

Inulinase Production by Agro-Industrial Residues: Optimization of Pretreatment of Substrates and Production Medium

Carla Sguarezi · Carine Longo · Gustavo Ceni ·
Gabriela Boni · Marcell F. Silva · Marco Di Luccio ·
Marcio A. Mazutti · Francisco Maugeri ·
Maria Isabel Rodrigues · Helen Treichel

Received: 18 October 2007 / Accepted: 29 November 2007 / Published online: 20 December 2007
© Springer Science + Business Media, LLC 2007

Abstract In this work, production of inulinase was studied. Media formulation was optimized by experimental design and response surface techniques, as well as the pretreatment of the agro-industry residues used in the formulation of fermentation medium. Two agro-industry residues were investigated: sugarcane molasses (SCM) and corn steep liquor (CSL). Pretreatment with sulfuric acid was the most effective for clarification of SCM (pH 5.0, 24 h of resting time and final pH 4.0). Clarification of CSL was accomplished with phosphoric acid (pH 3.0, 24 h of resting time and final pH 5.5). A color reduction of approximately 70% was achieved for both substrates. The highest production of inulinase was obtained in a medium containing 100 g l^{-1} of pretreated SCM, 100 g l^{-1} of pretreated CSL and 6 g l^{-1} of Prodex Lac (yeast hydrolysate), yielding $1,139 \text{ U ml}^{-1}$.

Keywords Agro-industrial residues · Experimental design · Inulinase · *Kluyveromyces marxianus* · Pretreatment · Sugarcane molasses · Corn steep liquor

Introduction

Inulinase (β -2,1-D-fructan fructanohydrolase, EC 3.2.1.7) targets on the β -2,1 linkage of inulin (a polyfructan consisting of linear β -2,1 linked fructose) and hydrolyzes it into fructose, producing fructose syrup in a single step (Wei et al. 1998).

Fructose production by inulin hydrolysis is more advantageous than conventional process based on starch, which includes the action of α -amylase, amyloglucosidase, and glucose isomerase, yielding only 45% of fructose in the final product, due to the thermodynamical equilibrium of the reaction. Inulinase based hydrolysis of inulin can yield products with 95% of fructose (Mazutti et al. 2006).

However, a more potential application of such enzymes in food industry is the production of fructooligosaccharides (FOS), by the transfructosilation of sucrose catalyzed by inulinase or by β -fructosyltransferase. The FOS present many interesting functional properties, besides their ability to increase shelf-life and flavor of many products (Sangeetha et al. 2005). Nevertheless, the industrial application of inulinase will only be viable when this enzyme is available in large quantities at competitive market prices. Hence, it is critical to define the most adequate microorganism and cultivation medium, as well as to specify the stirring rate, pH, temperature, and aeration conditions for feasible scale-ups of fermentation process.

The use of industrial media is a good alternative to produce inulinase at low cost, if the enzyme activity is improved or if the activity is at least the same obtained in a synthetic medium. The production of inulinase using sugarcane molasses (SCM) and corn steep liquor (CSL) may be an interesting alternative to add value to such

C. Sguarezi · C. Longo · G. Ceni · G. Boni · M. F. Silva ·
M. Di Luccio · M. A. Mazutti · H. Treichel (✉)
Department of Food Engineering,
Universidade Regional Integrada, URI-Campus de Erechim,
Av. Sete de Setembro, 1621,
Erechim, Rio Grande do Sul 99700-000, Brazil
e-mail: helen@uricer.edu.br

F. Maugeri · M. I. Rodrigues
Department of Food Engineering,
Universidade Estadual de Campinas, UNICAMP,
Campinas, Sao Paulo, Brazil

residues and lower enzyme production costs. Inulinase can be produced by solid state fermentation using untreated sugarcane bagasse, CSL and SCM as substrates, obtaining maximum inulinase activities of 390 and 444 U g⁻¹, respectively (Mazutti et al. 2006; Bender et al. 2006). Similar results were obtained using wheat bran with different supplements (Xiong et al. 2007).

