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ETHANOL PRECIPITATION AS A DOWNSTREAM PROCESSING STEP FOR CONCENTRATION OF XYLANASES PRODUCED BY SUBMERGED AND SOLID-STATE FERMENTATION

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Abstract – Xylanases have applications in different industries, being produced by microbial cultivations using submerged (SmF) or solid-state fermentation (SSF). Precipitation stands out as a potential method for the concentration of xylanases, especially with the use as ethanol as the precipitant due to its compatibility with the biorefinery concept. This paper presents a comparative laboratory scale study of ethanol precipitation of xylanases produced by *Aspergillus niger* cultivated under SSF and SmF. Precipitation conditions were selected according to a central composite design. Statistical analysis showed a significant effect of pH on the recoveries of total protein and xylanase activity. The kinetic profiles showed that a relatively short period of time (up to 15 min) was sufficient to recover most of the xylanase activity precipitated under the selected conditions. Xylanase recoveries of 65 and 79% were achieved for the SSF and SmF enzymatic complexes, respectively.

Keywords: Aspergillus niger; downstream processing; ethanol precipitation; xylanase.

INTRODUCTION

Hemicellulose is the second most abundant renewable natural polymer and, together with lignin and cellulose, forms the highly complex plant cell wall structure (Pauly et al., 2013; Shallom and Shoham, 2003; Wong et al., 1988). Hemicelluloses are branched heteropolysaccharide complex compounds made of D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-O-methyl-glucuronic acid. Removal of their side chains is required to increase the rate of enzymatic degradation (Santos et al., 2012). Xylan, the major component of hemicellulose, consists of a backbone of β-xylopyranose residues joined by β-1,4-glycosidic linkages, decorated with acetyl, arabinofuranosyl, and glucuronic or 4-O-methylglucuronic acid groups (Saha, 2003). The endo-1,4-β-xylanase (xylanase) enzyme cleaves the β-1,4-glycosidic linkage between xylose residues in the backbone of xylan and is essential for the depolymerization of hemicellulose (Dodd and Cann, 2009). Xylanase enzymes have applications in the food industry, as well as in other technological sectors such as pulp and paper production (Collins et al., 2005). In the biofuels sector, the action of xylanases improves cellulose conversion during the hydrolysis of biomass for cellulosic

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ethanol production by removing hemicellulose and increasing the accessibility of the substrate to cellulase enzymes (Gao et al., 2011; Kumar and Wyman, 2009). On an industrial scale, xylanases are mainly produced by filamentous fungi of the genera *Aspergillus* and *Trichoderma* (Park et al., 2002), using submerged fermentation (SmF) or solid-state fermentation (SSF). About 80-90% of all xylanases are produced in submerged culture (Polizeli et al., 2005). Nevertheless, the use of SSF is particularly advantageous for enzyme production by filamentous fungi, because it simulates the natural habitat of these microorganisms and, from an environmental perspective, enables the use of agro-industrial residues as sources of carbon and energy for microorganism growth and enzyme production (Farinas, 2015; Holker and Lenz, 2005).

Production of xylanases using strains of *Aspergillus* under both SmF and SSF have been widely reported (Betini et al., 2009; Chapla et al., 2010; Chipeta et al., 2008; Fang et al., 2010; Ghanem et al., 2000; Pirota et al., 2013; Venegas et al., 2013), as these fungi exhibit favorable fermentation characteristics, including high protein secretion rates and the ability to produce a wide range of extracellular enzymes (de Vries, 2003). However, most of the aforementioned studies have mainly focused on the enzyme production step, with evaluation of different fungal strains and the effects of the operational conditions used in the cultivation process. Given that both SSF and SmF cultivations usually result in enzymatic complexes at concentration levels too low for immediate application in industrial processes, there is an urgent need for studies focusing on downstream processing (DSP) unit operations. The concentration step is especially necessary in the development of processes for on-site production of enzymes, in order to improve the catalytic efficiency of the enzymatic cocktails.

