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Production of β-Galactosidase from Cheese Whey Using *Kluyveromyces marxianus* CBS 6556

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This work presents alternatives sources of carbon and nitrogen for production of β-galactosidase by *Kluyveromyces marxianus* CBS 6556. The main goal was to evaluate the production of this enzyme in the presence of cheese whey and corn step liquor in shake flasks cultures. Preliminary results from an experimental design (2^{5-1}) showed that Prodex-lac® and agitation weren't significant variables. For the purpose of optimizing the enzyme production, a new factorial design was used. The central composite rotational design (CCRD) had cheese whey (100 to 1,000 mL L^{-1}), corn step liquor (0 to 18 g L^{-1}) and temperature (25 to 45 ºC) as independent variables. The optimum temperature for the enzyme production was found to be 31 °C when associated with 820 mL L^{-1} of cheese whey and 14.36 g L^{-1} of corn step liquor, after 24 hours of culture. In lower corn step liquor concentration (3.64 g L^{-1}), the enzyme activity was 20.7% less, indicating that a nitrogen source is important to promote enzyme production.

1. Introduction

Lactose is a dominant carbohydrate in milks and to perform its hydrolysis β-galactosidase can be employed as catalyst converting into glucose and galactose. A large number of people do not digest lactose properly due to a lack of or inactivity of the intestinal β-galactosidase and they suffer from intestinal dysfunctions— gas, abdominal pain and diarrhea—if their diet contains lactose. Lactose-intolerance is associated with a deficit on the production of β-galactosidase (Swagerty et al., 2002).
The enzyme β-galactosidase is one among others enzymes with industrial potential used in the hydrolysis of lactose in milk and cheese whey, generating food with low levels of lactose, whose the result is a better solubility and digestibility of milk and dairy products, making them, ideal for consumers intolerant to this sugar (Husain, 2010).
The cheese production is a very common process around the world and it brings a considerable amount of cheese whey, which represents a serious environmental problem for its disposal. Usually the treatments consist in biological and chemical processes for the removal of organic matter in whey, in function of the chemical oxygen demand (COD), biochemical oxygen demand (BOD) and dissolved organic carbon removals. Nevertheless, the cheese whey contains high lactose concentration that could convert this sugar into a high value-added product. The fermentation of cheese whey using yeasts can be a sustainable process for the production of enzymes, like β-galactosidase (Manera et al., 2011) and bio-ethanol (Joshi et al., 2011).
Several cultures of yeasts and bacteria have been used to produce β-galactosidase using cheese whey as an alternative carbon source and the biggest challenge is obtain high-cell-density cultures and improve the productivity. Fermentation of cheese whey by using yeasts *Saccharomyces cerevisae* (Rech and Ayub, 2006) and *Kluyveromyces marxianus* (Rech and Ayub, 2007) has been reported to produce β-galactosidase, using fed-batch bioreactor.
The main objective of this work was to evaluate the composition of the culture medium for the production of the enzyme β-galactosidase from *Kluyveromyces marxianus* CBS 6556 using an experimental central

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composite rotational design (CCRD), using cheese whey as a source of carbon and corn steep liquor as a source of nitrogen. Temperature was also evaluated in order to obtain high levels of enzyme activity.

2. Materials and Methods

2.1 Substrates

The fresh cheese whey was obtained from Do Vale Industry in Palhoça city, Brazil. It was necessary to remove the protein of the whey. After heating for 15 minutes, the whey was vacuum filtered on 0.2 μm membrane (Manera et al., 2011) and the desproteinized cheese whey was kept frozen until use. The equivalent lactose concentration in whey was 48 g L^{-1}. The corn steep liquor was acquired from Corn Products in Trombudo Central city, Brazil and was kept frozen.

2.2 Yeast Strain

The yeast Kluyveromyces marxianus CBS 6566 originally obtained from Centraal-Büreau von Schimmelcultures (Amsterdam, The Netherlands), was kindly provided by the Biotechnology Laboratory in University of Joinville Region. The strain was maintained on agar-plates containing YPL medium at 4 °C.

