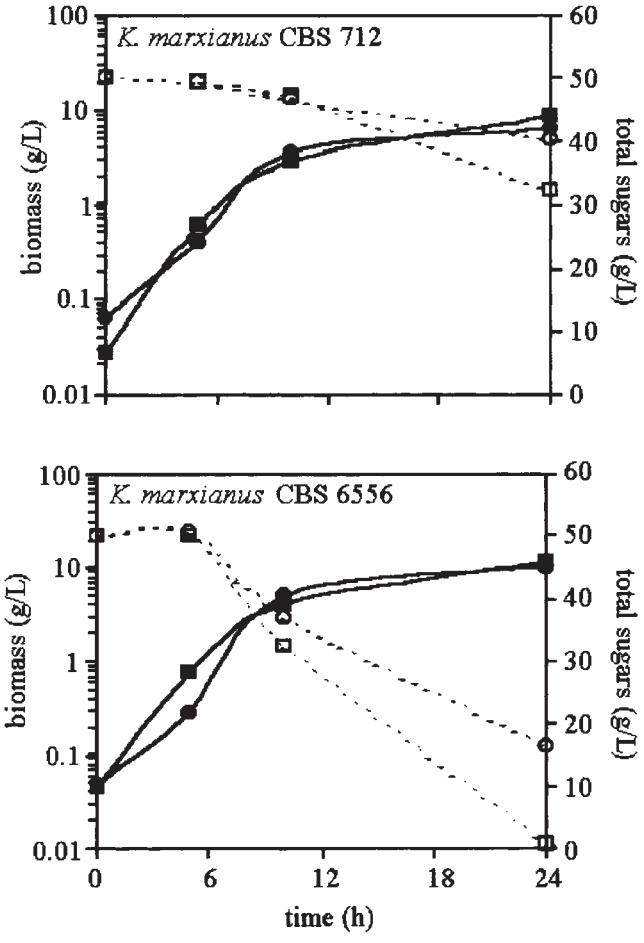

Figure 2 Growth of *Kluyveromyces marxianus* strains CBS 712 and CBS 6556 at 30°C and 37°C on an orbital shaker. Culture medium and conditions: cheese whey 70 g L^{-1}, pH 5.5, 200 rpm. ■ Biomass at 30°C; □ total sugars at 30°C; ● biomass at 37°C; ○ total sugars at 37°C.

marxianus strains tested in this work are thermostable, which is an industrial advantage, since thermostability decreases cooling costs and makes it possible to obtain temperature-resistant enzymes. Our results contrast with those of Furlan and co-workers [5] who reported maximum growth rate at 30°C for the same strains, concluding that an increase in temperature impairs cell growth. An explanation for this discrepancy may reside in the fact that in their work, aeration was limited while our culture conditions guaranteed a fully aerated system.

Growth medium: Cheese whey medium 70 g L^{-1} (M1), or M1 medium supplemented with 10 g L^{-1} urea (M2) or 10 g L^{-1} yeast extract (M3) were tested in order to determine the ideal growth medium.

The strains showed limited growth on M2. This is due to the alkalinization of the culture medium (pH 8.5, data not shown), which inhibited cell growth. Therefore, we concluded that urea was not a suitable nitrogen source for growing *K. marxianus* with hydrolyzed cheese whey, which contrasts with results from Hensing and co-workers [7] who found urea to be the ideal nitrogen source for yeast growth.

The results shown in Figure 3 suggest optimum cell growth in cheese whey medium with added yeast extract (M3). These findings are in accordance with those of Sonawat *et al* [16] and Wendorff *et al* [18], who showed that yeast extract had a positive effect on growth of *K. marxianus*. On medium M3, the yeasts consumed all sugars present in the culture medium. Both CBS 712 and CBS 6556 strains showed increased growth (64% and 92% respectively) in M3 when compared with M1.