The choice of DSP unit operations is a major challenge in defining a biotechnological process, because loss of the bioproduct in the multiple operations of separation, concentration, and purification can compromise the efficiency of the entire process. Furthermore, cost reduction is a priority in the case of industrial enzymes. Given these considerations, the precipitation technique can be very effective in concentrating xylanase preparations, especially since high purity (which is typically achieved using chromatographic processes) may not be required for applications in areas such as the biofuels sector (Farinas et al., 2011). Although there are numerous reports concerning the isolation and purification of xylanases, most only describe the precipitation step for a single specific condition, usually for the purposes of biochemical characterization (Ahmad et al., 2013; Bokhari et al., 2009; Chanwicha et al., 2015; Lu et al., 2008; Mander et al., 2014; Ninawe et al., 2008).

From a bioprocess engineering standpoint, identification of the optimal operational conditions for the precipitation of proteins involves the study of different process variables, including the type and concentration of the precipitant, temperature, aging time, agitation, pH, and so forth. In terms of the precipitant, the use of a renewable material such as ethanol is especially attractive, because it complies with the biorefinery concept (Kamm and Kamm, 2004), since it is a bioproduct of the biorefinery itself, available *in site* at low cost. Ethanol is one of the most important industrial precipitants, and its use can lead to a favorable balance between the solubility effect and an appropriate hydrophilicity, minimizing protein denaturation (Golunski et al., 2011). However, in order to achieve high precipitation recovery of xylanase activity, a relatively high ethanol concentration (≥80%, v/v) is usually required (Fadel, 2001; Marino et al., 2015; Varma et al., 1999). This increases cost as well as the risk of protein denaturation, hence compromising the feasibility of the DSP. Therefore, a systematic investigation of the precipitation operational parameters has to be undertaken to enable this process to be effectively applied for each specific system.

Considering the industrial importance of xylanase enzymes, together with the high availability and low cost of ethanol, the purpose of this study was to carry out a systematic comparative evaluation of the precipitation with ethanol of xylanases produced by *A. niger* under SSF and SmF, and to determine the feasibility of applying this process as a cost-effective DSP unit operation. Prior to the precipitation studies, preliminary evaluations were made of the thermal and pH stabilities of the xylanases from both sources, together with determination of the cloud points following ethanol addition. Experimental design methodology was then used as a tool to investigate the effects of ethanol concentration and pH on xylanase precipitation recovery.

MATERIALS AND METHODS

Microorganism

The *Aspergillus niger* A12 strain was obtained from Embrapa Food Technology (Rio de Janeiro, Brazil) (Couri and deFarias, 1995). The strain was kept at -18

ºC and was activated by incubation on slants of potato dextrose agar (PDA) medium for four days at 32 ºC. Suspensions of spores were prepared by the addition of 10 mL of Tween 80 (0.3%, v/v). Spore concentrations were determined using a Neubauer chamber.

Solid-state fermentation (SSF)

The solid-state fermentation cultivations were carried out in 250 mL conical flasks containing 5 g of dry wheat bran sterilized by autoclaving at 121 ºC for 15 min. The moisture content of the medium was then adjusted by the addition of 3 mL of a nutrient medium (Mandels and Sternberg, 1976). A concentration of 10^7 spores/g of dry solid substrate was added, and the cultivations were conducted under static conditions at 32 ºC for 72 h. The enzymes were extracted by the addition of 1:10 (w/v, mass of dry wheat bran to volume of extraction solution) 0.05 mol/L sodium acetate buffer solution (pH 4.5) and agitating the flasks in a shaker at 200 rpm, 32 ºC, for 30 min. The final enzymatic extracts were filtered, centrifuged at 12,900 *g* for 10 min, and kept frozen at -18 ºC prior to the analytical assays. All the cultivation experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

Submerged fermentation (SmF)

In the submerged fermentation procedure, the preculture was initiated with a 10^7 spores/mL conidial suspension, in 250 mL conical flasks containing 50 mL of nutrient medium (Mandels and Sternberg, 1976) enriched with 30 g/L of glucose. The incubation was carried out for 50 h in a shaker at 32 ºC, with stirring at 200 rpm. Aliquots (10 mL) of preculture suspension were transferred to 250 mL conical flasks containing 40 mL of the culture medium supplemented with 10 g/L of glucose and 1% (w/v) of wheat bran sterilized by autoclaving at 121 ºC for 15 min. The cultivations were performed for 72 h in a shaker at 32 ºC and 200 rpm. The cultivation broth was then filtered, centrifuged at 12,900 *g* for 10 min, and the resulting crude enzymatic extract was stored at -18 ºC prior to further analysis. All the cultivation experiments were performed in triplicate, and the data were calculated as means ± standard deviations.