2.3 Inoculum Medium

The inoculum medium was prepared with a single strain from Petri plates and incubated for 12 h at 37 °C with an orbital shaking velocity of 180 min^{-1}. All the experiments used 300 mL flask with 100 mL of medium, which was constituted by lactose 2%, peptone 1% and yeast extract 1%. The humidity of the lactose was removed in a greenhouse at 60 °C for 24 h.

2.4 Experimental Protocol

All the experiments have been carried out in 1,000 mL shake flasks containing 300 mL of culture medium considering 10% of inoculum. Each run has been performed within 24 h, with velocity of 220 min^{-1}.

2.5 Analytical Methods

Cell concentration was spectrophotometrically determined at λ= 600 nm (LKB Biochron –Novaspec II), with a curve calibration of biomass dry weight to co-relating readings. The cells were washed twice with distilled water and separated by centrifugation. The enzyme activity was carried out using ONPG (o-nitrophenol-β-D-galactopyranoside) as substrate (Lederberg, 1950), after cell disruption and the enzyme extraction. Total reducing sugar concentrations were measured by using the calorimetric method, using 3,5-Dinitrosalicylic acid.

2.6 Experimental Design

In a previous work, Scholz et al. (2011) studied the effect of temperature, cheese whey, corn steep liquor, Prodex-lac® and agitation for production of β-galactosidase by Kluyveromyces marxianus CBS 6556 using a 2^{5-1} factorial experimental design. It was verified that Prodex-lac® and agitation weren't significant variables. For the purpose of optimizing the enzyme production, a new factorial design was used. The variables temperature, cheese whey concentration and corn steep liquor effect has been considered to produce β-galactosidase in a central composite rotational design (CCRD). The total number of experiments has been 17 (8 factorial, 6 axial and 3 on the central point) and the data analysis of the experimental design was performed by the software STATISTICA 7.0.

The experimental conditions tested in CCRD were showed on Table 1and each level represents one of the variables: X_1 - cheese whey concentration - CW (mL L^{-1}) and lactose equivalent (g L^{-1}) in parantheses, X_2 - corn steep liquor concentration - CSL (g L^{-1}) and X_3 – temperature - T (°C).

Table 1: Coded levels and concentration of the variables in CCRD

Levels	X_1 (lactose g L^{-1})	X_2	X_3
-1.68	100 (4.81)	0	25
-1	280 (13.46)	3.64	31
0	550 (26.44)	9	35
1	820 (39.43)	14.36	39
1.68	1,000 (48.08)	18	45

3. Results and Discussion

The experimental conditions tested in central composite rotational design have studied the influence of the nutrients sources' concentration and temperature on biomass formation and β-galactosidase production. Figure 1 shows two experiments carried out at the same temperature (31 °C) and CW volume (39.43 g L^{-1} lactose equivalent) but using a different concentration of CSL.

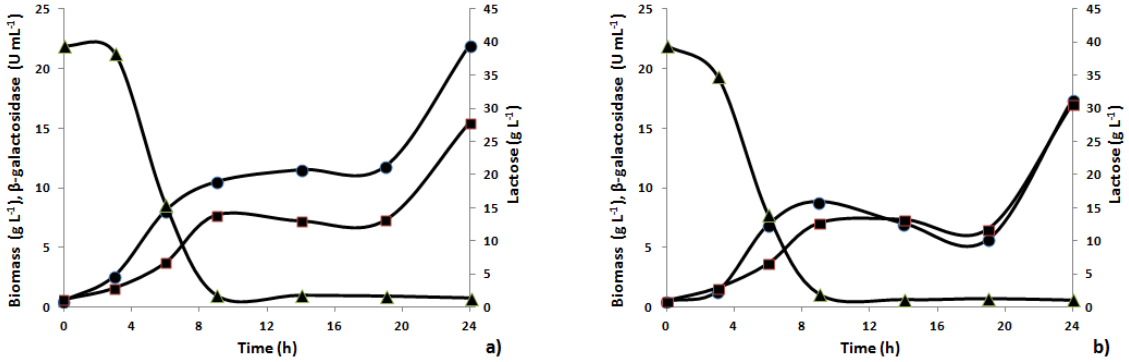

Figure 1: Batch experiments of cheese whey conversion carried out at T= 31 °C and CW= 820 mL L^{-1} (39.43 g L^{-1} lactose equivalent) and different corn steep liquor concentration, CSL: a) T= 31 °C, CW = 820 mL L^{-1}, CSL = 14.36 g L^{-1}; b) T= 31 °C, CW = 820 mL L^{-1}, CSL = 3.64 g L^{-1}. ●,β-galactosidase volumetric activity; ■, biomass; ▲, lactose.

