ISSN 1330-9862
(FTB-1834)

original scientific paper

Optimization of the Culture Medium for the Production of β-Galactosidase from *Kluyveromyces marxianus* CCT 7082

*Ana Paula Manera, Joana da Costa Ores, Vanessa Amaral Ribeiro, Carlos André Veiga Burkert and Susana Juliano Kalil**

Department of Chemistry, Federal University Foundation of Rio Grande, P.O. Box 474, BR-96201-900 Rio Grande, RS, Brazil

Received: December 18, 2006
Accepted: July 13, 2007

Summary

Seven strains of the genus *Kluyveromyces* were screened for β-galactosidase activity in a synthetic medium. *Kluyveromyces marxianus* CCT 7082 was selected as the best enzyme producer for optimization of the culture medium. A fractional factorial design 2^{4-1} was used to determine the most relevant variables of the culture medium composition for the enzyme production. The parameters studied were the concentrations of lactose, yeast extract and $(NH_4)_2SO_4$ as well as pH, and all were shown to have significant effects on the production of the enzyme. Based on the results from the first factorial design, lactose, yeast extract and $(NH_4)_2SO_4$ concentrations were selected to be utilized in a 2^3 central composite rotatable design (CCRD). This led to a further optimization of the fermentation conditions to achieve higher enzyme activities, which reached 10.6 U/mL.

Key words: β-galactosidase, *Kluyveromyces marxianus*, optimization, factorial design, response surface methodology

Introduction

Among the enzymes, hydrolases are receiving increasing industrial application. Especially β-galactosidase or β-D-galactoside galactohydrolase (EC 3.2.1.23), which catalyses the hydrolysis of lactose into galactose and glucose, has got special attention (*1*).

The importance of β-galactosidase is related to its use in milk and milk derivatives to decrease their lactose content, solving the problem of low lactose solubility and its low degree of sweetening. Furthermore, the economic interest in this enzyme is related to its use in food and pharmaceutical industries, because of the deficiency of β-galactosidase in many people (*2*).

The lactose-fermenting yeasts *Kluyveromyces marxianus* and *Kluyveromyces lactis* are both important industrial yeasts in classical applications with biomass, ethanol, enzymes and single-cell protein production (*3–5*). *Kluyveromyces marxianus* offers great advantages, such as: (*i*) good growth yield, which has an important economical impact in the food industry; (*ii*) acceptability as a safe microorganism, an important technical aspect when considering that the fermented products have food or pharmaceutical applications; and (*iii*) higher β-galactosidase activity than other yeasts (*6*).

Increased industrial demand for β-galactosidase requires good cost-effective production methods to ensure the economic viability of lactose hydrolysis at commercial scale (*7*). In order to improve the β-galactosidase production, several groups have made investigations to select microorganisms that have high activity (*8,9*), to evaluate substrates (*10,11*) and define optimized fermentation conditions for the chosen microorganism (*12*).

Optimization through factorial design and response surface methodology has been used in biotechnical processes, and several research works for the production

*Corresponding author; Phone: ++55 53 32 338 754; Fax: ++55 53 32 338 676; E-mail: dqmsjk@furg.br

of enzymes have applied this technique for the optimization of culture conditions, namely for the production of glucoamylase by *Aspergillus niger* (*13*), lipase by *Geotrichum candidum* (*14*), or inulinase by *Kluyveromyces marxianus* (*15,16*). The aim of optimization is to determine suitable conditions for the economically and/or technologically important process variables such as product concentration, yield, selectivity and raw material cost (*17*). Statistically designed experiments are very effective because the affecting parameters can be evaluated collectively, even with a limited number of experiments (*18*).

In the present work, the production of β-galactosidase from *Kluyveromyces* yeasts by submerged fermentation in agitated flasks was evaluated by using factorial design and response surface methodology. The best enzyme producer among seven strains of *Kluyveromyces lactis* and *Kluyveromyces marxianus* was selected. Afterwards, the culture medium was optimized. In order to establish the optimal conditions for the production of β-galactosidase, the pH and the concentrations of lactose, yeast extract and $(NH_4)_2SO_4$ were first evaluated in a fractional factorial design (2^{4-1}) followed by a 2^3 central composite rotatable design (CCRD) and response surface methodology.

Materials and Methods

Microorganisms

Five strains of *Kluyveromyces marxianus* (CCT 7080, CCT 7081, CCT 7082, NCYC 587, and var. *bulgaricus* ATCC 16045), and two strains of *Kluyveromyces lactis* (NRRL Y 8279 and NRRL Y 1564) were tested. The strains were maintained at 4 °C on yeast mold (YM) agar medium containing (in g/L): yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0 and agar 20.0.