On the other hand, the composition of industrial media based in agro-industrial residues is very complex and the presence of heavy metals (iron, zinc, magnesium, calcium, copper, manganese) may inhibit inulinase production (Lazarridou et al. 2002; Roukas 1998). For instance, SCM contains a large amount of melanin, which is toxic to microorganisms due to its antioxidant properties (Lee et al. 2000). Thus, a study of the upstream processing for removal of contaminants of the fermentation media for enzyme production may contribute to increase enzyme yields and even simplification of the downstream steps, which usually represents 50 to 80% of product final costs.

The present work aimed to study the pretreatment of agro-industrial residues used in the production of inulinase. Corn steep liquor (CSL) and sugarcane molasses (SCM) were pretreated to improve enzyme yields. Experimental design and response surface methodology were used to optimize the conditions of acid treatment of substrates and the formulation of fermentation medium.

Materials and Methods

Pretreatment

Sugarcane molasses (SCM) was obtained from a sugar refinery (Ester Sugar Refinery, Campinas SP, Brazil) and corn steep liquor (CSL) was obtained from Corn Products Brazil.

Preliminary tests indicated that treatment with phosphoric acid and sulfuric acid were the most effective methods for clarification of CSL and SCM, respectively. The preliminary results helped to define experimental designs to study the clarification of substrates. A complete factorial design with triplicate at the central point was used to study SCM clarification using sulfuric acid. A Central Composite Rotatable Design (CCRD) was used for optimization of CSL clarification by phosphoric acid. Table 1 present the investigated levels in both designs. The studied factors included initial pH of treatment, resting time and final pH.

At each experimental run, the pH of the substrate was adjusted with the respective acid and the solution was set to rest at 24°C for the time defined by the experimental design. The media was then centrifuged at 5,000×g for 15 min, and the final pH was adjusted with NaOH 10 M until the pH defined in the experimental design. The clarification of the medium was quantified by optical

Table 1 Range of factors for molasses and CSL clarification

Level	-1.68	-1	0	+1	+1.68
Molasses clarification by sulfuric acid					
Initial pH	–	3	4	5	–
Resting time (h)	–	24	36	48	–
Final pH	–	4	5	6	–
CSL clarification by phosphoric acid					
Initial pH	1.32	2	3	4	4.68
Resting time (h)	4	12	24	36	44
Final pH	4.66	5	5.5	6	6.34
Inulinase production					
Molasses	16	50	100	150	184
CSL	16	50	100	150	184
Prodex Lac	2.64	4	6	8	9.36

density measurements at 600 nm in a spectrophotometer (Agilent 8453). The analysis of data was performed using software Statistica® 5.1 (Statsoft).

Microorganism and Medium

The strain of *Kluyveromyces marxianus* NRRL Y-7571 was employed for inulinase production. The microorganism was grown on YM agar medium containing (g l⁻¹): yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0, agar 20.0, and was subcultured every 3 weeks.

Cell production for the preinoculum cultures was carried out in a medium containing 2% sucrose at pH 6.8 in 500 ml flasks with 100 ml of culture medium, at 30°C, 150 rpm for 24 h.

Submerged Fermentation

The inulinase production was carried out in 500 ml Erlenmeyer flasks with 100 ml pretreated culture medium. The fermentations started with 10% (v/v) inoculum, at initial pH 5.0, 36°C, and 150 rpm rotation in orbital shaker PSYCROTHERM (New Brunswick Scientific, NJ, USA). The activity was determined after 72 h of fermentation.

The composition of the medium was optimized for highest inulinase production. The optimization was performed using a Central Composite Rotatable Design (CCRD), with three replications on central point. Table 1 shows the range of factors investigated in this step. The analysis of data was performed using software Statistica® 5.1 (Statsoft).

Inulinase Assay

Activity was assayed as follows: 1 ml enzyme solution was mixed with 9 ml of 2% (m/m) sucrose or inulin on acetate buffer 0.1 M pH 4.6. The mixture was maintained at 50°C and the reducing sugars were determined by the 3,5-

dinitrosalicylic acid method (DNS) (Miller 1959). One unit of inulinase activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of sucrose per minute under the conditions mentioned above (sucrose as substrate).

Microbial Growth

The microbial growth was determined by an indirect dry weight method, by comparing the cell suspensions optical density at 600 nm to standard curve, which was previously prepared with the same microorganism produced in the same culture medium (Silva-Santisteban and Maugeri 2005).

