Journal of Chromatography B, 877 (2009) 3031–3037

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Use of response surface methodology to evaluate the extraction of *Debaryomyces hansenii* xylose reductase by aqueous two-phase system

Janaína Teles de Faria[a], Fábio Coelho Sampaio[b], Attilio Converti[c], Flávia M. Lopes Passos[d], Valéria Paula Rodrigues Minim[a], Luis Antônio Minim[a,*]

[a] Department of Food Technology, Federal University of Viçosa, Av. P. H. Rolfs s/n, 36571-000 Viçosa, Minas Gerais, Brazil
[b] Department of Pharmacy, Federal University of Vales do Jequitinhonha e Mucuri, Rodovia MGT 367 Km 583, 39100-000 Diamantina, Minas Gerais, Brazil
[c] Department of Chemical and Process Engineering, University of Genoa, via Opera Pia 15, 16145 Genoa, Italy
[d] Department of Microbiology, Federal University of Viçosa, Av. P. H. Rolfs s/n, 36571-000 Viçosa, Minas Gerais, Brazil

ARTICLE INFO

Article history:
Received 24 March 2009
Accepted 15 July 2009
Available online 21 July 2009

Keywords:
Enzyme extraction
Xylose reductase
Partition
Aqueous two-phase system
Response surface methodology

ABSTRACT

Xylose reductase (*XR*) from *Debaryomyces hansenii* was extracted by partitioning in aqueous two-phase systems (ATPS) composed of polyethylene glycol (PEG) 4000 in the presence of different salts, specifically sodium sulfate, lithium sulfate and potassium phosphate. Batch extractions were carried out under different conditions of temperature (25–45 °C) and tie-line length (TLL) for each system, according to a central composite design face-centered of 36 tests, and the response surface methodology was used to evaluate the results. Quadratic polynomial models were adjusted to the data to predict the behavior of four responses, namely the *XR* partition coefficient (K_{XR}), the selectivity (S), the purification factor (PF_T) and the activity yield (Y_T) in the top phase. The optimal extraction conditions were found using the PEG 4000/sodium sulfate system at 45 °C and TLL = 25.1, which ensured PF_T = 3.1 and Y_T = 131%. The ATPS proved effective for partial purification of *D. hansenii* xylose reductase in cell-free crude extract, and the response surface methodology revealed to be an appropriate and powerful tool to determine the best dominion of temperature and ATPS composition.

1. Introduction

Xylose-fermenting yeasts and moulds were found to follow the oxido–reductive route via xylitol in xylose conversion [1]. An alternative enzymatic approach for xylitol production is proposed that employs isolated xylose reductase (*XR*, EC 1.1.1.21) [2,3], the enzyme that catalyzes the first step of D-xylose metabolic pathway, i.e. the NAD(P)H-dependent reduction of D-xylose to xylitol [4,5]. In this case, however, the enzyme must be readily available and inexpensive.

Optimization of enzyme recovery and purification is very important for the scale-up of enzymatic processes; therefore, there is increased interest in developing efficient and cost-effective downstream operations to perform high yield-enzyme separation [6].

Aqueous two-phase systems (ATPSs) have been employed as an efficient tool in several biotechnological processes for the partition of proteins, among which α- and β-lactoalbumins [7], proteases [8] and α-toxin [9], nucleic acids, microorganisms, animal and plants cells [10,11], and even phenolic compounds present in hemicellulosic hydrolyzates [12]. Also, ATPSs were successfully used to purify α-galactosidase [6], glucose oxidase, β-galactosidase [13], α-amylase [14], xylanolytic complex [15] and chymosin [16].

These techniques, which are very cheap and ease to scale-up, have the advantages of ensuring high values of the purification parameters, preserving the targeted biomolecules, yielding satisfactory separation performance, and performing, by only one step, their clarification, concentration [17,18] and even partial purification. An ATPS forms when two incompatible polymers or one polymer and an inorganic salt are mixed in low concentrations so that two immiscible phases coexist [17]. In batch process some operating parameters need to be controlled, typically temperature, pH, size and concentration of the targeted biomolecule, and type of polymers and salts employed [19].

The classical method of optimization varying the level of one parameter at a time over a certain range, while holding the rest of the variables constant, is generally time-consuming and requires a large number of experiments to be carried out [20]. These restrictions can be overcome by the use of statistical experimental factorial designs, combined with response surface methodology (RSM).

RSM is a collection of mathematical and statistical techniques that are useful for modeling and analyzing systems where a response of interest is simultaneously influenced by several variables. Therefore, RSM aims at identifying a region of the factor space

* Corresponding author. Tel.: +55 31 3899 1617; fax: +55 31 3899 2208.
E-mail address: lminim@ufv.br (L.A. Minim).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.07.023

that is believed to include the optimum desired responses for a system under investigation [21].