Inoculum

The inoculum cultures were grown on a medium containing lactose 10 g, KH_2PO_4 5 g, $(NH_4)_2SO_4$ 1.2 g, $MgSO_4 \cdot 7H_2O$ 0.4 g and yeast extract 1 g in 1 L of 0.2 M potassium phosphate buffer, pH=5.5 (*19*). Sterilization of the medium was done at 121 °C for 15 min, lactose was sterilized by filtration (*3*). β-Galactosidase was produced in 500-mL Erlenmeyer flasks with 150 mL of culture medium. The culture was incubated in an orbital shaking incubator (Tecnal TE-420, Brazil) for 14 h at 180 rpm and 30 °C (*20*).

Submerged fermentation

In order to select the best β-galactosidase producer microorganism, submerged fermentations in Erlenmeyer flasks were done with the same inoculum culture medium. The fermentations were started with 10 % of inoculum (*7*). The operational conditions were 30 °C and 180 rpm (Tecnal TE-420, Brazil) during 72 h. After that time, the enzymatic activity started to decline. The fermentations were done in duplicate for each microorganism.

At appropriate intervals, culture samples were collected for analysis of β-galactosidase activity, biomass concentration, lactose concentration and pH.

Experimental design

The effects of pH and the concentrations of lactose, yeast extract and $(NH_4)_2SO_4$ on β-galactosidase production were studied by using a fractional design of 2^{4-1} trials plus 3 central points, which means a total of 11 trials.

A CCRD (2^3 plus axial and central points) with three replicates at the central point, a total of 17 trials, was utilized for the three selected variables from the fractional design, having β-galactosidase activity as response. Table 1 gives the values of the coded levels used in both the fractional and central composite rotatable designs.

Amounts of KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ were fixed at 5 and 0.4 g/L, respectively, for all the trials. The medium was prepared with 0.2 M potassium phosphate buffer varying the pH (Table 1). The software Statistica 6.0 was used to analyse the results.

Enzyme extraction

Samples of 1 mL of cell suspension containing 2.62 mg of dry cell in phosphate buffer (0.1 M, pH=7.3) were ultrasonicated (Unique MaxiClean 700, Brazil) for 10 min with 1.1 g of glass beads (r<0.4 mm). During this treatment, the temperature was kept between 0 and 4 °C. The suspension was centrifuged at 5200×*g* (Cientec CT--5000R, Brazil) for 10 min at 4 °C, and the supernatant was assayed for β-galactosidase activity.

Biomass content in the cell suspension was adjusted according to Numanoğlu and Sungur (*21*), using a dry cell calibration curve previously established.

Table 1. Values of coded levels and real values used in fractional design and CCRD

Factorial design	Coded variable level	γ(lactose) g/L	γ(yeast extract) g/L	$\gamma((NH_4)_2SO_4)$ g/L	pH
Fractional	–1	10	1	0	3.5
	0	40	6.5	4	4.8
	+1	70	12	8	6.0
CCRD	–1.68	10	5	4	–
	–1	28.2	8	5.2	–
	0	55	12.5	7	–
	+1	81.8	17	8.8	–
	+1.68	100	20	10	–

β-Galactosidase assay

The β-galactosidase activity was measured spectrophotometrically by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma-Aldrich) by the method of Food Chemical Codex (22). A sample of 0.5 mL was mixed with 2 mL of 8.3 mM ONPG in PEM buffer (27.2 g/L of KH_2PO_4, 37.2 mg/L of disodium EDTA dehydrate, and 20.3 mg/L of $MgCl_2 \cdot 6H_2O$), pH=6.5, and incubated for 15 min at 37 °C. The reaction was stopped by adding 1 mL of solution into 1 mL of 10 % sodium carbonate solution, then mixed by swirling, diluted to 10 mL with distilled water, and mixed again. Liberated *o*-nitrophenol (ONP) was measured spectrophotometrically (Quimis Q-108D, Brazil) at 420 nm. One unit of enzyme activity (U) was defined as the quantity of enzyme that will liberate 1 μmol of *o*-nitrophenol per min under the conditions of the assay.

Biomass concentration

Samples were collected and centrifuged at 5200×*g* (Cientec CT-5000R, Brazil) for 10 min at 4 °C. Following the removal of the supernatant, cells were washed once with distilled water, centrifuged again and resuspended in a known volume of phosphate buffer (0.1 M, pH=7.3). Absorbance was measured at 620 nm using a spectrophotometer (Quimis Q-108D, Brazil) and compared to a previously constructed standard curve for absorbance *vs.* cell dry mass.