The strains showed different behavior with regard to sugar consumption kinetics in medium M1. Strain CBS 712 stopped growing when there were 30 g L^{-1} of lactose remaining in the medium. This suggests that cheese whey lacks an essential nutrient for strain CBS 712. On the other hand, strain CBS 6556 consumed all sugars when growing in cheese whey *in natura*. This indicates a physiological difference between these two strains.

β-galactosidase production in the bioreactor

Yeast culture in an aerated bioreactor was carried out in order to study the production of β-galactosidase. Culture conditions were previously determined as follows: initial pH 5.5, temperature 37°C, cheese whey *in natura* for strain

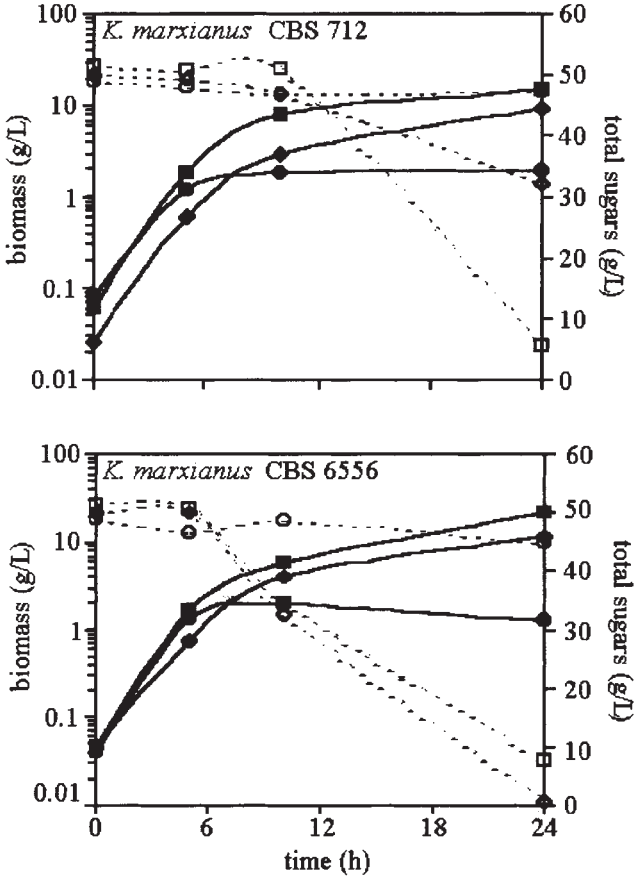

Figure 3 Growth of *Kluyveromyces marxianus* strains CBS 712 and CBS 6556 in different media on an orbital shaker. Conditions: pH 5.5, temperature: 30°C, 200 rpm. M1: cheese whey 70 g L^{-1}; M2: cheese whey 70 g L^{-1} and urea 10 g L^{-1}; M3: cheese whey 70 g L^{-1} and yeast extract 10 g L^{-1}. ■ M1, biomass; □ M1, total sugars; ● M2, biomass; ○ M2, total sugars; ◆ M3, biomass; ◇ M3, total sugars.

CBS 6556 and supplemented with yeast extract for strain CBS 712.

As previously demonstrated, strains showed no significant growth differences when cultured at 30°C and 37°C. In this way, temperature selection for bioreactor experiments (37°C) was chosen, aiming for the economy and the industrial advantages of a higher temperature (lower cooling costs and contamination risks).

Medium M1 was chosen for growth of strain CBS 6556, since it is a cheaper medium and is sufficient to support microbial growth. However, for strain CBS 712, medium M3 was chosen, since M1 did not produce good results for this strain.

