J Ind Microbiol Biotechnol (2009) 36:293–300
DOI 10.1007/s10295-008-0498-3

ORIGINAL PAPER

Optimal activity and thermostability of xylose reductase from *Debaryomyces hansenii* UFV-170

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Received: 7 August 2008 / Accepted: 23 October 2008 / Published online: 27 November 2008

Abstract Xylose reductase (XR) is the enzyme that catalyzes the first step of xylose metabolism. Although XRs from various yeasts have been characterized, little is known about this enzyme in *Debaryomyces hansenii*. In the present study, response surface analysis was used to determine the optimal conditions for *D. hansenii* UFV-170 XR activity. The influence of pH and temperature, ranging from 4.0 to 8.0 and from 25 to 55°C, respectively, was evaluated by a 2^2 central composite design face-centered. The *F*-test (ANOVA) and the Student's *t* test were performed to evaluate the statistical significance of the model and the regression coefficients, respectively. The NADPH-dependent XR activity varied from 0.502 to 2.53 U mL^{-1}, corresponding to 0.07–0.352 U mg^{-1}, whereas the NADH-dependent one was almost negligible. The model predicted with satisfactory correlation ($R^2 = 0.940$) maximum volumetric activity of 2.27 U mL^{-1} and specific activity of 0.300 U mg^{-1} at pH 5.3 and 39°C, which were fairly confirmed by additional tests performed under these conditions. The enzyme proved very stable at low temperature (4°C), keeping its activity almost entirely after 360 min, which corresponded to the half-time at 39°C. On the other hand, at temperatures ≥50°C it was lost almost completely after only 20 min.

Keywords Xylose reductase · *Debaryomyces hansenii* · Experimental design · Response surface methodology · Enzyme activity

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Introduction

Xylitol, a five carbon sugar alcohol, is manufactured by catalytic chemical reduction of xylose present in hemicellulosic hydrolysates [15]. Alternatively, this conversion can be performed by microbiological methods [40]. Nevertheless, the conversion process using microorganisms can lead to unsatisfactory production of xylitol due the influence of some culture conditions [5, 6, 39]. An alternative enzymatic approach was proposed that employs isolated xylose reductase (XR, EC 1.1.1.21) [22, 28], 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 [16, 23].

When released from the natural protective environment, XR activity is influenced by several parameters including pH and temperature [32]. However, 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 [33]. 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 situations in which a response of interest is influenced by

several variables, and the objective is to optimize this response. Therefore, aim of RSM is the determination of the optimum operating conditions for a system under investigation or of a region of the factor dominion within which the operating requirements are satisfied [20].

To identify the most suitable operating conditions for XR activity of *Debaryomyces hansenii* UFV-170, a 2^2 central composite design face-centered (CCF) was used in this work, varying pH and temperature in the ranges 4–8 and 25–55°C, respectively. The results collected for two response variables, specifically the volumetric and the specific activities, were worked out by RSM. The XR stability was also investigated after storage at different temperatures.

Materials and methods

Since the results of this work do not allow the specification whether the enzyme is an aldehyde or an aldose reductase, the term "xylose reductase" or "XR" will be used.

Microorganism and maintenance

The new strain *D. hansenii* UFV-170, isolated from samples collected in a dairy industry in Zona da Mata-MG, Brazil, and belonging to the collection of the UFV, was selected among others as a promising xylitol producer. The stock culture was maintained at −80°C on YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} 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^{-1} agar.

Growth conditions and preparation of cell extracts

Loopfuls of cells from the plates were transferred to 125-mL Erlenmeyer flasks containing 25 mL of the growth medium containing 50 g L^{-1} D-xylose, 0.62 g L^{-1} KH_2PO_4, 2.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} $(NH_4)_2SO_4$, 1.1 g L^{-1} $MgSO_4$ and 5.0 g L^{-1} 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. After inoculation of the flasks with an initial biomass concentration of 1.0 g L^{-1} (dry weight), cells were grown at 30°C under agitation at 200 rpm. Once achieved the early stationary phase (about 24 h), where cells exhibited the highest XR activity (unpublished results), they were harvested by centrifugation (4°C, 4,000*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^{-1} (dry 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. Cell debris was removed by 30-min centrifugation at 17,000*g*. The enzyme activities and protein concentration were determined in the supernatant.