For cell dry mass determination, culture samples containing cells were centrifuged at 5200×*g* (Cientec CT-5000R, Brazil) for 10 min at 4 °C (*4*). Following the removal of the supernatant, cells were washed once with distilled water and dried in a microwave oven (Panasonic) at 700 W output for 15 min (*23*).

Lactose concentration

Lactose concentration in the cell-free samples was determined by a colorimetric method using the phenol sulphuric acid reaction as described by Dubois *et al.* (*24*).

Results and Discussion

Screening of microorganism

Shake flask cultures were used to investigate the β--galactosidase production for the seven strains of *Kluyveromyces.* The experiments and analytical measurements were performed in duplicates.

As shown in Fig. 1, the maximum β-galactosidase activity occurred after 50 h of cultivation for *K. marxianus* CCT 7082 reaching values higher than 2.8 U/mL. The strains CCT 7080 and CCT 7081 reached maximum activity in 6 h of fermentation. The values obtained were 0.9 and 1.5 U/mL, respectively. The maximum activities reached by the strains ATCC 16045 and NCYC 587 were 0.8 U/mL, after 24 and 32 h of fermentation, respectively. For the *K. lactis* strains, the peaks of enzymatic activity occurred after 24 h of fermentation, presenting values of 0.7 and 1.5 U/mL for the NRRL Y 1564 and NRRL Y 8279, respectively.

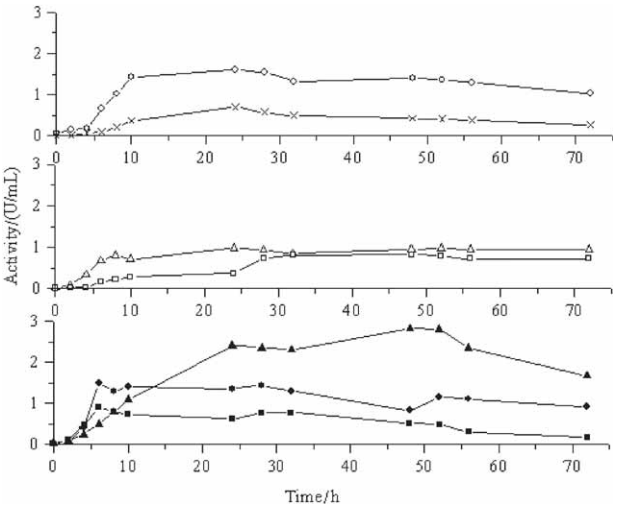

Fig. 1. Enzymatic activity of the seven microorganisms tested during fermentation: ○ *K. lactis* NRRL Y 8279, × *K. lactis* NRRL Y 1564, △ *K. marxianus* var. *bulgaricus* ATCC 16045, □ *K. marxianus* NCYC 587, ▲ *K. marxianus* CCT 7082, ● *K. marxianus* CCT 7081, ■ *K. marxianus* CCT 7080

Fig. 2 shows the enzymatic activity, biomass concentration, lactose consumption and pH variation for *Kluyveromyces marxianus* CCT 7082 during fermentation. Maximum enzymatic activity was reached with minimum pH, total lactose consumption and maximum biomass concentration. The same behaviour was observed for the other six microorganisms tested.

For both species, the obtained values for the enzymatic activity were in the same range or higher than the ones found by Inchaurrondo *et al.* (*3*). They also fermented *K. lactis* and *K. marxianus* strains in a medium containing lactose as carbon source. The values they obtained for the enzymatic activity were between 0.7 and 1.3 U/mL for the strains of *K. lactis* and between 0.7 and 1.0 U/mL for the strains of *K. marxianus.*

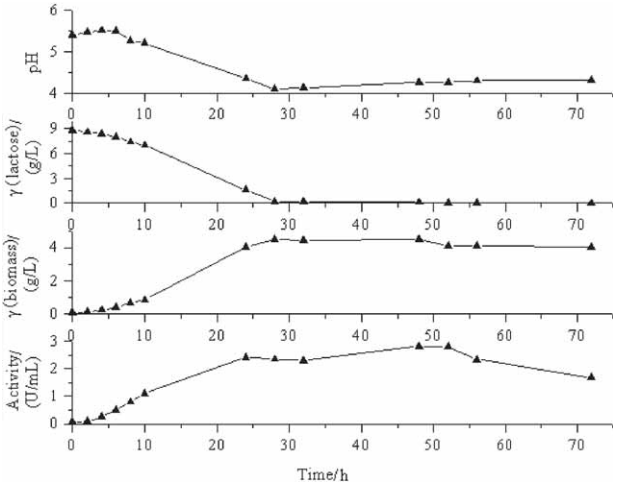

Fig. 2. Fermentation time profile for *Kluyveromyces marxianus* CCT 7082 using lactose as carbon source

From the seven microorganisms tested, *K. marxianus* CCT 7082 was selected for the optimization trials of the culture medium because it presented the highest enzyme production.