It can be noticed that all lactose was consumed until 9h. Between 9h and 19h the biomass concentration was stable and after 19h another growth was observed. This can be explained in yeasts Kluyveromyces, due to ethanol consumption. Silveira et al. (2005) notice the occurrence of oxidative and fermentative pathway at a low temperature of 32 °C. Also the fermentative pathway can happen to produce ethanol when cheese whey is used as a carbon source (Sansonetti et al., 2010). The use of ethanol as substrate is possible due to the presence of alcohol dehydrogenase (Flores et al. 2000). Furlan et al. (2001) reported that at lower temperatures, such as, 28 °C and 30 °C, ethanol consumption still occurs even when there is the initial substrate.

It is noteworthy that the volumetric activities of β-galactosidase (U mL^{-1}) was influenced by the concentration of CSL (nitrogen source) whereas with 14.36 mL L^{-1} the enzyme production was 21.99 U mL^{-1} (Figure 1a) and with 3.64 mL L^{-1} the enzyme production was 17.43 U mL^{-1} (Figure 1b), showing that the enzyme volumetric activity was 20.7% less with a lower concentration of CSL. According to Manera et al. (2011), the concentration of CSL was also considered statistically significant for production of β-galactosidase by Kluyveromyces marxianus CCT 7082 in the presence of cheese whey.

The analysis on the ANOVA test has been performed with 95% confidence to assay the statistical significance of the analysis based on β-galactosidase specific activity (U g-1) and volumetric activity (UmL-1). The square sum (SS), the degree of freedom (df), the medium square (MS), calculated F and the probability of the significance of values ($p < 0,05$) are shown on table 2:

Table 2: Analysis of variance (ANOVA) demonstrating the significant variation on the parameter: corn steep liquor concentration (CSL) on β-galactosidase specific activity (U g^{-1})

Factor	SS	df	MS	F	p
CW (L)	171,754	1	171,754	0.984999	0.354028
CW (Q)	442,776	1	442,776	2.539293	0.155073
CSL (L)	**1,494,834**	**1**	**1,494,834**	**8.572785**	**0.022089***
CSL (Q)	46,401	1	46,401	0.266107	0.621844
T (L)	208,622	1	208,622	1.196436	0.310241
T (Q)	550,503	1	550,503	3.157100	0.118846
CW CSL	114,305	1	114,305	0.655531	0.444788
CW T	1,870	1	1,870	0.010727	0.920416
CSL T	127,761	1	127,761	0.732702	0.420341
Error	1,220,588	7	174,370		
Total SS	4,440,053	16			

* = significant, L = linear and Q = quadratic.

It was found that only the CSL concentration had significant influence (linear) over β-galactosidase specific activity. The model adjustment was also expressed by the correlation coefficient R^2 which was 0.7251, indicating that 72.51% of the variability in the response. The response function to β-galactosidase specific activity has been defined through statistical analysis and the model equation for coded variables can be written as in Eq (1):

$$\beta - galactosidase\left(U\ g^{-1}\right) = 1{,}792.15 + 112.19\mathrm{CW} - 198.45\mathrm{CW}^2 + 330.99\mathrm{CSL} + \\ + 64.24\mathrm{CSL}^2 + 123.65\mathrm{T} - 221.28\mathrm{T}^2 - 119.53\mathrm{CW.CSL} - 15.29\mathrm{CW.T} + 126.37\mathrm{CSL.T} \quad (1)$$

Considering this equation it is possible to generate a response surface to analyze different values of CW and CSL concentration for production of β-galactosidase. The conditions that resulted in the highest enzymatic specific activity were the conditions which indicate higher values of CSL concentration, about 18 g L^{-1} (Figure 2).