In this work, to identify the most suitable operating conditions for purification of *Debaryomyces hansenii* UFV-170 *XR*, a 2^2 central composite design face-centered was used, varying temperature and tie-line length as the independent variables. The results collected for four response variables, specifically the *XR* partition coefficient, purification factor in the top phase, activity yield and selectivity, were analyzed by RSM.

2. Experimental

Since the results from the present study did not allow establishing whether the enzyme is an aldehyde or an aldose reductase, the term "xylose reductase" or "*XR*" was used.

2.1. Microorganism and maintenance

The new strain *D. hansenii* UFV-170, isolated from samples collected in a dairy industry located in Zona da Mata-MG, Brazil, and belonging to the Collection of the Federal University of Viçosa (UFV), was selected among others as a good xylitol producer. The stock culture was maintained at −80 °C on YPD medium (10 g/l yeast extract; 20 g/l peptone and 20 g/l D-glucose) containing 40% glycerol. Before each experiment, cells were transferred and grown for 48 h at 30 °C on Petri plates containing the above YPD medium supplemented with 15 g/l agar.

2.2. Growth conditions and preparation of crude enzyme extract

Loopfuls of cells from the plates were transferred to 125 ml Erlenmeyer flasks containing 25 ml of the growth medium consisting of 50 g/l D-xylose, 0.62 g/l KH_2PO_4, 2.0 g/l K_2HPO_4, 1.0 g/l $(NH_4)_2SO_4$, 1.1 g/l $MgSO_4$ and 5.0 g/l yeast extract, pH 6.0. Solutions of D-xylose, yeast extract, $MgSO_4$ and the rest of salts were sterilized separately by autoclaving at 121 °C for 20 min. The flasks were maintained at 30 °C under agitation at 200 rpm for 24 h after inoculation with an initial cell concentration of 1.0 g/l (dry cell weight). Cells from a preceding fermentation were harvested by centrifugation (4 °C, 4000 × *g*, 5 min) and washed twice with 0.1 M potassium phosphate buffer, pH 7.2. The final pellet, having a cell mass concentration of about 7–8 g/l (dry cell weight), was resuspended in 5 ml 0.1 M potassium phosphate, pH 7.2. Cells were disrupted passing the suspension three times through a French Pressure Mini-cell (Urbana, IL, USA) at 19,000 psi and 5 °C. During this operation, the suspension was kept cooled at 4 °C to prevent overheating. Cells debris was removed by 30 min centrifugation at 17,000 × *g*. The enzyme activities and protein concentration were determined in the supernatant.

2.3. ATPS preparation

Aqueous two-phase systems were prepared using different tie-line lengths (TLL) according to the phase diagram presented by Carvalho et al. [22]. Stock solutions of PEG with 4000 g/mol molecular weight (PEG 4000) (60%, w/w), sodium sulfate (25%, w/w), lithium sulfate (25%, w/w) and potassium phosphate (25%, w/w) were prepared. The pH values of sodium and lithium sulfate solutions were very close to 7.0, so they did not need any adjustment, whereas the pH of mixtures containing potassium phosphate was adjusted to 7.0 by adding mono and dibasic potassium phosphate in the proportion of 1:1.82, respectively. Aliquots of the salt stock, water, and PEG stock were mixed in 50 mL graduated centrifuge tubes and weighed, to achieve the desired composition of the system. The total mass of the system was kept at 40 g. The resulting mixtures were centrifuged at 2000 × *g* for 20 min to speed up the phase separation and maintained at different temperatures for 24 h to allow equilibration. After this period, 5.0 ml of each phase were collected to prepare the new 10 ml systems.

2.4. Partitioning of XR in ATPS

Aliquots of 100 μl of crude enzyme extract were added to the ATPS. After homogenization, the mixtures were centrifuged at 1800 × *g* for 5 min to allow equilibration. In accordance with the central composite design face-centered (CCF), they were then exposed to different temperatures in a thermostated bath for 8 h to allow separation. After measurement of their volumes, top and bottom phases were separated and analyzed for determinations of enzyme activities and protein concentration.

2.5. Partition parameters

The *XR* partition coefficient (K_{XR}) was defined as the ratio of *XR* (E.C. 1.1.1.21) volumetric activity in the top phase ($[U]_T$) to that in the bottom phase ($[U]_B$) (see Section 2.7):

$$K_{XR} = \frac{[U]_T}{[U]_B} \tag{1}$$

The protein partition coefficient (K_{ptn}) was calculated as ratio of the protein concentration in the top phase, $[ptn]_T$, and to that in the botton phase ($[ptn]_B$):

$$K_{ptn} = \frac{[ptn]_T}{[ptn]_B} \tag{2}$$

The selectivity (S) was defined, according to Mayerhoff et al. [23], as the ratio of K_{XR} to K_{ptn}:

$$S = \frac{K_{XR}}{K_{ptn}} \tag{3}$$

The purification factor in the top phase (PF_T) was calculated as the ratio of the specific activity in the top phase ($[U]_T/[ptn]_T$) to that in the crude extract ($[U]_{CE}/[ptn]_{CE}$):

$$FP_T = \frac{[U]_T/[ptn]_T}{[U]_{CE}/[ptn]_{CE}} \tag{4}$$

where $[ptn]_{CE}$ is the protein concentration in the crude extract.