Enzyme thermal and pH stability

The thermal stability of the xylanase was evaluated by measuring the residual enzymatic activity after incubation of 1.0 mL of the crude fermentation broth (SmF) or extract (SSF) with 1.0 mL of a 0.2 mol/L sodium acetate pH 5.0 buffer solution (for pH adjustment) at 15, 30, and 45 ºC for up to 180 min. For the pH stability study, the pH of the enzyme preparations was adjusted to pH 3.0, 4.0, 5.0, and 6.0 using 0.2 mol/L sodium citrate buffer, and to pH 6.0, 7.0, and 8.0 using 0.2 mol/L sodium phosphate buffer. These pH-adjusted enzyme preparations (1.0 mL volumes) were incubated at 30 ºC for a total period of 180 min. In each of these studies, sampling was performed after time intervals of 10, 30, 60, 120, and 180 min. At the end of the incubation period, the samples were centrifuged at 12,900 *g* for 10 min and the supernatants were used for total protein concentration and xylanase activity analyses. All the experiments were carried out in triplicate, and the data were calculated as means ± standard deviations. The data obtained were used to do linear fits for protein concentration and xylanase activity ($Prot|_t$ and $Activ|_t$, respectively) as a function of time.

Cloud point determination

For determination of the cloud point (the minimum concentration of ethanol added to the enzyme preparation required to cause visual clouding of the solution), the pH of the enzyme preparation (2.0 mL volume) was adjusted to 5.5 by adding 9 volumes of a 2.0 mol/L sodium citrate pH 5.5 buffer in a 15 mL Falcon tube. The tube was weighed and then placed in an ice bath, followed by dropwise addition of ethanol at -7 ºC, at a flow rate of 1 mL/min, until clouding was visually detected. The mixture was allowed to rest for 10 min, and if the turbidity was maintained, the tube was weighed again in order to determine the final mass of ethanol required to reach the cloud point. Ten replicate determinations were performed for each enzyme preparation (SSF and SmF).

Experimental design

A full factorial design followed by response surface analysis was used to evaluate the effects of two variables (pH and ethanol concentration, individually and in interaction) on the precipitation of the xylanases. The experimental design selected was a central composite design (CCD) comprising 11 runs, corresponding to four cube points, four axial points, and three central points, with the experiments carried out in random order. The pH varied from 3.4 to 7.6 and the ethanol concentration varied from 78 to 92% (v/v) (a preliminary study showed that, below 70% ethanol, recovering of activity and protein in the precipitate were too low for precise quantification). The dependent

variables (responses) were xylanase activity recovery, total protein recovery, specific activity, and purification factor. Statistica v. 8.0 software (Statsoft) was used to analyze the experimental data, perform analysis of variance (ANOVA) calculations, and plot the response surfaces.

For this entire set of precipitation experiments, the pH of the enzyme preparations was first adjusted to the values established in the experimental design, using 2.0 mol/L sodium citrate buffer at a 1:9 (v/v) sample:buffer ratio. A 1.0 mL volume of each sample was transferred to a 50 mL Falcon tube. The tubes were then continuously stirred in an ice bath at -7 ºC, while ethanol was added dropwise, at a flow rate of 1 mL/min, until the desired concentration was reached. The tubes were then incubated at 15 ºC for 3 h, followed by centrifugation at 12,900 *g* for 10 min. The precipitate was suspended in 5.0 mL of 0.2 mol/L sodium citrate buffer (pH 5.0) for the quantification of total protein and xylanase activity. All the experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