Experimental design

The RSM combined with a 2^2 CCF was used to point out the relationship existing between the response functions and the process variables as well as to determine the conditions of these variables able to optimize the XR activity. Three central points were added to estimate the experimental error and to investigate the suitability of the proposed model. The two independent variables investigated in this study, i.e., the initial pH and temperature, were chosen on the basis of our knowledge on their effects on XR activity [19] and coded according to the equation:

$$x_i = \frac{(X_i - X_o)}{\Delta X_i} \quad i = 1, 2, 3 \tag{1}$$

where x_i and X_i are the dimensionless and the actual values of the independent variable i, X_o the actual value of the independent variable i at the central point, and ΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value.

Volumetric (U mL^{-1}) and specific activity (U mg^{-1}) were chosen as the dependent variables or responses. The behavior of the system can be described by the following second-order polynomial:

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \tag{2}$$

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 and without taking into account the statistically insignificant terms.

Xylose reductase stability

The XR stability was evaluated at the optimal pH for this enzyme (pH 5.3). Extract samples free of cells were kept for 360 min at 4 and 39°C, being the former temperature representative of cold storage and the latter the optimum one for the enzyme activity [19]. After this storage period, the samples were exposed for 20 min at 1, 7, 20, 30, 35, 40, 47 and 50°C and the residual enzyme activity was determined.

Analytical methods

The optical density at 600 nm (OD_{600}) was determined by a spectrophotometer, series 600 (Beckman, Fullerton, CA, EUA), and then related to cell concentration, X (g dry weight L^{-1}) through a calibration curve ($OD_{600} = 1.6717X$). XR (EC 1.1.1.21) volumetric activities were assayed by following the oxidation of the coenzymes NADH or NADPH at 340 nm with the same instrument. To this purpose, the methodology described by Cortez [7] was employed with modification, and the activities were expressed as U mL^{-1} of free cell extract. For each temperature, reagents were maintained for 10 min in a water bath. Assays were carried out in 1.30 mL-cuvettes containing 570 µL distilled water, 80 µL 1.0 M phosphate buffer, 150 µL 0.1 M mercaptoethanol, 100 µL 1.3 mM NADPH or NADH, 200 µL cell-free extract and 200 µL 0.5 M xylose. The reaction was started by addition of xylose. The protein content was determined by the method of Bradford [4]. One enzyme unit was defined as the amount of XR that oxidized 1 µmol NADPH or NADH per minute under the assay conditions. All reaction rates were proportional to the amount of enzymes added in the assay. The specific activities were expressed as U mg^{-1} of total proteins. All tests were performed in triplicate and expressed as mean values.

Results and discussion

Volumetric and specific XR activities

As extensively reviewed by Parajó et al. [25], pentose-fermenting yeasts are the best xylitol producers, and among these those belonging to the genera *Candida* and *Debaryomyces* are by far the microorganisms with the highest XR activities; for this reason we have selected the new promising *D. hansenii* UFV-170 isolate to perform this work.

The enzyme XR is responsible for the first step in the metabolism of xylose, thereby reducing such a pentose to xylitol. Depending on the microorganism, this reaction can use NADPH or NADH as a cofactor. According to Gírio et al. [11] and Roseiro et al. [29] the accumulation of xylitol by yeasts grown in xylose is associated with the complete absence of NADH-dependent XR activity.

Many studies carried out with free cell-crude extract have demonstrated that XR has a greater or even absolute preference for NADPH as a cofactor. Suzuki and Onishi [36] identified many forms of aldose reductase in *Pichia quercuum* that had activities restricted to NADPH. Sirisansaneeyakul et al. [35] observed that *Candida mogii* ATCC 18364 reduces D-xylose to xylitol by preferentially using a NADPH-dependent XR, and Nolleau et al. [24] proved the same cofactor specificity in *Candida parapsilosis*, *Candida guilliermondii* and *Candida* sp. XRs. These results were confirmed for *C. guilliermondii* FTI 20037 [34] and *C. guilliermondii* ATCC 20118 [12]. XRs from *Pichia stipitis* [38] and *Candida tenuis* [21] were shown to be active using both NADH and NADPH. Finally, even cell extracts of the yeast-like fungus *Aureobasidium pullulans* grown in media with different carbon sources exhibited rigorously NADPH-dependent XR activity [18].