The activity yield (Y_T) was calculated as the ratio of total activity in the top phase to that in the crude extract and expressed as percentage:

$$Y_T = \frac{[U]_T V_T}{[U]_{CE} V_{CE}} \times 100 \tag{5}$$

where V_T and V_{CE} are the volumes of the top phase and the crude extract, respectively.

2.6. Experimental design

The response surface methodology (RSM) combined with a 2^2 CCF was used to optimize the *XR* partition in three different ATPSs (PEG 4000/sodium sulfate, PEG 4000/lithium sulfate and PEG 4000/potassium phosphate). Four central points were added to calculate the experimental error and to investigate the suitability of the proposed model. The two independent variables investigated in this study, the temperature and TLL (Table 1), were chosen on the basis of our knowledge about their important effects on *XR* partition [23].

In particular, the TLL was defined as:

$$\mathrm{TLL} = \{([S]_T - [S]_B)^2 + ([P]_T - [P]_B)^2\}^{1/2} \tag{6}$$

where $[S]_T$, $[S]_B$, $[P]_T$, and $[P]_B$ are the salt and PEG mass fractions in the top and the bottom phases, respectively. So, the TLL, expressed

Table 1
Factors and levels investigated for *D. hansenii XR* partitioning by different ATPSs according to the CCF.

Coded values	Independent variable	Range and level		
		−1	0	+1
x_1	Temperature (°C)	25	35	45
x_2	TLL (mass fraction)			
	(i) PEG4000/sodium sulfate	22.0	34.0	46.0
	(ii) PEG4000/lithium sulfate	33.0	43.0	53.0
	(iii) PEG4000/potassium phosphate	34.0	43.5	53.0

in mass fraction (dimensionless), is directly related to both the total polymer mass fractions and the interactions between the two polymers.

Salt and PEG mass fractions in the top and bottom phases were obtained from the phase diagram presented by Carvalho et al. [22].

K_{XR}, FP_T, Y_T and S were chosen as the dependent variables or responses, whose actual values are listed in Table 2. The behavior of the system can be described by the following second-order polynomial:

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (7)$$

where Y is the predicted response, β_o is the interception coefficient, β_i are the linear terms, β_{ii} are the quadratic terms, β_{ij} are the interaction terms, and x_i and x_j are the coded levels of the independent variables.

The Student's t-test permitted us to check the statistical significance of the regression coefficients. The Fisher's test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model. The "Statistica" software (trial version 6.0, StatSoft, Tulsa, OK) and the "Design Expert" software (trial version 6.0.10, Stat-Ease, Minneapolis, MN) were employed for the regression analysis and the graphical optimization, respectively.

The models of the two responses were expressed in terms of coded variables, without taking into account the statistically insignificant terms.

2.7. Analytical methods

The volumetric activity of *XR* was assayed by following the oxidation of the coenzyme NADPH at 340 nm with a spectrophotometer, series 600 (Beckman, Fullerton, CA). To this purpose, the methodology described by Cortez et al. [24] was employed with modification, and the activity was expressed as U/ml of cell free extract. The reagents were maintained in a water bath for 10 min at 39 °C [25]. Assays were carried out in 1.30 ml-cuvettes containing 570 μl of distilled water, 80 μl of 1.0 M phosphate buffer, 150 μl of 0.1 M mercaptoethanol, 100 μl of 1.3 mM NADPH, 200 μl of cell-free extract and 200 μl of 0.5 M xylose. The reaction was started by the addition of xylose.

The protein content was determined by the method of Bradford [26]. One enzyme unit was defined as the amount of *XR* that transforms 1 μmol NADPH per minute under the assay conditions. All reaction rates were proportional to the amount of enzymes added in the assay.

The specific activity was expressed as U/mg of total proteins. All tests were performed in quadruplicate and expressed as mean values. The central point was repeated four times to estimate the experimental error as well as to investigate the suitability of the proposed models. All experimental data differed from the mean by less than 5%.

3. Results and discussion

Table 2 shows the experimental results obtained for the PEG 4000/sodium sulfate, PEG 4000/lithium sulfate and PEG 4000/potassium phosphate systems at different levels of the above independent variables, the tie-line length (TLL) and the temperature (T), according to the 2^2 central composite design face-centered (CCF) depicted in Table 1.