Fractional factorial design

Experiments using 2^{4-1} fractional factorial design were carried out with three values of each independent variable (Table 1). Enzymatic activities were measured every 24 h of the fermentation for 120 h. The response of the experimental designs was the maximum enzymatic activity for each trial. An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from low (–1) to high (+1) levels of the corresponding variable.

Activities varied according to the fermentation conditions, from 0.7 to 11.6 U/mL. As can be seen in Table 2, all variables had statistically significant effect at 95 % confidence at the maximum enzymatic activity. Changes in the variables from level –1 to level +1 led to an increase in the maximum enzymatic activity. Maximum β-galactosidase production was more significantly affected (significance at the 0.05 level) by yeast extract (average positive effect of 4.18 U/mL), followed by lactose, pH and $(NH_4)_2SO_4$.

From these results the concentrations of lactose, yeast extract and $(NH_4)_2SO_4$ were selected for further optimization of the medium composition in a CCRD. The pH was fixed at 6.0, corresponding to the pH value of the best trial in the fractional factorial design (11.6 U/mL).

Central composite rotatable design (CCRD)

The results of the enzymatic activity in the CCRD for the three studied variables: lactose, yeast extract and $(NH_4)_2SO_4$ concentrations, and the values predicted by the model provided by the Eq. 1 are presented in Table 3.

Table 2. Effect estimates for β-galactosidase activity[a] using fractional factorial design

Factor	Effect/(U/mL)	Standard error	t(2)[c]	p	Conf. limit[b] (–95 %)	Conf. limit[b] (+95 %)
Mean	4.66	0.06	78.32	0.00016	4.40	4.90
γ(lactose)/(g/L)	2.55	0.14	18.30	0.00297	1.95	3.15
γ(yeast extract)/(g/L)	4.18	0.14	29.97	0.00111	3.58	4.78
$\gamma((NH_4)_2SO_4)$/(g/L)	1.60	0.14	11.44	0.00754	0.99	2.19
pH	1.65	0.14	11.87	0.00701	1.05	2.25

[a]All numerical values are significant factors (95 % confidence level); [b]Conf., confidence; [c]Degrees of freedom according to central points

$$\text{Activity/(U/mL)} = 7.81 + 0.27(\text{yeast extract}) + 0.8(\text{yeast extract})^2 - 0.20((NH_4)_2SO_4)^2 - 0.85(\text{lactose} \times \text{yeast extract}) - 0.65(\text{lactose} \times (NH_4)_2SO_4) + 0.25(\text{yeast extract} \times (NH_4)_2SO_4) \quad /1/$$

Table 3. Real values and coded values (in parentheses), experimental and predicted values of enzymatic activity in the CCRD

Trial	γ(lactose) g/L	γ(yeast extract) g/L	$\gamma((NH_4)_2SO_4)$ g/L	Maximum enzymatic activity U/mL	Predicted activity U/mL
1	28.2 (–1)	8.0 (–1)	5.2 (–1)	7.1	6.9
2	81.8 (+1)	8.0 (–1)	5.2 (–1)	10.1	9.9
3	28.2 (–1)	17.0 (+1)	5.2 (–1)	7.8	8.6
4	81.8 (+1)	17.0 (+1)	5.2 (–1)	8.7	8.2
5	28.2 (–1)	8.0 (–1)	8.8 (+1)	7.6	7.7
6	81.8 (+1)	8.0 (–1)	8.8 (+1)	9.3	8.1
7	28.2 (–1)	17.0 (+1)	8.8 (+1)	10.6	10.4
8	81.8 (+1)	17.0 (+1)	8.8 (+1)	7.6	7.4
9	10.0 (–1.68)	12.5 (0)	7.0 (0)	7.6	7.8
10	100.0 (+1.68)	12.5 (0)	7.0 (0)	7.0	7.8
11	55.0 (0)	5.0 (–1.68)	7.0 (0)	8.9	9.6
12	55.0 (0)	20.0 (+1.68)	7.0 (0)	10.7	10.5
13	55.0 (0)	12.5 (0)	4.0 (–1.68)	7.8	7.2
14	55.0 (0)	12.5 (0)	10.0 (+1.68)	6.2	7.2
15	55.0 (0)	12.5 (0)	7.0 (0)	8.0	7.8
16	55.0 (0)	12.5 (0)	7.0 (0)	7.8	7.8
17	55.0 (0)	12.5 (0)	7.0 (0)	8.2	7.8