However, there is no systematic work in the literature on the purification and characterization of *D. hansenii* XR. Therefore, we investigated in this study both NADH- and NADPH-dependent XR activities.

Table 1 shows the volumetric and specific NADPH-dependent XR activities of free cell-enzymatic extract of *D. hansenii* UFV-170 grown in semi-synthetic culture medium containing xylose as the sole source of carbon and energy. Maximum volumetric activities of 0.323 ± 0.017 U mL^{-1} and 0.015 ± 0.009 U mL^{-1} and maximum specific activities of 0.096 ± 0.005 U mg^{-1} and 0.006 ± 0.002 U mg^{-1} were observed with NADPH and NADH, respectively. Although these results demonstrate that *D. hansenii* UFV-170 XR can utilize both cofactors, consistently with the above-discussed literature, it is evident that the former resulted to be always negligible; therefore, it will be not taken into consideration anymore.

D-xylose fermenting *P. stipitis* and *Candida shehatae* were shown to produce only one XR active either with NADPH or NADH [14]. On the other hand, two isoenzymes were observed either in *Pachysolen tannophilus* [37] or in the fungus *Neurospora crassa* [42]; however, in the former both isoenzymes were NADPH- and NADH-dependent, while in the latter one of them was exclusively

Table 1 Xylose reductase activity in the crude cell extract of *D. hansenii* UFV-170 grown on D-xylose

Type	Volumetric activity (U mL^{-1})	Specific activity (U mg^{-1})
NADPH-dependent	0.323 ± 0.017	0.096 ± 0.005
NADH-dependent	0.015 ± 0.009	0.006 ± 0.002

NADH-dependent. The present results, however, do not allow establishing if the observed NADPH-dependent XR activity was due to only one enzyme or to the presence of multiple isoenzymes.

High XR activity is a fundamental requisite to develop a catalytic conversion process using the enzyme directly [28]. However, this activity is influenced by various operating conditions such as pH and temperature. Therefore, to study the simultaneous influence of several independent variables on one or more responses, the use of statistical methodologies is recommended. Experimental designs have been successfully applied to optimization of bioprocess steps [8, 26, 27] or enzyme reactions [2, 3, 17, 19].

Table 2 shows the matrix of both the actual and coded values of the selected independent variables, together with the results of tests planned according to the 2^2 CCF. Each test was performed in triplicate and the central point was repeated four times (lines 3–4, 11–12) to estimate the experimental error as well as to investigate the suitability of the proposed model. The NADPH-dependent XR volumetric activity ranged between 0.502 and 2.53 U mL^{-1} and the specific one from 0.070 and 0.352 U mg^{-1}, depending on the conditions. The highest value was observed under acidic conditions (pH 6.0) at 40°C, whereas the lowest one under alkaline conditions (pH 8.0) at 25°C.

The quadratic models calculated for the two dependent variables Y_1 (volumetric activity) and Y_2 (specific activity), after the elimination of the non-statistically significant terms ($P > 0.05$), were:

$$Y_1 = 2.11 - 0.50x_1 + 0.045x_2 - 0.84x_1^2 + 0.26x_1 \cdot x_2 \quad (3)$$

$$Y_2 = 0.29 - 0.069x_1 + 0.006167x_2 - 0.12x_1^2 + 0.036x_1 \cdot x_2 \quad (4)$$

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

The analysis of variance (ANOVA) presented in Table 3 showed that the model was significant. The Fisher's test was conducted on the experimental data to evaluate the statistical significance of the model. *F*-values of 16.66 and 16.64 for Y_1 and Y_2, respectively, as well as the corresponding *P*-value and R^2 (0.0011 and 0.940 for both models) demonstrate that the regressions were statistically significant at 94.0% confidence level.

The Student *t* test was used to assess the significance of the regression coefficients. Both Y_1 and Y_2 responses were influenced by the linear contributions of x_1 (pH) and x_2 (temperature), the quadratic contribution of x_1 and the interaction between these two variables ($x_1 \cdot x_2$). Only the quadratic contribution of temperature (x_2^2) was not statistically significant ($P > 0.05$). After elimination of this term, the model coefficients were adjusted and are presented in Eqs. 3 and 4.