Depending on the conditions, the values of the *XR* partition coefficient (K_{XR}), selectivity (S), purification factor in the top phase (PF_T) and activity yield (Y_T) varied in the ranges 0.54–4.4, 0.52–6.0, 0.23–3.1 and 49.0–147%, respectively, in the PEG 4000/sodium sulfate system; 0.64–2.9, 0.55–2.5, 0.27–1.9 and 63.5–223% in the PEG 4000/lithium sulfate system; and 0.24–1.3, 0.35–3.0, 0.28–1.1 and 58.3–159% in the PEG 4000/potassium phosphate one. As suggested by Mayerhoff et al. [23], yields higher than 100% are the likely result of elimination of inhibitors from the systems, which enhances the enzymatic activity. Values of K_{XR} greater than unity indicate effective partitioning in the aqueous two-phase system. Besides, negatively charged proteins usually prefer the top phase in PEG/salt systems, and the partition coefficient consequently increases [17,27]. Although the isoelectric point (p*I*) of *D. hansenii XR* is unknown, those produced by similar microorganisms have p*I* around 4.1 [28]. So, to ensure always a negative net charge in *XR*

Table 2
Design matrix and results of *D. hansenii XR* partitioning by PEG/lithium sulfate, PEG/lithium sulfate and PEG/potassium phosphate ATPSs according to the CCF.

Run	T	TLL	PEG/sodium sulfate				PEG/lithium sulfate				PEG/potassium phosphate			
	x_1	x_2	K_{XR}	S	PF_T	Y_T (%)	K_{XR}	S	PF_T	Y_T (%)	K_{XR}	S	PF_T	Y_T (%)
1	−1	−1	0.54	0.61	0.30	53.0	1.0	1.3	0.56	63.5	0.32	0.60	0.52	61.9
2	−1	0	1.1	0.92	0.25	60.1	1.3	1.3	0.73	78.0	0.24	0.35	0.28	58.3
3	−1	+1	0.65	0.52	0.23	49.0	2.0	1.4	0.27	81.2	0.36	0.58	0.35	72.9
4	0	−1	0.87	0.93	0.79	79.5	0.64	0.78	1.4	89.5	0.35	0.49	1.1	80.9
5	0	0	1.1	1.2	0.52	70.0	1.8	1.6	1.6	195	0.70	1.2	0.78	148
6	0	0	0.80	0.72	0.48	70.7	2.6	2.2	1.9	223	0.64	1.7	1.0	133
7	0	0	1.0	1.2	0.51	67.8	1.7	1.5	1.6	193	0.67	1.1	0.78	148
8	0	0	0.59	0.53	0.42	61.4	2.9	2.5	1.9	219	0.67	1.7	1.1	147
9	0	+1	1.4	1.1	0.43	78.2	2.4	1.4	1.2	217	1.3	3.0	0.89	159
10	+1	−1	4.4	6.0	3.1	124	1.2	0.75	1.2	112	0.26	0.66	0.73	81.1
11	+1	0	3.1	1.7	1.3	147	0.78	0.55	0.67	96.8	0.22	0.40	0.37	68.7
12	+1	+1	1.9	1.7	0.68	123	1.2	0.78	0.56	107	0.36	0.76	0.37	72.4

during partitioning, the pH was maintained in this work at 7.0.

The development of a purification procedure using ATPS requires the variation of several factors to get the optimum enzyme separation. In this study, we utilized the Response Surface Methodology as a tool to identify the ATPS composition and temperature able to maximize *XR* partitioning.

A regression analysis was carried out to fit mathematical models to the experimental data aiming at identifying an optimal region for a given response. Such models, describing the relationships among selected independent variables and responses, took into account only the statistically significant (quadratic) terms. The regression was accepted when the *F*-value of the model was higher than the corresponding critical *F*-value or when the *P*-value was lower than the significance level (<0.01). However, if none of theses conditions was fulfilled by a given model, it was only accepted when the coefficient of determination (R^2) was higher than 0.90, which means that more than 90% of the data were explained by it.

According to these criteria, only the PF_T and Y_T models resulted to be significant for all the three systems under consideration. The analysis of variance (ANOVA) indicated no significance of linear and quadratic models for *S* at 90% confidence level and lack of fit in both cases; therefore, its statistical evaluation was omitted. Since the same took place for the K_{XR} models, except for the sodium sulfate system (data not shown), they were not taken into consideration as well.

3.1. PEG 4000/sodium sulfate system

The statistical significance of the two models for the PEG 4000/sodium sulfate system was evaluated by the *F*-test, which revealed that this regression was statistically significant (*P*-value <0.01) at a confidence level of 99% (Table 3). Moreover, the fit of the models was checked by R^2, which was found to be 0.918 and 0.940 for PF_T and Y_T, respectively.