Results and Discussion

Pretreatment

Table 2 presents the results of the complete factorial design used for definition of the pretreatment of molasses with sulfuric acid. Optical density (OD) of molasses solution without the pretreatment was 0.749. The results show that sulfuric acid treatment is effective for color removal of molasses, since a decrease in the OD was detected in all runs. The condition in run 2 presented the highest removal of color (70%). The clarification action of sulfuric acid is possibly related to oxidation of suspended organic matter in the medium, since sulfuric acid is known as an oxidizing and dehydrating agent (Atkins and Jones 2005).

The results in Table 2 were statistically analyzed, yielding an empirical model as a function of the resting time and the final pH of the medium. The model was validated by analysis of variance (ANOVA) presented in Table 3. The factors that were not significant ($p < 0.05$) were added to the lack of fit for the analysis of variance (ANOVA). The ANOVA shows a high R (0.87, correlation coefficient) and a good performance of the F test for

Table 2 Matrix of the experimental design (coded and real values) with responses in terms of optical density (OD) for clarification of molasses

Trial	Initial pH	Resting time (h)	Final pH	OD
1	3.0 (-1)	24.0 (-1)	4.0 (-1)	0.230
2	5.0 (+1)	24.0 (-1)	4.0 (-1)	0.220
3	3.0 (-1)	48.0 (+1)	4.0 (-1)	0.486
4	5.0 (+1)	48.0 (+1)	4.0 (-1)	0.630
5	3.0 (-1)	24.0 (-1)	6.0 (+1)	0.394
6	5.0 (+1)	24.0 (-1)	6.0 (+1)	0.500
7	3.0 (-1)	48.0 (+1)	6.0 (+1)	0.687
8	5.0 (+1)	48.0 (+1)	6.0 (+1)	0.60
9	4.0 (0)	36 (0)	5.0 (0)	0.509
10	4.0 (0)	36 (0)	5.0 (0)	0.529
11	4.0 (0)	36 (0)	5.0 (0)	0.571

Table 3 ANOVA for molasses clarification as response

Source of variation	Sum of squares	Degrees of freedom	Mean square	F test
Regression	0.227	2	0.114	28.5
Residual	0.029	8	0.004	
Lack of fit	0.027	6		
Pure error	0.002	2		
Total	0.256	10		

$$R=0.94; F_{0.05; 2; 8}=4.46$$

regression (calculated value about six times the listed one). Therefore, Eq. 1 is predictive of SCM clarification in the investigated range of factors, and consists in a first order function of resting time (t) and final pH (pH_{final}).

$$\text{OD} = 0.49 + 0.14 \times t + 0.09 \times \text{pH}_{\text{final}} \quad (1)$$

Equation 1 shows that the initial pH does not influence clarification of molasses in the investigated range (3.0–5.0). Clarification is then a function of resting time and final pH. The final pH of treatment and resting time presented positive main effects on optical density. This means that an increase on these factors would result in a lower clarification of the medium. Therefore, these factors were set to the minimum level in medium optimization. A Central Composite Rotatable Design (CCRD) was unnecessary in this case because a sufficient color removal was obtained in this design. (Rodrigues and Iemma 2005).

Table 4 presents the matrix of the results of pretreatment of CSL with phosphoric acid. Optical density of the medium without treatment was 0.835. For runs 4, 7, and 8, an increase in OD is observed, showing that these conditions were inadequate for clarification of CSL. Good color removal (70%) was obtained at runs 15–17, which correspond to the central point of the experimental design, i.e., initial pH 3.0, 24 h of resting time, final pH 5.5.

The results presented in Table 4 were submitted to statistical analysis, yielding a second order empirical model (Eq. 2). The model was validated by ANOVA, presented in Table 5. The factors that were not significant ($p < 0.05$) were added to the lack of fit for the analysis of variance (ANOVA). The ANOVA shows a high R (0.87, correlation coefficient) and a good performance of the F test for regression (calculated values about 1 times the listed one). Equation 2 is predictive for clarification of CSL in the range investigated and explains 87.0% of total variation of the experiments.

$$\begin{aligned} \text{OD} = & 0.23 + 0.07 \times \text{pH}_{\text{initial}} + 0.11 \times \text{pH}_{\text{initial}}^2 + 0.14 \\ & \times t + 0.13 \times t^2 + 0.03 \times \text{pH}_{\text{final}} + 0.15 \\ & \times \text{pH}_{\text{final}}^2 + 0.03 \times t \times \text{pH}_{\text{final}} \quad (2) \end{aligned}$$