Kinetics of xylanase precipitation with ethanol

For the kinetic study of xylanase precipitation with ethanol under the selected process conditions, the pH values of the crude SSF and SmF enzyme preparations were adjusted to pH 5.5 (as described before) and 1.0 mL volumes were transferred to 15 mL Falcon tubes. The tubes were kept under stirring in an ice bath at -7.0 ºC and ethanol was added dropwise until reaching a concentration of 85% (v/v). The tubes were then incubated at 15 ºC, withdrawn at different times, and centrifuged at 12,900 *g* for 10 min. The precipitates were dissolved in 5.0 mL of 0.2 mol/L sodium citrate buffer (pH 5.0) for the quantification of total protein and xylanase activity. All the experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

Enzyme activity assay

The xylanase activity was measured according to the methodology described by Bailey and Poutanen (Bailey and Poutanen, 1989), with incubation of 1.0 mL of the suitably diluted samples at 50 ºC for 30 min with a substrate of 1% beechwood xylan (Sigma, USA) solution prepared in 0.20 mol/L sodium acetate buffer (pH 5.0). Here, one unit of xylanase activity corresponds to 1 µmol of xylose released per minute at pH 5.0 and 50 ºC. Quantification of the reducing groups was performed according to the dinitrosalicylic acid (DNS) method (Miller, 1959).

Total protein

The total protein concentrations in the enzymatic samples were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Thermal and pH stability of xylanase

A preliminary set of experiments was carried out to evaluate the thermal and pH stability of the xylanase present in the crude enzymatic preparations obtained by cultivation of *A. niger* under SSF and SmF. These analyses were required in order to establish the ranges of pH and temperature that would be appropriate in the subsequent precipitation studies. The xylanase stability results are presented in terms of the linear fitting parameters obtained by plotting the residual activity as a function of incubation time of the crude enzyme preparations at different temperatures and pH.

In the case of thermal stability (Table 1), no significant changes in xylanase activity or protein concentration were detected up to 180 min of incubation at 15, 30, or 45 ºC, for both SSF and SmF enzyme preparations, as shown by the very low angular coefficient values obtained for all the temperatures tested. These results indicated that the xylanases produced by *A. niger* presented good thermal stability (for the temperatures and time periods tested). In addition, it is noteworthy that no difference was observed between the thermal stabilities of the xylanases obtained from the different cultivation systems.

These findings are in agreement with literature reports on the thermal stability of xylanases from different *Aspergillus* strains. For instance, Guimarães et al. (2013) found that the xylanase produced by *A. niger* under SmF retained over 85% activity after 2 h of incubation at 50 ºC. Xylanase produced by *A. terreus* under SmF showed no significant decrease in activity during 3 h incubation at 30 and 45 ºC (Bakri et al., 2010). Similar thermal stability has also been reported for xylanases produced under SSF by *A. niger*, *A. ochraceus*, and *A. niveus*, which remained stable for up to 1 h at 50 ºC (Betini et al., 2009).

Table 2 presents the pH stability results obtained for incubation of the SSF and SmF enzyme preparations at different pH. Similar to the previous results for thermal stability, no significant changes were observed

Table 1. Thermal stability study of xylanase enzymes produced by *A. niger* under SSF and SmF: parameters of the linear fits for residual enzyme activity ($Ativ|_t$) or protein concentration ($Prot|_t$), as a function of time.

	Temperature (°C)	Xylanase activity(IU/mL)			Protein concentration (mg/mL)		
		$\mathbf{Activ}|_0$	$\mathbf{a_{activ}}(10^{-3})$	$\mathbf{R^2}$	$\mathbf{Prot}|_0$	$\mathbf{a_{prot}}(10^{-5})$	$\mathbf{R^2}$
	15	6.50	2.6	0.970	0.23	1.0	0.983
SSF	30	6.84	2.1	0.975	0.22	20.0	0.880
	45	6.90	-1.1	0.984	0.22	2.0	0.964
	15	6.22	-1.2	0.980	0.07	-4.0	0.957
SmF	30	6.34	-1.1	0.983	0.05	8.0	0.931
	45	6.39	1.3	0.977	0.05	10.0	0.906

Values from linear fitting of the plots of protein concentration or xylanase activity, as a function of time. $Ativ|_t = Ativ|_0 + a_{ativ}t$ and $Prot|_t = Prot|_0 + a_{prot}t$. $Ativ|_0$ and $Prot|_0$, linear coefficients; $a_{ativ}t$ and $a_{prot}t$, angular coefficients; R^2, correlation coefficient.

in xylanase activity or protein concentration after incubation of the crude enzyme preparations for 180 min at 30 ºC, using pH ranging from 3.0 to 8.0. It is interesting to note that for pH 6.0, similar stability results were obtained using two different buffers, indicating that the nature of the salts present in the buffers did not affect the analysis.