The Student's *t*-test was used to assess the significance of the regression coefficients. The quadratic and linear contributions of temperature were significant for both responses, while the linear contribution of TLL and the interaction between TLL and temperature were significant only for PF_T .

After elimination of statistically insignificant terms (*P*-value >0.01), the coefficients were adjusted, and the models resulted to be:

$$PF_T = 0.55 + 1.02x_1 - 0.50x_2 - 0.57x_1x_2 + 0.74x_1^2 \quad (8)$$

$$Y_T = 71.26 + 38.57x_1 + 21.34x_1^2 \quad (9)$$

where x_1 and x_2 are the coded values of temperature and TLL, respectively.

Fig. 1 illustrates the combined effects of these independent variables on PF_T (A) and Y_T (B) as surface plots. It should be noticed that, whereas PF_T increased with simultaneously increasing temperature and decreasing TLL, Y_T only increased with temperature beyond 30–35 °C.

3.2. PEG 4000/lithium sulfate system

The ANOVA summarized in Table 3 for the PEG 4000/lithium sulfate system shows that the models calculated for PF_T and Y_T were significant. *F*-values of 17.05 and 10.74 and *P*-values of 0.0009 and 0.0041, respectively, did in fact demonstrate that the regressions performed by these equations were statistically significant at 99% confidence level. Moreover, the values of R^2 (0.791 and 0.705, respectively) demonstrate that the models explained 79.1% and 70.5% of the total variations in the responses.

The Student's *t*-test evidenced that both PF_T and Y_T were influenced only by the linear and quadratic contributions of temper-

Table 3
Results of ANOVA for *D. hansenii XR* partitioning by PEG/sodium sulfate, PEG/lithium sulfate and PEG/potassium phosphate ATPSs according to the CCF.

Source	PEG/sodium sulfate					PEG/lithium sulfate					PEG/potassium phosphate				
	DF[a]	SS[b]	MS[c]	*F*-value	*P*-value	DF[a]	SS[b]	MS[c]	*F*-value	*P*-value	DF[a]	SS[b]	MS[c]	*F*-value	*P*-value
PF_T															
Model	4	6.38	1.59	19.56	0.0007	2	2.70	1.35	17.05	0.0009	2	0.80	0.40	15.41	0.0012
Residual	7	0.57	0.082			9	0.71	0.079			9	0.23	0.026		
Lack of fit	4	0.56	0.14	69.71	0.0027	6	0.63	0.10	3.69	0.1556	6	0.13	0.022	0.67	0.6886
Pure error	3	6.075×10^{-3}	2.025×10^{-3}			3		0.028			3	0.099	0.033		
Total	11	6.95				11	3.42				11	1.03			
Y_T															
Model	2	10293.81	5146.91	71.07	<0.0001	2	31224.75	15612.37	10.74	0.0041	2	13514.29	6757.15	14.54	0.0015
Residual	9	651.81	72.42			9	13084.66	1453.85			9	4183.19	464.80		
Lack of fit	6	597.38	99.56	5.49	0.0953	6	12337.85	2056.31	8.26	0.0557	6	4022.76	670.46	12.54	0.0314
Pure error	3	54.43	18.14			3	746.81	248.94			3	160.43	53.48		
Total	11	10945.62				11	44309.41				11	17697.49			

[a] Degrees of freedom.
[b] Sum of square.
[c] Mean square.