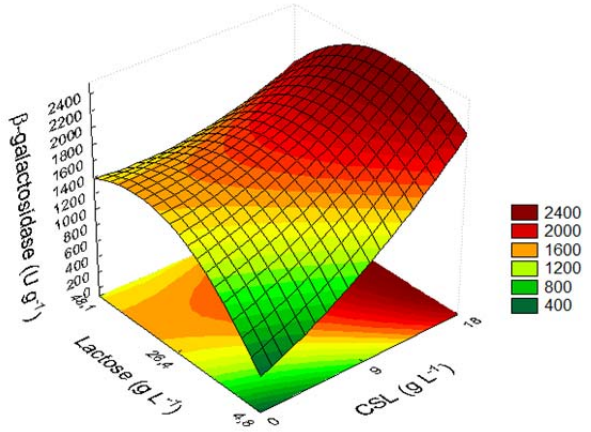

Figure 2: Response surface for β-galactosidase specific activity (U g^{-1}) based on lactose concentration and (CSL) corn steep liquor concentration.

On the other hand, the conditions that resulted in higher β-galactosidase volumetric activities (U mL^{-1}) were observed in higher values of CW concentration, about 1,000 mL L^{-1} (48 g L^{-1} lactose equivalent) and lower temperatures. Based on statistical analysis, Equation (2) was obtained which has been validated by analysis of variance (Table 3) in which the coefficient correlation was 0.8554.

Table 3: Analysis of variance (ANOVA) demonstrating the significant variation on the parameters: cheese whey concentration (CW) and temperature (T) on β-galactosidase volumetric activity (U mL^{-1})

Factor	SS	df	MS	F	p
CW (L)	**103.0204**	**1**	**103.0204**	**19.13369**	**0.003257***
CW (Q)	2.3601	1	2.3601	0.43833	0.529114
CSL (L)	15.6621	1	15.6621	2.90887	0.131862
CSL (Q)	2.3830	1	2.3830	0.44260	0.527177
T (L)	**58.0836**	**1**	**58.0836**	**10.78769**	**0.013404***
T (Q)	1.5152	1	1.5152	0.28141	0.612188
CW CSL	0.7555	1	0.7555	0.14032	0.719051
CW T	29.7278	1	29.7278	5.52127	0.051107
CSL T	7.2181	1	7.2181	1.34060	0.284903
Error	37.6897	7	5.3842		
Total SS	260.6330	16			

* = significant, L = linear and Q = quadratic.

$$\beta - galactosidase\left(U\ mL^{-1}\right) = 11.34 + 2.75\mathrm{CW} - 0.45\mathrm{CW}^2 + 1.07\mathrm{CSL} + \\ + 0.46CSL^2 - 2.06\mathrm{T} - 0.37\mathrm{T}^2 + 0.31\mathrm{CW.CSL} - 1.92\mathrm{CW.T} - 0.95\mathrm{CSL.T} \quad (2)$$

It was found from the variables tested that only CW concentration (linear) and temperature (linear) have significant influence. From the equation it is possible to generate a response surface (Figure 3) to analyze CW concentration and temperature for higher production of β-galactosidase (U mL^{-1}).

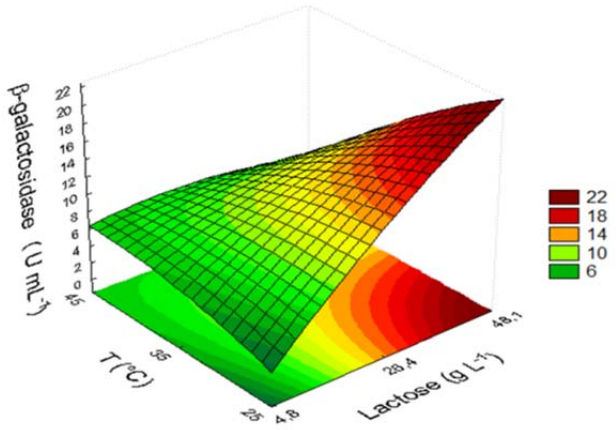

Figure 3: Response surface for β-galactosidase volumetric activity (U mL^{-1}) due to: lactose concentration and (T) temperature.