Results of yeast growth in the aerated bioreactor are shown in Figures 4 and 5. Both strains reached the final growth phase after 11 h. The maximum specific growth rate, μ_{max}, the doubling time, t_d, the yield of biomass formation, $Y_{X/S}$, specific product, $Y_{P/X}$, and product per substrate, $Y_{P/S}$, calculated at the final growth phase, are shown in Table 1. Although strain CBS 712 exhibited values of $Y_{X/S}$ and $Y_{P/S}$ twice as great as those for CBS 6556, there were

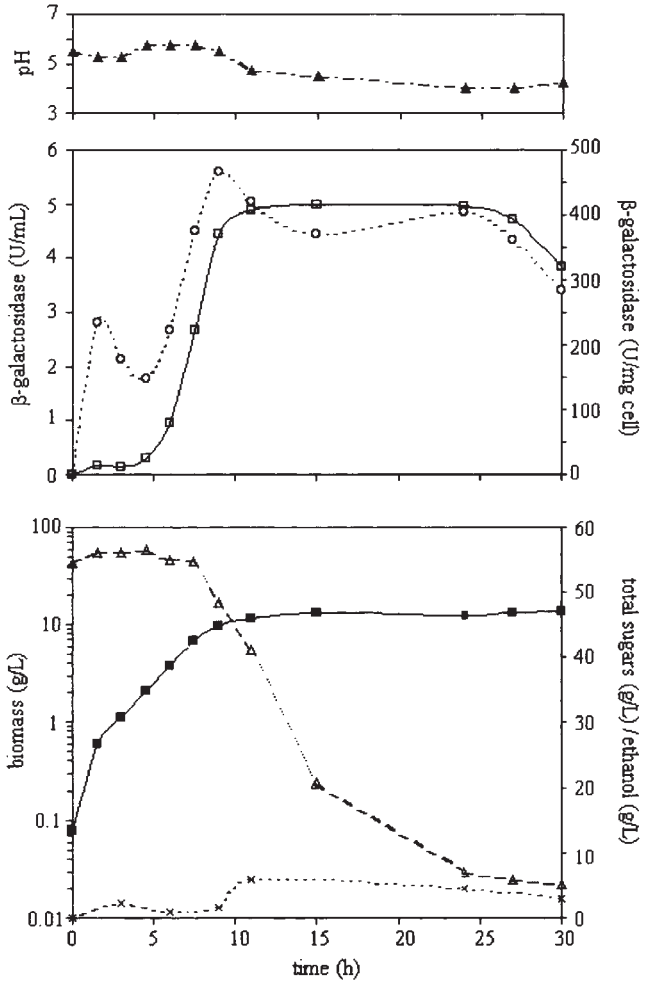

Figure 4 Growth of *Kluyveromyces marxianus* strain CBS 712 in an aerated bioreactor. Medium: cheese whey 70 g L^{-1} and yeast extract 10 g L^{-1}, pH: 5.5, temperature: 37°C, air flow: 3 L min^{-1}, 500 rpm. ▲ pH; ● β-galactosidase (U mg $cells^{-1}$); □ β-galactosidase (U ml^{-1}); △ total sugars; ■ biomass; × ethanol.