In this second design, enzyme production varied from 6.2 up to 10.7 U/mL. The best value for the activity occurred in trials 7 and 12. The conditions of trial 7 were: concentrations of lactose at level –1 (28.2 g/L), yeast extract at level +1 (17 g/L) and $(NH_4)_2SO_4$ at level +1 (8.8 g/L). In this trial the activity reached 10.6 U/mL after 96 h of fermentation, with a productivity of 0.11 U/(mL·h). In trial 12 the conditions were: concentrations of lactose at the central point (55 g/L), yeast extract at level +1.68 (20 g/L) and $(NH_4)_2SO_4$ at the central point (7 g/L). In this trial the maximum enzymatic activity was 10.7 U/mL after 120 h of fermentation, resulting in a productivity of 0.09 U/(mL·h).

The analysis of variance (ANOVA) was employed in the enzymatic activity presented in Table 4. ANOVA consists of classifying and cross-classifying statistical results and testing whether the means of a specified classification differ significantly. This was carried out using Fisher's statistical test for the analysis of variance. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model (*25*).

The correlation coefficient was 0.9 and the F-value around twice higher than the listed value for 90 % of confidence. Consequently, the model was found to be adequate to describe the response surface of β-galactosidase production (Eq. 1). An overview of Eq. 1 indicates that the activity is a first order function for yeast extract concentration, a second order function for yeast extract and $(NH_4)_2SO_4$ concentrations, and an interaction between lactose and yeast extract, lactose and $(NH_4)_2SO_4$, yeast extract and $(NH_4)_2SO_4$. The equation model fitted by regression analysis is given by Eq. 1.

The model for β-galactosidase activity was used to construct the response surfaces, which can be seen in Fig. 3, to understand the interaction of the medium components and the optimum concentration of each component required for optimum β-galactosidase production.

Figs. 3a and 3b show the effect of lactose and yeast extract concentrations on the production of β-galactosidase. When the lactose concentration was low and the yeast extract concentration was high, the production of the enzyme remained around 11–12 U/mL. When the lactose concentration was high and $(NH_4)_2SO_4$ concentration was low, or when the lactose concentration was low and $(NH_4)_2SO_4$ concentration was high, the activity was above 8 U/mL (Figs. 3c and 3d). Figs. 3e and 3f show the effects of yeast extract and $(NH_4)_2SO_4$ concentrations on the activity. The production of the enzyme reached values of about 10 U/mL when the concentration of yeast extract was high (20 g/L) and $(NH_4)_2SO_4$ was higher than 7 g/L.

The surfaces indicated that high enzymatic activity can be obtained when using culture medium with 28.2 g/L of lactose, 20 g/L of yeast extract and 8.8 g/L of

Table 4. ANOVA for β-galactosidase activity for CCRD

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Regression	20.32	6	3.38	4.82
Residual	5.35	10	0.70	
Lack of fit	5.27	8		
Pure error	0.08	2		
Total	25.67	16		