The maximum specific activity (2,853 U g^{-1} cell) was obtained after 24 h of cultivation when associated with 550 mL L^{-1} of cheese whey, 14.36 g.L^{-1} of corn steep liquor and 35 °C. This specific activity is 1.65 times higher than that obtained in batch cultures of *K. marxianus* CCT 7082 using 70 g L^{-1} of cheese whey and 100 g L^{-1} of corn steep liquor as culture medium at 30 °C after 48 h, by Manera et al. (2011). The authors suggested that the lower enzyme activity possibly occurred due to an oxygen limitation in the medium (180 min^{-1}), which is caused by the high oxidative metabolism of this yeast (Rech and Ayub, 2007), whereas this work utilized 220 min^{-1}.

Not considering the cell growth, the maximum β-galactosidase volumetric activities was obtained at 31 °C and 820 mL L^{-1} of cheese whey (Figure 1a) and the ANOVA analysis showed both variables statistically significant (Table 3). In face of it, the higher the cheese whey concentration and the lower the temperature, the best results of enzyme per ml medium were achieved as shown in Figure 3. Although, the corn steep liquor concentration is not a significant variable, it also may contribute to the maximum enzyme volumetric activity at higher concentrations.

The Figure 4 shows that the experimental values were observed versus the predicted values by the models for β-galactosidase volumetric and specific activity. The residual distribution is normal and its distribution is random and independent of the model, without showing trends, which shows reliability of the experimental data and the proposed models.

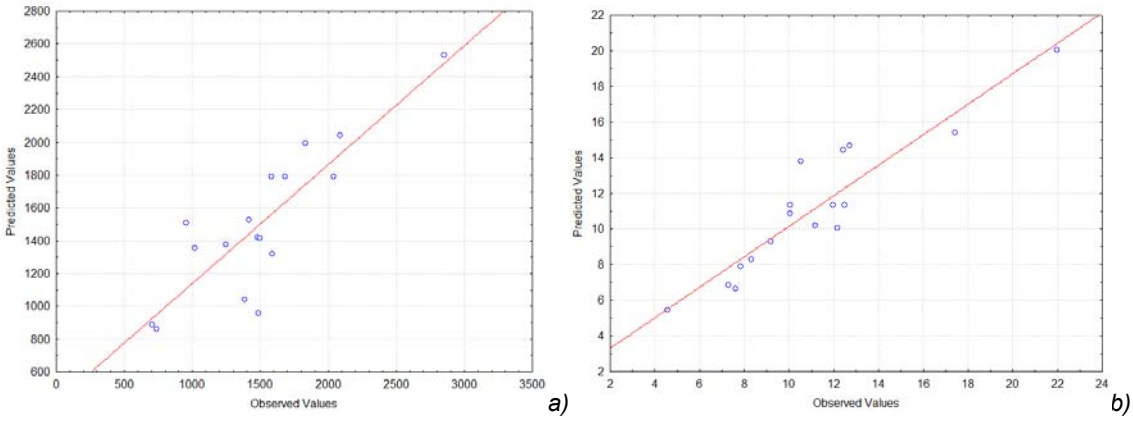

Figure 4: Comparison between the experimental values of: a) β-galactosidase specific activity (U g^{-1}); b) β-galactosidase volumetric activity (U mL^{-1}) and those predicted values.

A large number of works have been developed evaluating biomass and products yields, in addition to enzyme specific activity. It is important to notice that as high as the volumetric enzyme activity is, it is better for an industrial process application. Pure lactose and yeast extract provides high yields though they are an expensive source of nutrients. Nevertheless, cheese whey and corn steep liquor are wastes that can cause damage to the environment and are available at low prices in large quantity.

4. Conclusions

The highest β-galactosidase activity 21.99 U mL^{-1} was found with 39.43 g L^{-1} of lactose (820 mL L^{-1} cheese whey), 14.36 mL L^{-1} of corn steep liquor and 31 ºC after 24 h. However, based on CCRD, only cheese whey concentration and temperature were statistically significant. In this fermentation condition, it was observed that corn steep liquor has a positive effect on β-galactosidase activity as a complement in the medium.

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