Table 4 Matrix of the experimental design (coded and real values) with responses in terms of optical density (OD) for clarification of CSL

Trial	Initial pH	Resting time (h)	Final pH	OD
1	2.0 (-1)	12.0 (-1)	5.0 (-1)	0.304
2	4.0 (+1)	12.0 (-1)	5.0 (-1)	0.433
3	2.0 (-1)	36.0 (+1)	5.0 (-1)	0.552
4	4.0 (+1)	36.0 (+1)	5.0 (-1)	0.970
5	2.0 (-1)	12.0 (-1)	6.0 (+1)	0.345
6	4.0 (+1)	12.0 (-1)	6.0 (+1)	0.506
7	2.0 (-1)	36.0 (+1)	6.0 (+1)	0.888
8	4.0 (+1)	36.0 (+1)	6.0 (+1)	1.016
9	1.32 (-1.68)	24.0 (0)	5.5 (0)	0.450
10	4.68 (+1.68)	24.0 (0)	5.5 (0)	0.540
11	3.0 (0)	4.0 (-1.68)	5.5 (0)	0.458
12	3.0 (0)	44.0 (+1.68)	5.5 (0)	0.517
13	3.0 (0)	24.0 (0)	4.7 (-1.68)	0.650
14	3.0 (0)	24.0 (0)	6.3 (+1.68)	0.620
15	3.0 (0)	24.0 (0)	5.5 (0)	0.251
16	3.0 (0)	24.0 (0)	5.5 (0)	0.214
17	3.0 (0)	24.0 (0)	5.5 (0)	0.252

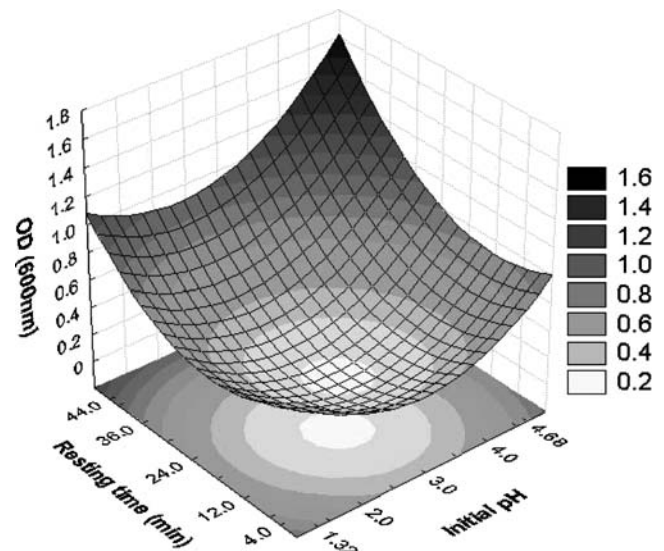
The fitted response surface for the clarification of CSL obtained by Eq. 2 is presented in Fig. 1. The results show that the clarification of CSL is optimized, since the minimum OD was obtained at the treatment conditions of the central point of the CCRD.

Many studies dealing with pretreatment of molasses are reported in literature. Some reports showed that only 80% of the glucose present in the molasses could be used by the microorganism in the medium without pretreatment, while 99% was consumed in the medium that was filtered with membrane (Kasseno and Kokugan 1997). The optimization of the production of xanthan gum using molasses as substrate, when the medium was formulated with molasses without any treatment xanthan resulted in a production of 46.9 g L⁻¹ in 48 h of fermentation. For a medium containing molasses treated with sulfuric acid at pH 4.0, production was 36.9 g L⁻¹. The pretreatment did not show a significant effect (Kalogiannis et al. 2003). Treatment with sulfuric acid and activated carbon for removal of heavy metals and colored compounds from molasses can be used for production of pullulan (Lazarridou et al. 2002).