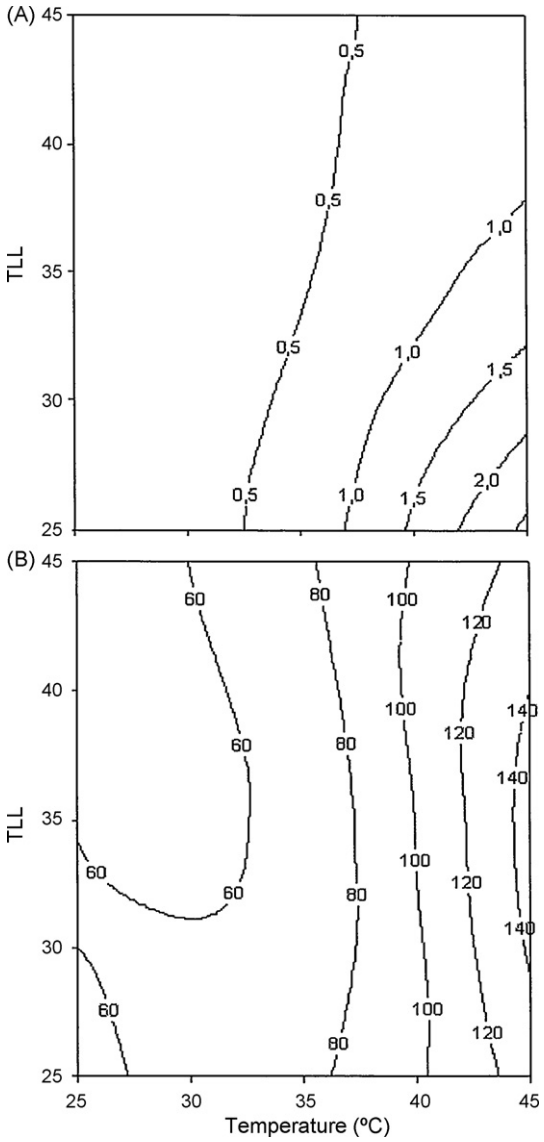

Fig. 1. Response surfaces of the purification factor in the top phase (A) and the activity yield (B) for the PEG/sodium sulfate system, as simultaneous functions of TLL (mass fraction) and temperature (°C).

ature. After elimination of statistically insignificant terms (P-value >0.01), the coefficients were adjusted and the models resulted to be:

$$PF_T = 1.59 + 0.15x_1 - 0.931x_1^2 \quad (10)$$

$$Y_T = 189.4 + 15.54x_1 - 99.63x_1^2 \quad (11)$$

Fig. 2 shows the response surfaces for PF_T (A) and Y_T (B) obtained by these models. Within the factor space investigated in this study, when temperature was higher than 35 °C an increase in TLL led to a decrease in PF_T, whereas below 35 °C it led to a decrease in PF_T for TLL ≥ 44 and an increase for TLL ≤ 44. On the other hand, whereas Y_T always rose with TLL, for a given value of TLL it increased with temperature up to a temperature threshold and then decreased. Nevertheless, such a temperature threshold was about 35 °C for TLL ≥ 42 and progressively increased at lower TLL values.

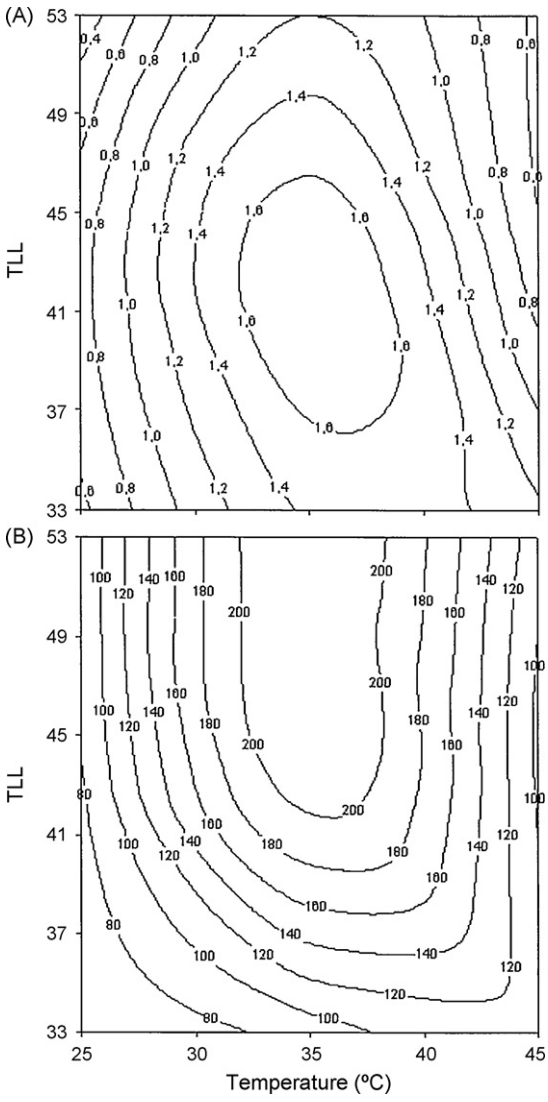

Fig. 2. Response surfaces of the purification factor in the top phase (A) and the activity yield (B) for the PEG/lithium sulfate system, as simultaneous functions of TLL (mass fraction) and temperature (°C).

3.3. PEG 4000/potassium phosphate system

The ANOVA presented in Table 3 for the PEG 4000/potassium phosphate system showed that also in this case the models for PF_T and Y_T were significant. F-values of 15.41 for PF_T and 14.54 for Y_T as well as their corresponding P-values (0.0012 and 0.0015) and determination coefficients (R^2 = 0.774 and 0.764) demonstrate that the regressions were statistically significant at 77.4% and 76.4% confidence levels.