Regression coefficient: 0.9
$F_{0.9;\ 6;\ 10}$: 2.46

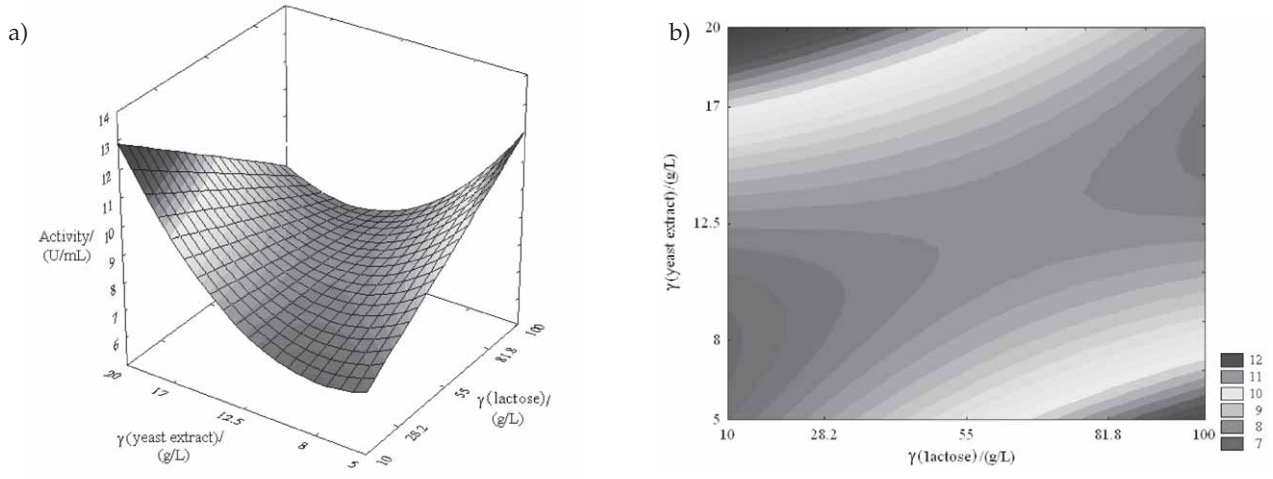

Fig. 3. Response surface and contour diagrams of β-galactosidase activity as a function of the concentrations of lactose and yeast extract (a, b)

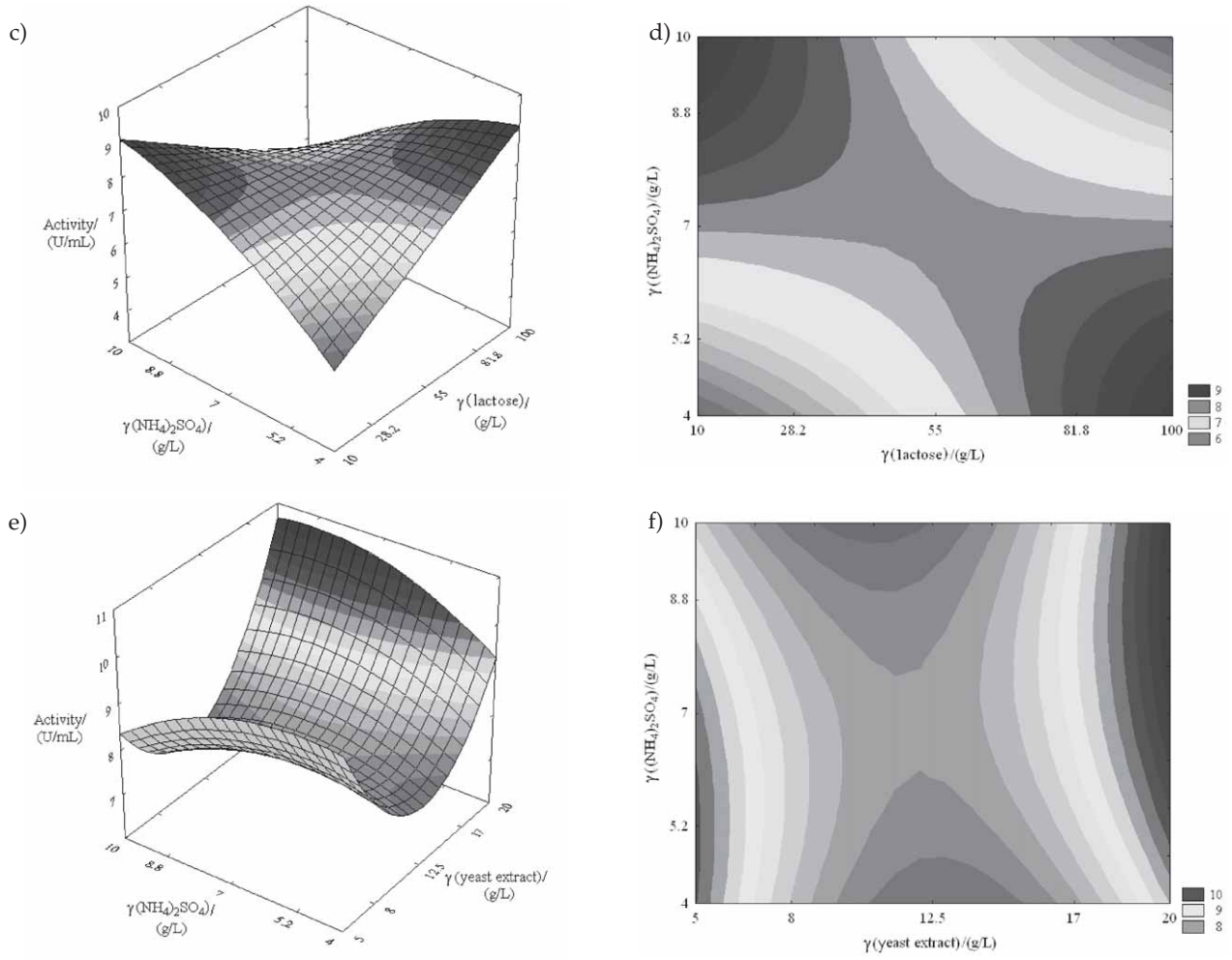

Fig. 3. Response surface and contour diagrams of β-galactosidase activity as a function of the concentrations of: lactose and $(NH_4)_2SO_4$ (c, d), and yeast extract and $(NH_4)_2SO_4$ (e, f)

$(NH_4)_2SO_4$. Fig. 4 shows the average results of fermentation carried out in triplicate, using the fermentation conditions already described.