Table 5 ANOVA for CSL clarification as response

Source of variation	Sum of squares	Degrees of freedom	Mean square	F test
Regression	0.712	7	0.102	3.96
Residual	0.231	9	0.026	
Lack of fit	0.001	7		
Pure error	0.943	2		
Total		16		

$R=0.87$; $F_{0.05; 7; 9}=3.29$

**Fig. 1** Response surface for clarification of CSL as a function of resting time and initial pH

Optimization of Inulinase Production

Table 6 presents the results of the CCRD used to study the influence of concentration of SCM, CSL and Prodex Lac on inulinase production. Experimental runs 10 and 13 showed the lowest enzyme productions, yielding activities 178 and 156.9 U ml⁻¹, respectively. The highest inulinase yield was found at the central point of the CCRD (1,139 ± 111 U ml⁻¹), showing that the experimental design was effective to optimize the process conditions. In the present work, a single CCRD was enough to optimize of inulinase production using the following concentration of pretreated

Table 6 Matrix of the experimental design (coded and real values) for inulinase production (72 h of fermentation)

Trial	Molasses (g l ⁻¹)	CSL (g l ⁻¹)	Prodex Lac (g l ⁻¹)	Activity (U ml ⁻¹)
1	50.0 (-1)	50.0 (-1)	4.0 (-1)	830.2
2	150.0 (+1)	50.0 (-1)	4.0 (-1)	450.0
3	50.0 (-1)	150.0 (+1)	4.0 (-1)	900.4
4	150.0 (+1)	150.0 (+1)	4.0 (-1)	653.3
5	50.0 (-1)	50.0 (-1)	8.0 (+1)	750.3
6	150.0 (+1)	50.0 (-1)	8.0 (+1)	305.6
7	50.0 (-1)	150.0 (+1)	8.0 (+1)	760.2
8	150.0 (+1)	150.0 (+1)	8.0 (+1)	656.9
9	16.0 (-1.68)	100.0 (0)	6.0 (0)	681.2
10	184.0 (+1.68)	100.0 (0)	6.0 (0)	178.0
11	100.0 (0)	16.0 (-1.68)	6.0 (0)	239.8
12	100.0 (0)	184.0 (+1.68)	6.0 (0)	562.3
13	100.0 (0)	100.0 (0)	2.64 (-1.68)	156.9
14	100.0 (0)	100.0 (0)	9.36 (+1.68)	397.6
15	100.0 (0)	100.0 (0)	6.0 (0)	1180.6
16	100.0 (0)	100.0 (0)	6.0 (0)	1,013.4
17	100.0 (0)	100.0 (0)	6.0 (0)	1,222.5

Table 7 ANOVA for inulinase production as response

Source of variation	Sum of squares	Degrees of freedom	Mean square	F test
Regression	1,152,541	4	288,135.3	6.05
Residual	570,653	12	47,554.4	
Lack of fit	546,175	10		
Pure error	24,478	2		
Total	1,723,194	16		

$$R=0.82; F_{0.05; 4; 12}=3.26$$

substrates: 100 g l⁻¹ CSL, 100 g l⁻¹ SCM, and 6 g l⁻¹ of Prodex Lac. In this condition, the activity of the enzyme on inulin was determined. The ratio between the activity on sucrose and the activity on inulin (S/I) was lower than 50, characterizing this enzyme as an inulinase rather than an invertase (Ettalibi and Baratti 1987).

The results show a large variability, suggesting that the concentration of such substrates present a great influence in the enzyme production, probably due to their complex composition of nutrients. A detailed composition of CSL and SCM before and after pretreatment with activated carbon is presented in literature (Valduga et al. 2007). Even after the treatment, residual metal cations were detected, like: zinc, manganese, copper, iron, magnesium, potassium, phosphorous, which can act as inhibitors above certain limit concentrations.

The inulinase activity obtained in the present work is the highest reported in literature so far. Studies about the effect of stirring rate, aeration, and the type of impeller in the production of inulinase found a maximum inulinase activity of 176 U ml⁻¹ (Silva-Santisteban and Maugeri 2005). The use

of synthetic media based in sucrose for inulinase production by *K. marxianus* can yield maximum inulinase production of 127 U ml⁻¹ (Kalil et al. 2001). The use of inulin as carbon source and *K. marxianus* ATCC 52466 resulted in 470 U l⁻¹ of inulinase (Selvakumar and Pandey 1999).