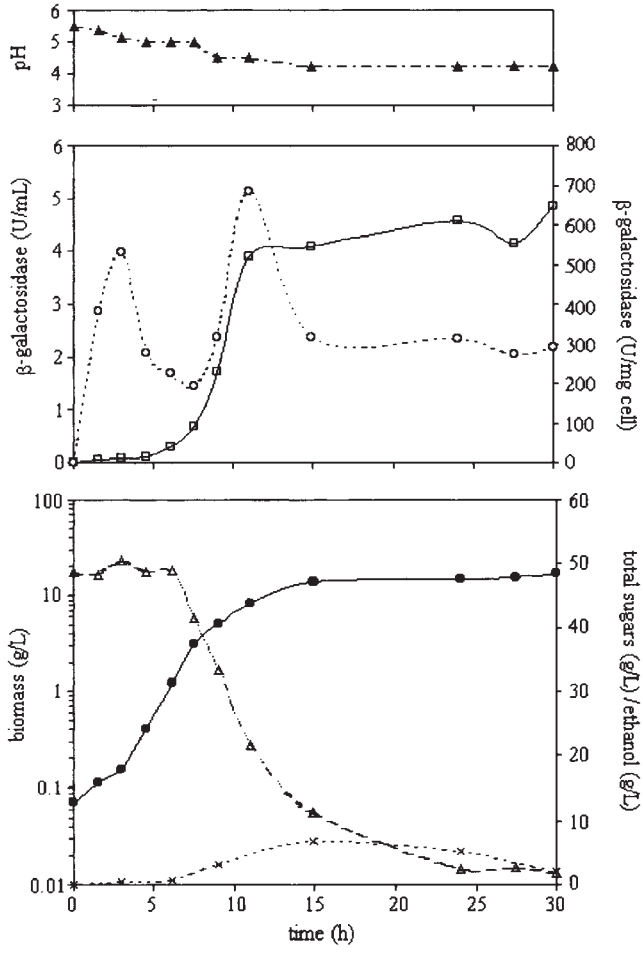

Figure 5 Growth of *Kluyveromyces marxianus* strain CBS 6556 in an aerated bioreactor. Medium: cheese whey 70 g L^{-1}, pH: 5.5, temperature: 37°C, air flow: 3 L min^{-1}, 500 rpm. ▲ pH; ● β-galactosidase (U mg $cells^{-1}$); □ β-galactosidase (U ml^{-1}); △ total sugars; ■ biomass; × ethanol.

Table 1 Growth parameters of *K. marxianus* after 11 h of growth

Parameter	Strain		Units
	CBS 712	CBS 6556	
μ_{max}	0.49	0.61	h^{-1}
t_d	1.42	1.15	h
$Y_{X/S}$	0.707	0.287	g cells g $lactose^{-1}$
$Y_{P/X}$	458.5	441.8	U_{ONPG} mg $cells^{-1}$
$Y_{P/S}$	333.8	129.7	U_{ONPG} g $lactose^{-1}$

no significant differences for the specific β-galactosidase activities, $Y_{P/X}$, between the two strains.

Figures 4 and 5 show similar β-galactosidase specific activity profile curves for both strains. The curves exhibit two distinct sharp points at the beginning and at the end of the growth phase. The β-galactosidase activity follows the pattern of the growth curve. During the stationary phase, β-galactosidase activity remained approximately constant, even exhibiting a slight increase for strain CBS 6556. These results differ from the observations made by Mahoney *et al* [11] for *K. marxianus* and Gonzales-Siso [6] for *K.*