The Student's t-test showed that both PF_T and Y_T were influenced only by the linear and quadratic contributions of temperature. After elimination of statistically insignificant terms (P-value >0.01), the coefficients were adjusted, and the models became:

$$PF_T = 0.95 + 0.053x_1 - 0.51x_1^2 \quad (12)$$

$$Y_T = 135.99 + 4.84x_1 - 66.77x_1^2 \quad (13)$$

A detailed presentation of the optimum values predicted by the models using the isoresponse contour is given in Fig. 3. Within the factor space investigated in this study, both PF_T and Y_T reached their maximum values at about 35 °C and decreased either below or beyond this temperature threshold. However, apart some

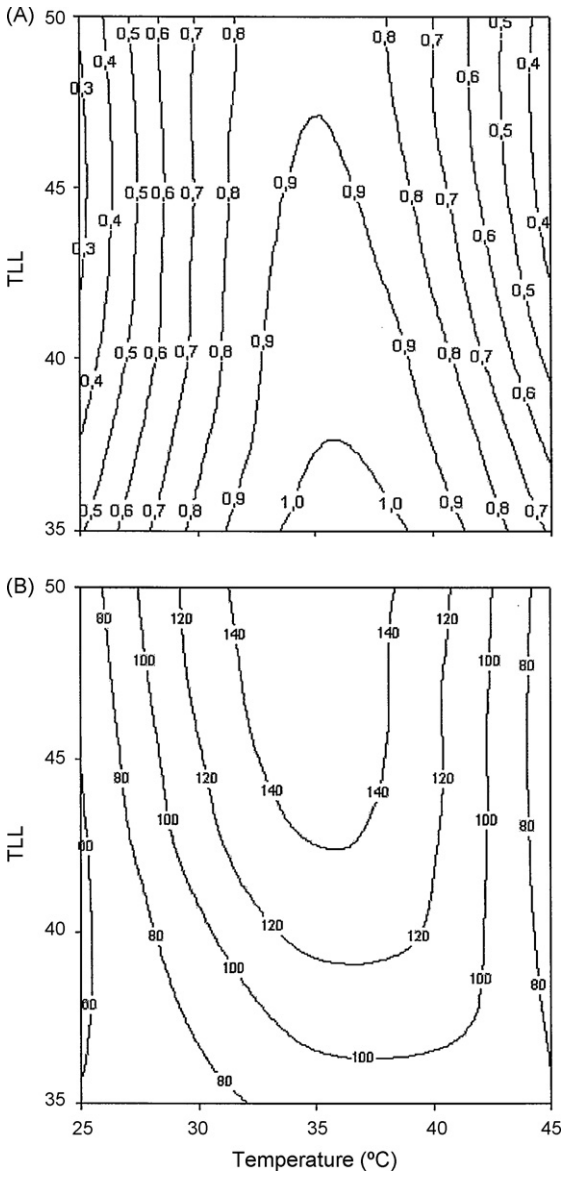

Fig. 3. Response surfaces of the purification factor in the top phase (A) and the activity yield (B) for the PEG/potassium phosphate system, as simultaneous functions of TLL (mass fraction) and temperature (°C).

points below 27 °C, PF_T increased with decreasing TLL, whereas Y_T decreased.

3.4. Numerical optimization

With the aim of definitively pointing out the optimal conditions for *XR* partial purification, a numerical optimization was carried out using the "Design Expert" software. Such a methodology essentially consists of overlaying the curves of the four models, obtained from the CCF, according to the specific criteria imposed.

The optimal working conditions are presented in Table 4 for the three systems. It should be noticed that the best results were obtained with the PEG/sodium sulfate system, for which the following constraints were imposed $PF_T = 3.1$ and $Y_T = 131\%$. On the other hand, the values PF_T and Y_T were for the PEG/lithium sulfate system 1.6 and 190%, respectively, and for the PEG/potassium phosphate one only 0.95 and 136%, respectively. In fact, although the overall performance of a partitioning system should be established on the basis of the results of all the selected responses, in the first system

Table 4
Optimal values of temperature and TLL estimated by overlaying plots of the response surfaces of the purification factor (PF_T) and activity yield (Y_T) in the top phase in three different ATPSs.

System	T (°C)	TLL (mass fraction)	PF_T (−)	Y_T (%)
PEG/sodium sulfate	45	25.1	3.1	131
PEG/lithium sulfate	36	42.1	1.6	190
PEG/potassium phosphate	35	43.4	0.95	136

the purification factor in the top phase, which is well accepted as the most important one, was about twice that of the second and more than 3-fold that of the third.

To confirm that the optimum operating conditions established for the PEG/sodium sulfate system can indeed provide desired outcome in larger scale, one confirmation experiment was performed in duplicate using 2 ml cell free extract, which yielded results practically coincident to those obtained with smaller volume (200 μl). In similar study, using overlay plot of response surface, Mayerhoff et al. [23], found that the best conditions for ATPS partition of *XR* from *Candida mogii* ($PF_T = 1.89$ and $Y_T = 103.5\%$) were obtained with PEG 1000 and Na/K phosphate at TLL = 34.