Enzymatic activity increased until 120 h of fermentation, reaching 10.6 U/mL. It was observed that there was no increase in the enzymatic activity when compared to the activities of trials 7 and 12 of CCRD, *i.e.* the conditions employed in this design comprised a broad range, permitting maximum enzyme production, being possible to consider the composition of trial 7 (28.2 g/L of lactose, 17 g/L of yeast extract and 8.8 g/L of $(NH_4)_2SO_4$) as the best condition for the production of β-galactosidase enzyme by *K. marxianus* CCT 7082.

A significant improvement of β-galactosidase activity was achieved with the optimization of the culture medium. The optimization of the medium resulted in an enzymatic activity four times higher than in an unoptimized medium (Fig. 2). Ramírez Matheus and Rivas (*12*) optimized the production of β-galactosidase from *Kluyveromyces lactis* by using deproteinized whey as fermentation medium. Four factors were evaluated: temperature, pH, agitation speed and fermentation time. The enzymatic activities were between 1.3 and 8.5 U/mL. Comparison of the results with other studies using *Kluyveromyces marxianus* is difficult due to differences in yeast strains, growth conditions and β-galactosidase assay technique.

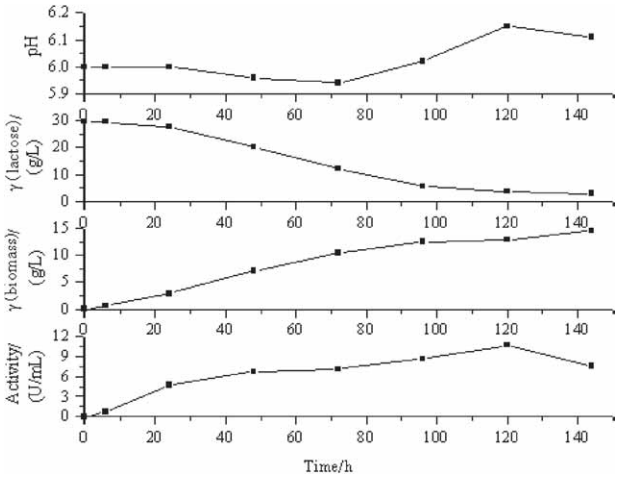

Fig. 4. Fermentation time profile for *Kluyveromyces marxianus* CCT 7082 in optimized medium

Conclusions

In the screening of seven strains of *Kluyveromyces* tested in this work, it was observed that *Kluyveromyces marxianus* CCT 7082 presented the highest production of β-galactosidase. This strain was selected for further optimization studies of the culture medium for enzyme production through the methodologies of Central Composite Rotatable Design (CCRD) and response surface techniques.

Statistical analysis proved to be a useful and powerful tool in developing optimum fermentation conditions. The optimum conditions established for the production of β-galactosidase were lactose concentration of 28.2 g/L, yeast extract concentration of 17.0 g/L, $(NH_4)_2SO_4$ concentration of 8.8 g/L and pH=6.0. Under these conditions the enzymatic activity was 10.6 U/mL.

Acknowledgements

The present work was carried out with the financial support of CNPq and CAPES, entities of the Brazilian government focused on qualifying human resources.