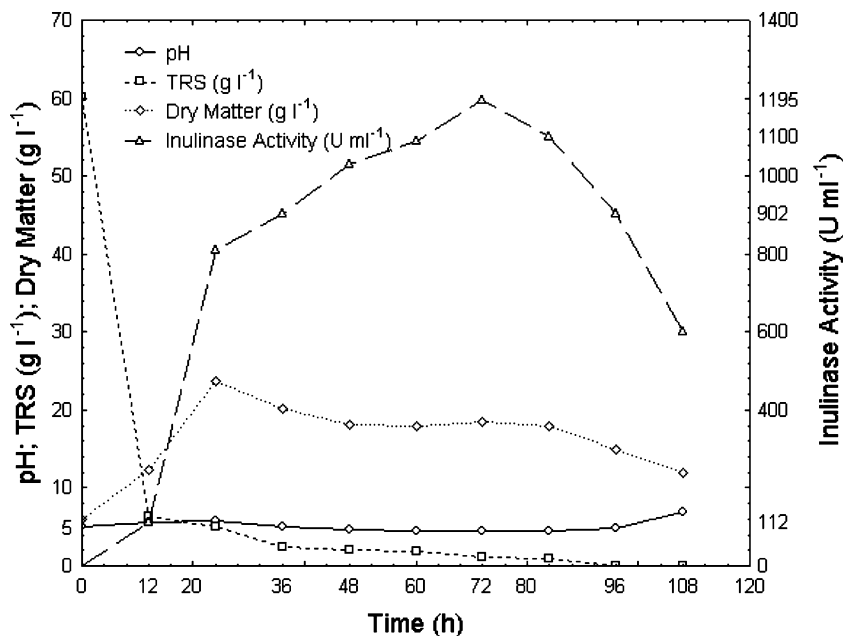
In the present work, production of 1,139±111 U ml⁻¹ shows the feasibility to produce inulinase using low cost substrates as SCM, CSL, and yeast hydrolysate.

The results presented in Table 6 were submitted to statistical analysis, yielding a second order empirical model of activity as function of concentration of molasses (SCM), corn steep liquor (CSL) and Prodex Lac (PL). The model was validated by ANOVA, presented in Table 7. The factors that were not significant ($p < 0.05$) were added to the lack of fit for the analysis of variance (ANOVA). The ANOVA shows a high R (0.93, correlation coefficient) and a good performance of the F test for regression (calculated values about 2 times the listed one). Therefore, Eq. 3 is predictive for inulinase production in the investigated range of factors.

$$\begin{aligned} \text{Activity} = & 1116.2 - 149.4 \times M - 175.4 \times M^2 \\ & - 185.5 \times \text{CSL}^2 - 229.3 \times \text{PL}^2 \end{aligned} \quad (3)$$

Equation 3 shows that the three factors studied present second order significant ($p < 0.05$) parameters. This means that there is an optimum point for inulinase production as a function of concentration of sugarcane molasses, corn steep liquor and Prodex Lac. Maximum inulinase production was obtained at 100 g l⁻¹ SCM, 100 g l⁻¹ CSL, and 6.0 g l⁻¹ of Prodex Lac. Different media compositions yielded lower inulinase activities.

Fig. 2 Kinetics of inulinase production at optimum conditions (100 g l⁻¹ SCM, 100 g l⁻¹ CSL, and 6.0 g l⁻¹ of Prodex Lac). TRS Total reducing sugars



The relative difference between the predicted and the experimental responses was not higher than 10% in all experimental designs. This is a reasonable result for bioproductions using residues.

Figure 2 shows the kinetic behavior of inulinase production in the optimized fermentation condition. The pH of the medium was kept nearly constant during fermentation. Maximum growth occurred at 24 h, and the biomass decreased during the rest of fermentation run. A steep decrease in sugar concentration is observed in the first 12 h of fermentation. It is interesting to note that inulinase production is more evident after 12 h and keeps increasing until 72 h, when maximum production is observed. The results then suggest that inulinase production is partially associated to growth, since the highest rate of production occurred when microbial growth is negligible.

Conclusions

The results presented in this work show the technical feasibility of inulinase production using low cost agro-industrial residues, after some pretreatment. Maximum inulinase activity obtained was 1139 U ml^{-1} , which is the highest inulinase yield ever reported so far. The treatment with sulfuric acid was effective in the clarification of sugarcane molasses (SCM) and treatment with phosphoric acid was effective for clarification of corn steep liquor (CSL). The acid treatment was able to reduce up to 80% of color of both substrates. This is an important result, since this upstream step can simplify the steps of recovery and purification of the enzyme, which may contribute from 50 to 80% of total enzyme production costs.