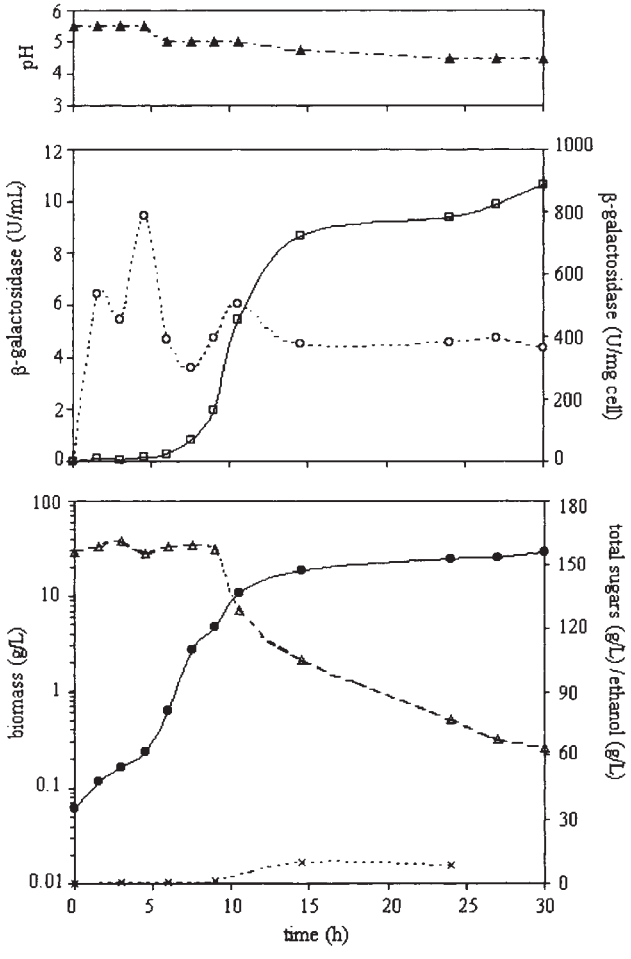

Figure 6 Growth of *Kluyveromyces marxianus* strain CBS 6556 in an aerated bioreactor. Medium: cheese whey 210 g L^{-1}, pH: 5.5, temperature: 37°C, air flow: 3 L min^{-1}, 500 rpm. ▲ pH; ● β-galactosidase (U mg $cells^{-1}$); □ β-galactosidase (U ml^{-1}); △ total sugars; ■ biomass; × ethanol.

lactis, who reported a decrease of β-galactosidase activity in the stationary phase. One explanation for this difference might be the stability of the enzyme produced by strains CBS 712 and CBS 6556, as results shown in this work indicate.

Growth in concentrated cheese whey

Aiming to improve β-galactosidase production, *K. marxianus* CBS 6556 was grown in concentrated cheese whey medium (210 g L^{-1}). Figure 6, and Table 2 present a comparison of growth parameters and yields obtained for growth at cheese whey concentrations of 70 g L^{-1} and 210 g L^{-1}. An F-test applied to the two conditions showed that there is no significant difference between the growth parameters.

Table 2 Growth parameters of *K. marxianus* strain CBS 6556 cultured in cheese whey

Parameter	Whey 70 g L^{-1}	Whey 210 g L^{-1}	Units
μ_{max}	0.61	0.65	h^{-1}
t_d	1.15	1.08	h
$Y_{X/S}$	0.287	0.320	g cells g $lactose^{-1}$
$Y_{P/X}$	441.8	480.3	U_{ONPG} mg $cells^{-1}$
$Y_{P/S}$	129.7	156.1	U_{ONPG} g $lactose^{-1}$

Partial characterization of the β-galactosidase produced by strain CBS 6556

Optimum temperature and thermostability of the β-galactosidase produced by *K. marxianus* CBS 6556 were tested in order to assess its suitability for industrial applications. The enzyme for these tests was obtained from bioreactor cultures, with cheese whey (70 g L^{-1}) at 37°C and pH 5.5.

Figure 7 shows the enzymatic activity of β-galactosidase as a variable of temperature. The highest enzymatic activity occurred around 37°C. Above 40°C enzymatic activity fell quickly. Thus β-galactosidase from *K. marxianus* CBS 6556 is less active at high temperatures when compared with β-galactosidase from strain IBM3, which exhibits maximum enzymatic activity between 45 and 50°C [3].

It is important to note that at 4°C this enzyme retained up to 40% of its maximum activity, contrasting with β-galactosidases from other sources, which keep only about 5% of their activity [3,8]. This allows the hydrolysis of lactose in milk, to produce lactose-reduced milk, at low storage temperatures.

Enzyme stability at 30°C is shown in Figure 8. The experiment shows that β-galactosidase activity decreases quickly and linearly with time, and, after 10 h, activity was only 40% of its initial value.

Figure 9 shows β-galactosidase stability at storage temperatures of 4°C (refrigerator), −4°C and −18°C (freezer). At 4°C, enzymatic activity fell linearly with time, attaining 65% of its initial value after 9 weeks. The curves at −4°C and −18°C show negligible activity loss after 9 weeks of storage.

Comparing these data with the enzymatic stability assay performed by Bales and Castillo [1] with lyophilized β-galactosidase from *Candida pseudotropicalis* grown in cheese whey, the lyophilized enzyme is more stable under the same storage conditions, compared with enzyme solutions.