The variation of the two responses evaluated as functions of the independent variables is shown in Fig. 1. Both responses increased when the system passed from alkaline environment (pH 8.0) to an acidic one, reached maximum values (~2.2 U mL^{-1} and ~0.3 U mg^{-1}, respectively) at pH 5–6, and fell down at pH < 5. Under alkaline

Table 2 Results of NADPH-dependent XR activity in the cell-free crude extract of D-xylose-grown *D. hansenii* UFV-170 collected from tests planned according to the 2^2 central composite design face-centered

Run	Independent variables				Responses	
	Coded values		Actual values		Volumetric activity (U mL^{-1})	Specific activity (U mg^{-1})
	x_1^a	x_2^b	pH	Temperature (°C)		
1	+1	0	8	40	0.722	0.101
2	0	+1	6	55	1.87	0.261
3	0	0	6	40	2.26	0.315
4	0	0	6	40	2.11	0.294
5	−1	0	4	40	1.83	0.254
6	−1	−1	4	25	1.97	0.274
7	+1	−1	8	25	0.502	0.070
8	0	−1	6	25	1.72	0.239
9	−1	+1	4	55	1.50	0.209
10	+1	+1	8	55	1.08	0.150
11	0	0	6	40	2.15	0.299
12	0	0	6	40	2.53	0.352

[a] Coded values of initial pH

[b] Coded values of temperature

Table 3 Results of analysis of variance (ANOVA) for XR volumetric (U mL^{-1}) and specific (U mg^{-1}) activities according to the 2^2 central composite design face-centered

Response	Source	SS[a]	DF[b]	MS[c]	*F*-value	*P*-value
Volumetric activity	Model	3.90	4	0.970	16.66	0.0011
	Residual	0.41	7	0.059		
	Lack of fit	0.30	4	0.076	2.15	0.2783
	Pure error	0.11	3	0.035		
	Total	4.31	11			
Specific activity	Model	0.075	4	0.019	16.64	0.0011
	Residual	0.008	7	0.001		
	Lack of fit	0.006	4	0.001	2.13	0.2806
	Pure error	0.002	3	<0.001		
	Total	0.083	11			

[a] Sum of squares

[b] Degrees of freedom

[c] Mean square

conditions or close to the neutrality, the increase in temperature led to a slight increase in XR activity. On the other hand, under acidic conditions, the activity increased when temperature was in the range 35–45°C.

Based on the model proposed for XR specific activity, a new region of operating conditions was selected (pH 5.3 and 39°C), where it could be possible to achieve the maximum value of this activity (0.300 U mg^{-1}). Under the conditions defined by the model, the predicted value was confirmed experimentally (0.324 U mg^{-1}) with satisfactory accordance.

The pH (5.3) and temperature (39°C) selected for the activity of XR are in reasonable agreement with previous results on the production of xylitol by the same yeast [30].

Although the intracellular activity of XR was reported to depend on the pH of the cultivation medium and the incubation temperature in most of pentose-fermenting yeasts [25], other physiological characteristics may have been influenced by the cultivation conditions and thus led to the highest accumulation of xylitol.

The optimum pH and temperature for the activity of aldose (xylose) reductase of various microorganisms show a very large species-dependent variability. To make only a few examples, Neuhauser et al. [21] found pH 8.0 and 50°C as the best conditions for the activity of an aldose reductase purified from *C. tenuis*, while Mayerhoff et al. [19] reported pH 6.5 and 38°C for XR of *C. mogii* extracted by aqueous two-phase system (ATPS). Under standard assay conditions, Verduyn et al. [38] observed an optimum pH of 6.0 for purified XR from *P. stipitis* CBS 5773 as well as an Arrhenius plot of NADPH-linked XR activity in the range 20–38°C. XR purified from *P. tannophilus* showed an optimum pH of 7.0, although no less than 60% of maximum activity was observed between pH 4.5 and 9.0 [9].