Acknowledgements The authors thank CAPES/PROCAD and CNPq for the financial support of this work and scholarships.

References

- Atkins, P., & Jones, L. (2005). *Chemical principles: The quest of insight*. New York, USA: Freeman.
- Bender, J. P., Mazutti, M. A., Oliveira, D., Di Luccio, M., & Treichel, H. (2006). Inulinase production by *Kluyveromyces marxianus* NRRL Y-7571 using solid state fermentation. *Applied Biochemistry and Biotechnology*, 129, 951–958.

- Ettalibi, M., & Baratti, J. C. (1987). Purification, properties and comparison of invertase, exoinulinase and endoinulinase of *Aspergillus ficuum*. *Applied Microbiology Biotechnology*, 26, 13–20.
- Kalil, S. J., Suzan, R., Maugeri, F., & Rodrigues, M. I. (2001). Optimization of inulinase production by *Kluyveromyces marxianus* using factorial design. *Applied Biochemistry and Biotechnology*, 94(3), 257–264.
- Kalogiannis, S., Iakovidou, G., Kyriakides, M. L., Kyriakidis, D. A., & Skaracis, G. N. (2003). Optimization of xanthan gum production by *Xanthomonas campestris* grown in molasses. *Process Biochemistry*, 39, 249–256.
- Kasseno, L., & Kokugan, T. (1997). The effect of molasses pretreatment by ceramic micro filtration membrane on ethanol fermentation. *Journal of Bioscience and Bioengineering*, 83(6), 577–582.
- Lazarridou, A., Roukas, T., Biliaderis, C. G., & Vaiakousi, H. (2002). Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. *Enzyme and Microbial and Technology*, 31, 122–132.
- Lee, T. H., Aoki, H., & Sugano, Y. (2000). Effect of molasses on the production and activity of dye-decolorizing peroxidase from *Geotrichum candidum* Dec. *Journal of Bioscience and Bioengineering*, 6, 545–549.
- Mazutti, M. A., Bender, J. P., Di Luccio, M., & Treichel, H. (2006). Optimization of inulinase production by solid-state fermentation using sugarcane bagasse as substrate. *Enzyme and Microbial Technology*, 39, 56–59.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426–428.
- Rodrigues, M. I., & Iemma, A. F. (2005). *Planejamento de Experimentos e Otimização de Processos*. Campinas, Brazil: Editora Casa do Pão.
- Roukas, T. (1998). Pretreatment of beet molasses to increase pullulan production. *Process Biochemistry*, 33, 805–810.
- Sangeetha, P. T., Ramesh, M. N., & Prapulla, S. G. (2005). Recent trends in the microbial production, analysis and application of fructooligosaccharides. *Trends in Food Science and Technology*, 16, 442–457.
- Selvakumar, P., & Pandey, A. (1999). Comparative studies on inulinase synthesis by *Staphylococcus* sp. and *Kluyveromyces marxianus* in submerged culture. *Bioresource Technology*, 69, 123–127.
- Silva-Santisteban, B., & Maugeri, F. (2005). Agitation, aeration and shear stress as key factors in inulinase production by *Kluyveromyces marxianus*. *Enzyme and Microbial Technology*, 36(5–6), 717–724.
- Valduga, E., Treichel, H., Valério, A., Jacques, R., Furigo Júnior, A., & Di Luccio, M. (2007). Pré-Tratamento de Melaço de cana-de-açúcar e água de maceração de milho para a bioprodução de carotenóides. *Química Nova* http://quimicanova.s bq.org.br/qn/Prelo_QN_Artigos.htm.
- Wei, W., Zheng, Z., Liu, Y., & Zhu, X. (1998). Optimizing the culture conditions for higher inulinase production by *Kluyveromyces* sp. Y-85 and scaling-up fermentation. *Journal of Fermentation and Bioengineering*, 86, 395–399.
- Xiong, C., Jinhua, W., & Dongsheng, L. (2007). Optimizations of solid-state medium for the production of inulinase by *Kluyveromyces* S120 using response surface methodology. *Biochemical Engineering Journal*, 34, 179–184.