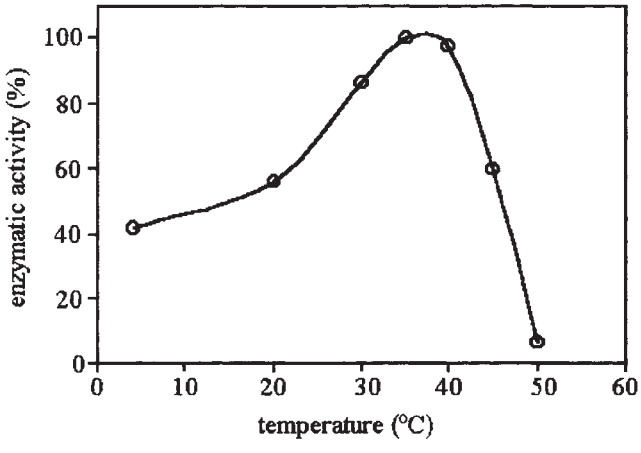

Figure 7 *Kluyveromyces marxianus* strain CBS 6556 β-galactosidase enzymatic activity variation with temperature.

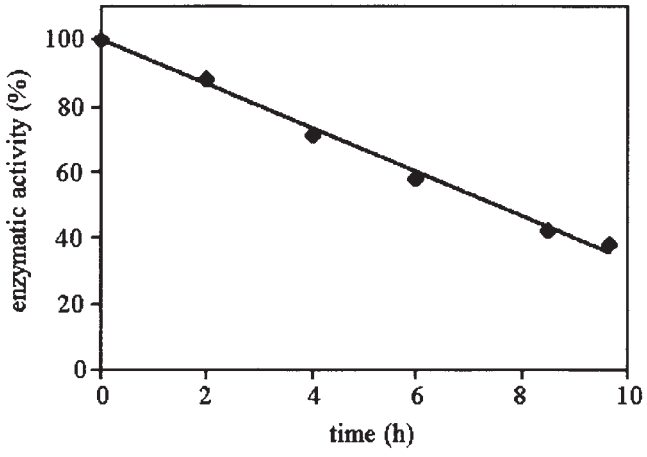

Figure 8 *Kluyveromyces marxianus* strain CBS 6556 β-galactosidase enzymatic stability assay at room temperature (30°C).

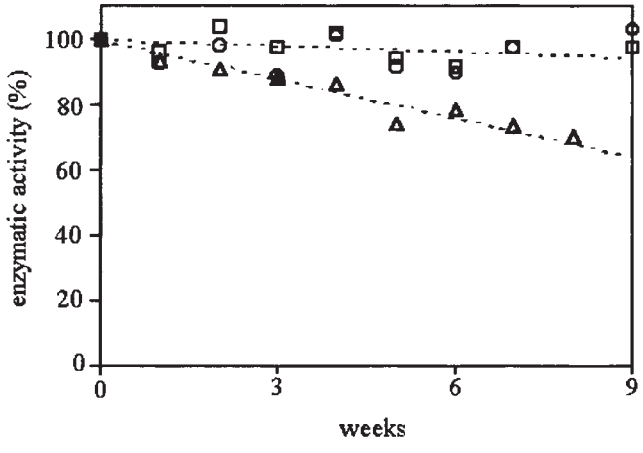

Figure 9 *Kluyveromyces marxianus* strain CBS 6556 β-galactosidase enzymatic stability assay at different storage temperatures. ○ –18°C; □ –4°C; △ 4°C.

Conclusions

The results obtained in this work suggest that β-galactosidase production by *Kluyveromyces marxianus* CBS 6556, using cheese whey as a culture medium, has the potential to be used in industrial processes. Cheese whey is a cheap, potentially-polluting by-product of the dairy industry, which makes it a very attractive medium for microbial cultures, since its cost is very low. *K. marxianus* CBS 6556 has a high specific growth rate, a shorter culture time and increased process productivity when compared to other industrially important yeasts such as *S. cerevisiae* and *K. lactis*. Further research is in progress in our laboratory to assess the potential use of recombinant *K. marxianus* for production of β-galactosidase.

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