The results corroborated the findings of other studies concerning the pH stability of xylanase from *Aspergillus*. Guimarães et al. (2013) found that *A. niger* xylanase retained over 95% of the initial activity after incubation at pH in the range from 3.0 to 8.0 for up to 1 h. Xylanases from *A. ochraceus* and *A. terricola* presented stability greater than 70% when incubated in the pH range from 2.5 to 8.0 for up to 1 h (Michelin et al., 2010). Xylanase from *A. niger* was stable at pH in the range from 2.0 to 7.0, retaining over 75% activity after 1 h, while xylanases of *A. ochraceus* and *A. niveus* were stable at pH from 3.0 to 7.0, with more than 70% activity retained (Betini et al., 2009).

In light of all the previous results concerning the thermal and pH stability of *A. niger* xylanase from both SSF and SmF cultivation systems, temperatures between 15 and 45 ºC and pH values in the range from 3.0 to 8.0 were selected for evaluation of ethanol precipitation. The xylanases were quite stable over these ranges of temperature and pH, within the period studied (up to 3 h). This information is important in order to avoid any misinterpretation of the following ethanol precipitation results.

Table 2. Stability, at different pH, of xylanase enzymes produced by *A. niger* under SSF and SmF: parameters of the linear fits for residual enzyme activity or protein concentration, as a function of time.

	pH	Xylanase activity (IU/mL)			Protein concentration (mg/mL)		
		$\mathbf{Activ}|_0$	$\mathbf{a_{activ}}(10^{-3})$	$\mathbf{R^2}$	$\mathbf{Prot}|_0$	$\mathbf{a_{prot}}(10^{-5})$	$\mathbf{R^2}$
	3.0[a]	8.78	3.6	0.911	0.16	9.0	0.790
	4.0[a]	9.09	-4.2	0.821	0.21	-2.0	0.964
	5.0[a]	7.61	4.6	0.917	0.24	-20.0	0.931
SSF	6.0[a]	8.59	5.8	0.950	0.24	-10.0	0.918
	6.0[b]	9.65	0.8	0.972	0.25	-5.0	0.988
	7.0[b]	9.32	-3.3	0.967	0.25	-10.0	0.945
	8.0[b]	8.94	-1.0	0.909	0.26	-4.0	0.930
	3.0[a]	7.67	-0.6	0.861	0.06	0.7	0.931
	4.0[a]	8.96	-4.6	0.841	0.07	3.0	0.890
	5.0[a]	6.93	8.3	0.971	0.08	-9.0	0.897
SmF	6.0[a]	8.72	6.6	0.898	0.08	-6.0	0.949
	6.0[b]	8.58	0.8	0.895	0.08	1.0	0.970
	7.0[b]	8.67	-1.7	0.967	0.09	-3.0	0.937
	8.0[b]	8.17	-2.7	0.926	0.10	-5.0	0.929

Values from linear fitting of the plots of protein concentration or xylanase activity, as a function of time. $Ativ|_t = Ativ|_0 + a_{ativ}t$ and $Prot|_t = Prot|_0 + a_{prot}t$. $Ativ|_0$ and $Prot|_0$, linear coefficients; $a_{ativ}t$ and $a_{prot}t$, angular coefficients; R^2, correlation coefficient.

[a]Buffer: 0.2 mol/L sodium citrate.

[b]Buffer: 0.2 mol/L sodium phosphate.