4. Conclusions

This study showed that aqueous two-phase systems were efficient for partial purification for *D. hansenii* xylose reductase in cell free crude extract, showing the possibility of pre-purification by the removal of some contaminants. The response surface methodology combined to an adequate factorial experimental design proved to be an appropriate and powerful tool to determine the best dominion of temperature and ATPS composition. After numerical optimization, it was possible to conclude that PEG 4000/sodium sulfate was the most adequate system to perform *XR* partition at $T = 45$ °C and TLL = 25.1. Under these experimental conditions, the predicted values of *XR* purification factor in the top phase and activity yield were 3.1 and 131%, respectively, and these results were confirmed using a 10-fold crude extract volume.

Acknowledgements

The authors wish to thank the Brazilian agencies CNPq and FAPEMIG for the financial support.

References

[1] C. Chiang, S.G. Knight, Nature 188 (1960) 79.
[2] B. Nidetzky, W. Neuhauser, D. Haltrich, K.D. Kulbe, Biotechnol. Bioeng. 52 (1996) 387.
[3] S.M.A. Rosa, M.G.A. Felipe, S.S. Silva, M. Vitolo, Appl. Biochem. Biotechnol. 70–72 (1998) 127.
[4] V. Nolleau, L. Preziosi-Belloy, J.P. Delgenes, J.M. Navarro, Curr. Microbiol. 65 (1993) 3594.
[5] M. Kern, D. Haltrich, B. Nidetzky, K.D. Kulbe, FEMS Microbiol. Lett. 149 (1997) 31.
[6] K. Naganagouda, V.H. Mulimani, Process Biochem. 43 (2008) 1293.
[7] L.A. Minim, O.S. Monteiro, J.S.R. Coimbra, J.A. Oliveira, L.H.M. Silva, J. Chromatogr. B 866 (2008) 1.
[8] T.S. Porto, G.M. Silva, C.S. Porto, M.T.C.V. Soares, B. Barros-Neto, J.L. Lima-Filho, A. Converti, A.L.F. Porto, A. Pessoa Jr., Chem. Eng. Proc. 47 (2008) 716.
[9] M.T.C.V. Soares, T.S. Porto, B. Barros-Neto, J.L. Lima-Filho, A.L.F. Porto, A. Pessoa Jr, J. Chem. Technol. Biotechnol. 83 (2008) 158.
[10] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Application to Biotechnology, Academic Press, Orlando, FL, 1985.
[11] M.E.D. Silva, T.T. Franco, J. Chromatogr. B 743 (2000) 287.
[12] F.A. Hasmann, V.C. Santos, D.B. Gurpilhares, A. Pessoa Jr., I.C. Roberto, J. Chem. Technol. Biotechnol. 83 (2008) 167.
[13] S.B. Dhoot, J.M. Dalal, V.G. Gaikar, Sep. Sci. Technol. 42 (2007) 1859.
[14] W. Zhi, J. Song, F. Ouyang, J. Bi, J. Biotechnol. 118 (2005) 157.
[15] S.A. Costa, A. Pessoa Jr., I.C. Roberto, J. Chromatogr. B 43 (2000) 339.
[16] G. Reh, D. Spelzini, G. Tubío, G. Picó, B. Farruggia, J. Chromatogr. B 860 (2007) 98.

[17] P.A. Albertsson, Partition of Cell Particles and Macromolecules, 3rd ed, John Wiley & Sons, New York, 1986.
[18] H. Hustedt, K.H. Kroner, N. Papamichale, Process Biochem. (1988) 129.
[19] R.M. Banik, A. Santhiagu, B. Kanari, C. Sabarinath, S.N. Upadhyay, World J. Microbiol. Biotechnol. 19 (2003) 337.
[20] R. Sen, T. Swaminathan, Appl. Microbiol. Biotechnol. 47 (1997) 358.
[21] D.C. Montgomery, Design and Analysis of Experiments, 5th ed., John Wiley and Sons, New York, 2001.
[22] C.P. Carvalho, J.S.R. Coimbra, I.A.F. Costa, L.A. Minim, H.M.S. Silva, M.C. Maffia, J. Chem. Eng. Data 52 (2007) 351.
[23] Z.D.V.L. Mayerhoff, I.C. Roberto, T.T. Franco, Biochem. Eng. J. 18 (2004) 217.
[24] E.V. Cortez, M.G.A. Felipe, I.C. Roberto, A. Pessoa Jr., M. Vitolo, Appl. Biochem. Biotechnol. 91/93 (2001) 753.
[25] F.C. Sampaio, J.Y. de Faria, F.M. Lopes Passos, A. Converti, L.A. Minin, J. Ind. Microbiol. Biotechnol. 36 (2009) 293.
[26] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[27] P.A. Albertsson, A. Cajarville, D.E. Brooks, F. Tjerneld, Biochim. Biophys. Acta 926 (1987) 87.
[28] H. Lee, Yeast 14 (1998) 977.