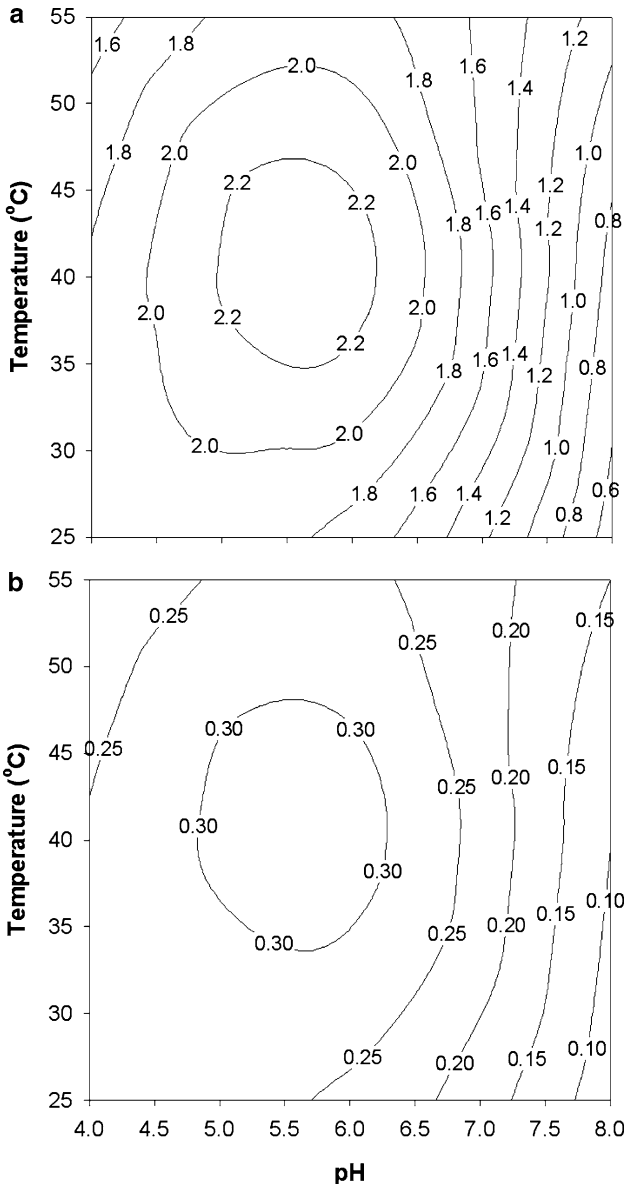

Fig. 1 Isoresponse contour plot of NADPH-dependent XR volumetric (**a**) and specific (**b**) activities as simultaneous functions of temperature and pH

For XR purified from *P. quercuum* [36] and *Candida utilis* [31], the optimum pH was between 5.5–7.6 and 6.2, respectively. However, it is necessary to emphasize that the physico-chemical characteristics that determine the activity of a purified or semi-purified enzyme may be quite different from those of the enzyme contained in crude extracts, because there is the influence of other components such as proteins, enzyme and so on, which may be interacting with the enzyme under investigation.

A final question is whether or not other effects may have influenced the enzyme activity when varying temperature and pH. Indeed, it is well known that the maximal initial rates of enzyme reactions and Michaelis constants depend

on the concentration of the buffer ions. These data may be interpreted in terms of the ionization degree of the amino acids at the reactive site as well as the thermodynamic water activity (i.e., the enzyme hydration level), which are critical parameters for the catalytic activity, prevent excess enzyme dehydration or promote an adequate enzyme ionization state [13, 41]. For phosphate buffers the anion concentration is dependent on pH since the relative concentrations of $H_2PO_4^-$ and HPO_4^{2-} vary with it [1, 10]. So, the initial protonation state of the enzyme is expected to be dictated by the pH of the aqueous buffer. Unfortunately, however, there is not enough data describing the influence of pH and buffer concentration/type on the kinetic properties of XR from *D. hansenii*. Without such an information, in the present study the concentration of buffer was maintained always the same for the different treatments, and such secondary effects were omitted for the sake of simplicity.

Thermal stability of XR

Besides the optimum temperature for the enzyme reaction, also the thermal stability of the enzyme is of great concern to define and select the storage conditions of the cell-free crude extract. The stability of XR in such an extract was evaluated at storage temperature varying from 4 to 39°C. Figure 2 shows the results of relative activity assuming the starting volumetric activities as reference values (1.49 and 1.51 U mL^{-1} at 4 and 39°C, respectively).

At 4°C, which is representative of cold storage, the enzyme remained stable for 360 min, while at the optimum temperature of the enzyme (39°C) a 50% reduction of the enzyme activity was observed after the same time.