Determination of the cloud point

The proteins from SSF and SmF showed slightly different behaviors in terms of the minimum concentration of ethanol required for detection of a visible haze in the medium (the cloud point). The crude enzyme preparation from SSF required 50.8% (v/v) of ethanol to reach the cloud point, while the SmF preparation required an average of 56.2% (v/v) of ethanol. This could have been due to the higher concentration of proteins in the SSF medium (0.24 mg/mL), compared to the SmF medium (0.11 mg/mL), which enabled supersaturation to be achieved at a lower ethanol concentration. A higher initial concentration of proteins in the medium leads to higher supersaturation, at a given concentration of ethanol, which in turn leads to increased protein precipitation (Nakadai and Nasuno, 1989). Therefore, it was important to know the cloud point in order to determine the minimum concentration of ethanol required in the subsequent precipitation studies.

Ethanol precipitation of xylanases

An overall analysis of the results of xylanase precipitation using ethanol, performed according to the 2^2 central composite design, showed that protein recoveries in the precipitates from SSF were mostly higher than in those from SmF (Table 3). This could have been due to the 2.4-fold higher initial concentration of proteins in the SSF medium, compared to the SmF medium. The same rationale could be extended to the xylanase activity, since the highest recoveries were obtained from the SmF medium, with initial activity values of 7.09 and 5.16 IU/mL for SmF and SSF, respectively. The crude enzyme preparations from SSF and SmF also presented different initial xylanase specific activities (17.79 IU/mg for SSF and 59.08 IU/mg for SmF), as well as different final xylanase specific activities in the precipitates, hence resulting in different purification factors.

For the precipitation with ethanol using the SSF medium, the highest recoveries of protein (between 78.9 and 86.2%) and xylanase activity (between 59.8 and 64.4%) were obtained in test runs 7-11, in which the initial pH was 5.5, irrespective of the concentration of ethanol used (Table 3). In the case of the precipitations carried out with the SmF broth, adjustment of the pH to 5.5 and increasing the ethanol concentration from 78 to 92% (v/v) resulted in 22% and 16% increases in the recoveries of protein and xylanase activity, respectively.

Pareto chart analysis was used to determine the degree of significance of the effect of each variable on the recoveries of xylanase activity and protein, for SSF (Figure 1) and SmF (Figure 2). For SSF, only the effect of pH was statistically significant ($p<0.05$) for the recoveries of both xylanase activity (Figure 1A) and protein (Figure 1B). For xylanase recovery from precipitation using the SmF broth, only the quadratic pH effect was significant, within the range evaluated (Figure 2A). For protein recovery from SmF (Figure 2B), the quadratic pH effect and the linear ethanol concentration effect were both significant at the 95% confidence level ($p<0.05$). This quadratic effect - that leads to a pH of maximun recovery for a fixed ethanol concentration - is due to the charge of the

Table 3. Matrix of coded and real values of the central composite design (CCD) and responses for ethanol precipitation of the xylanase enzymes produced by *A. niger* under SSF and SmF.

Run	pH	Ethanol (%)	SSF				SmF			
			X_r(%)	P_r(%)	A_e(IU/mg)	P_F	X_r(%)	P_r(%)	A_e(IU/mg)	P_F
1	-1(4.0)	-1(80)	42.6	59.4	14.9	0.83	54.2	54.6	27.3	0.46
2	-1(4.0)	1(90)	45.1	63.0	14.8	0.83	54.6	68.3	53.4	0.90
3	1(7.0)	-1(80)	56.1	83.0	14.0	0.78	65.4	61.0	51.1	0.86
4	1(7.0)	1(90)	55.1	76.4	15.0	0.84	55.9	76.5	48.9	0.83
5	-1.41(3.4)	0(85)	48.6	45.1	22.4	1.25	58.3	46.3	40.7	0.69
6	1.41(7.6)	0(85)	46.6	69.8	13.8	0.77	51.9	51.1	60.0	1.02
7	0(5.5)	-1.41(78)	60.0	78.9	15.8	0.88	57.8	63.3	60.3	1.02
8	0(5.5)	1.41(92)	59.8	85.6	14.5	0.81	73.5	85.3	46.0	0.78
9	0(5.5)	0(85)	62.1	81.4	15.8	0.88	65.8	72.1	48.7	0.82
10	0(5.5)	0(85)	64.4	86.2	14.9	0.83	68.7	76.9	47.7	0.80
11	0(5.5)	0(85)	61.8	82.7	15.6	0.87	66.4	73.4	48.3	0.82