References

1. S.A. Furlan, A.L.S. Schneider, R. Merkle, M.F. Carvalho-Jonas, R. Jonas, Formulation of a lactose-free, low-cost culture medium for the production of β-D-galactosidase by *Kluyveromyces marxianus*, *Biotechnol. Lett. 22* (2000) 589–593.
2. V. Gekas, M. López-Leiva, Hydrolysis of lactose: A literature review, *Process Biochem. 20* (1985) 1–12.
3. V.A. Inchaurrondo, O.M. Yantorno, C.E. Voget, Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium, *Process Biochem. 29* (1994) 47–54.
4. R. Rech, C.F. Cassini, A. Secchi, M. Ayub, Utilization of protein-hidrolyzed cheese whey for production of β-galactosidase by *Kluyveromyces marxianus*, *J. Ind. Microbiol. Biotechnol. 23* (1999) 91–96.
5. M. Rubio-Texeira, Endless versatility in the biotechnological applications of *Kluyveromyces* LAC genes, *Biotechnol. Adv. 24* (2006) 212–225.
6. M.A.F. Belem, B.H. Lee, Production of bioingredients from *Kluyveromyces marxianus* grown on whey: An alternative, *Crit. Rev. Food Sci. Nutr. 38* (1998) 565–598.
7. Z.M. Nor, M.I. Tamer, M. Mehrvar, J.M. Scharer, M. Moo-Young, E.J. Jervis, Improvement of intracellular β-galactosidase production on fed-batch culture of *Kluyveromyces fragilis*, *Biotechnol. Lett. 23* (2001) 845–849.
8. A.A. Thigiel, T. Deak, Selection of strains and extraction procedures for optimum production of galactosidase from *Kluyveromyces* strains, *Zbl. Mikrobiol. 144* (1989) 465–471.
9. A.F. Furlan, M.F. Carvalho-Jonas, R. Merkle, G.B. Bértoli, R. Jonas, Application of the microtiter reader system for screening of β-D-galactosidase producing microorganisms, *Braz. Arch. Biol. Technol. 38* (1995) 1261–1268.
10. C.F. Grubb, A.J. Mawson, Effects of elevated solute concentrations on the fermentation of lactose by *Kluyveromyces marxianus* Y-113, *Biotechnol. Lett. 15* (1993) 621–626.
11. L. Domingues, C. Oliveira, I. Castro, N. Lima, J.A. Teixeira, Production of β-galactosidase from recombinant *Saccharomyces cerevisiae* grown on lactose, *J. Chem. Technol. Biotechnol. 79* (2004) 809–815.
12. A.O. Ramírez Matheus, N. Rivas, Production and partial characterization of β-galactosidase from *Kluyveromyces lactis* grown in deproteinized whey, *Arch. Latinoam. Nutr. 53* (2003) 194–201 (in Spanish).
13. S.T. Silveira, M.S. Oliveira, J.A.V. Costa, S.J. Kalil, Optimization of glucoamylase production by *Aspergillus niger* in solid-state fermentation, *Appl. Biochem. Biotech. 128* (2006) 131–140.
14. J.F.M. Burkert, R.R. Maldonado, F. Maugeri, M.I. Rodrigues, Comparison of lipase production by *Geotrichum candidum* in stirring and airlift fermenters, *J. Chem. Technol. Biotechnol. 80* (2005) 61–67.
15. S.J. Kalil, R. Suzan, F. Maugeri, M. I. Rodrigues, Optimization of inulinase production by *Kluyveromyces marxianus* using factorial design, *Appl. Biochem. Biotech. 94* (2001) 257–264.
16. D. Vranešić, Ž. Kurtanjek, A.M.P. Santos, F. Maugeri, Optimization of inulinase production by *Kluyveromyces bulgaricus*, *Food Technol. Biotechnol. 40* (2002) 67–73.
17. D. Weuster-Botz, Experimental design for fermentation media development: Statistical design or global random search?, *J. Biosci. Bioeng. 90* (2000) 473–483.
18. M.C.T. Damaso, C.M.M.C. Andrade, N. Pereira Jr., Production and properties of the cellulase-free xylanase from *Thermomyces lanuginosus* IOC-4145, *Braz. J. Microbiol. 33* (2002) 333–338.
19. R. Pinheiro, I. Belo, M. Mota, Growth and β-galactosidase activity in cultures of *Kluyveromyces marxianus* under increased air pressure, *Lett. Appl. Microbiol. 37* (2003) 438–442.
20. T. Lukondeh, N.J. Ashbolt, P.L. Rogers, Fed-batch fermentation for production of *Kluyveromyces marxianus* FII 510700 cultivated on a lactose-based medium, *J. Ind. Microbiol. Biotechnol. 32* (2005) 284–288.
21. Y. Numanoğlu, S. Sungur, β-galactosidase from *Kluyveromyces lactis* cell disruption and enzyme immobilization using a cellulose-gelatin carrier system, *Process Biochem. 39* (2004) 705–711.
22. *Food Chemical Codex: General Tests and Apparatus*, National Academy Press, Washington, USA (1981) pp. 491–492.
23. M. Hensing, H. Vrouwenvelder, C. Hellinga, R. Baartmans, H. van Dijken, Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluyveromyces marxianus*, *Appl. Microbiol. Biotechnol. 42* (1994) 516–521.
24. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem. 28* (1956) 350–356.
25. F. Francis, A. Sabu, K.M. Nampoothiri, S. Ramachandran, S. Ghosh, G. Szakacs, A. Pandey, Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*, *Biochem. Eng. J. 15* (2003) 107–115.