The stability of XR was also evaluated after keeping the cell-free crude extract for 20 min at different temperatures in the wide range of 1–50°C (Fig. 3). As expected, the highest residual activity was detected at the lowest temperature (0.99 U mL^{-1} at 1°C), and then it was taken as a reference value (100%) for comparison. The relative XR activity showed a small reduction up to 47°C (by about 20%), then it fell down at 50°C (0.025 ± 0.012 U mL^{-1}), and practically vanished at 58 and 70°C (results not shown).

Mayerhoff et al. [19] observed almost complete thermal stability of XR from *C. mogii* after exposition of the enzyme at 4 and 38°C for 180 min, whereas the residual activity decreased markedly (by about 20%) after 240 min and showed a half-life of almost 300 min.

In disagreement with these and our results, the activity of a purified aldose (xylose) reductase of *C. tenuis* increased linearly from 25 up to 50°C, and its stability after 48 h-incubation started to decrease at 30–35°C [21].

Conclusions

This study showed that the RSM combined to an adequate factorial experimental design was an appropriate and powerful tool to determine the best conditions of pH (5.3) and temperature (39°C) to measure the activity of NADPH-dependent XR from *D. hansenii* UFV-170. In cell-free crude extract, the enzyme showed a good stability after exposition at 4°C for 360 min, which is a quite promising

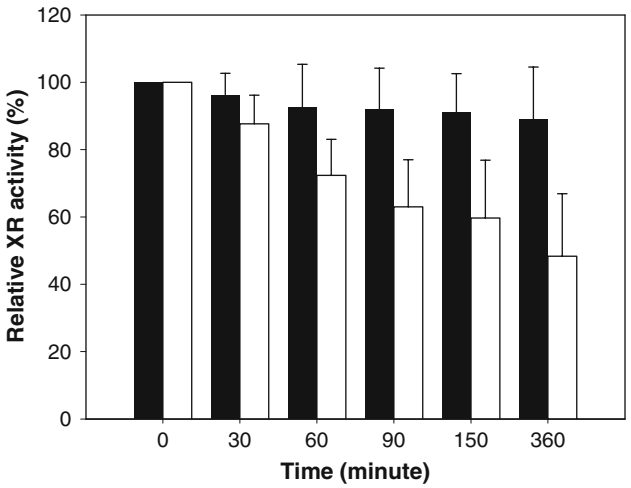

Fig. 2 Relative XR activity in cell-free extracts of *D. hansenii* UFV-170 maintained at 4°C (*filled bar*) and 39°C (*open bar*) along the time. Initial activities at 4°C (1.49 U mL^{-1}) and 39°C (1.51 U mL^{-1}) were assumed as 100%

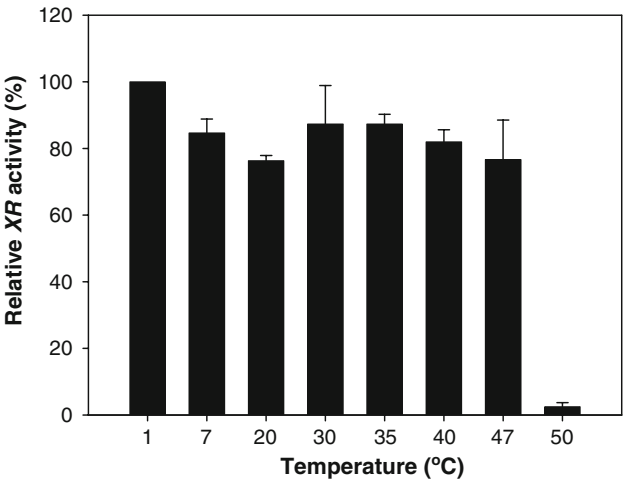

Fig. 3 Relative XR residual activity in cell-free extracts of *D. hansenii* UFV-170 maintained at temperatures between 1 and 50°C for 20 min. The activity at 1°C (0.99 U mL^{-1}) was assumed as 100%

feature to avoid activity loss during the manipulation of the extract for industrial purposes.

The results of the present study add knowledge to the development of a new bioprocess in which the cell-free crude extract containing *D. hansenii* XR may be used to perform the industrial xylose-to-xylitol bioconversion. Besides, they could be used as a starting basis for new studies devoted to the semi-purification or purification of this and similar enzymes, which will be the target of next studies.

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

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