X_r, xylanase activity recovered; P_r, recovered protein concentration; A_e, specific xylanase activity in the precipitate; P_F, purification factor. SSF reference values: initial xylanase activity, 5.16 IU/mL; initial protein concentration, 0.29 mg/mL; specific activity of the initial xylanase, 17.79 IU/mg. SmF reference values: initial xylanase activity, 7.09 IU/mL; initial protein concentration, 0.12 mg/mL; specific activity of the initial xylanase, 59.08 IU/mg.

molecules. Most of proteins have their pI in the range of neutrality to slightly acidic pH. Values of pH distant from this range lead to charged molecules that tend to repel each other.

Table 4 provides the coefficients of the mathematical model and the statistical parameters obtained by analysis of the CCD responses for the xylanase activity and protein recoveries after ethanol precipitation of the SSF and SmF crude preparations. The low value for the coefficient of determination (R) for acitivity recovery may be explained by the fact that *A. niger* xylanases precipitated are not a single type of molecule, but xylanases with different molecular masses and pI values.

In general, the pH effect was greater than the ethanol concentration effect, as can be seen from the coefficient values listed in Table 4. The ANOVA correlation coefficient and F-test values indicated satisfactory prediction by the models used to describe the response surface plots of the xylanase activity and protein recoveries for SSF (Figure 3) and SmF (Figure 4). The response surface plots showed that, in the case of ethanol precipitation of the enzyme complex produced by SSF, higher xylanase activity and total protein recoveries were obtained for an initial extract pH in the range from 5.5 to 7.0. Under these conditions, there was no significant influence of the ethanol concentration (Figure 3). For the ethanol precipitation using the SmF broth extract, the results showed that, for both xylanase activity and total protein, the best recovery was obtained when the pH was adjusted to the center point value, 5.5 (Figure 4). However, unlike the precipitation using the SSF extract, the concentration of the precipitant affected the precipitation process of the SmF broth extract, at any pH, with the effect being greater at lower pH.

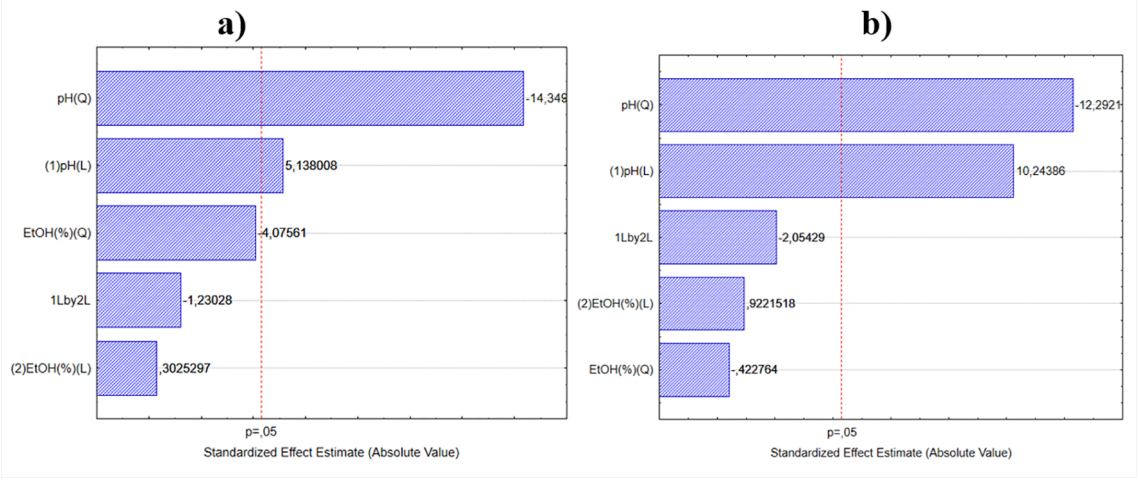

Figure 1. Pareto chart for the effects of the variables ethanol concentration and pH on the recovery of xylanase activity (A) and total protein (B) for the enzymatic complex from *A. niger* cultivated under SSF.

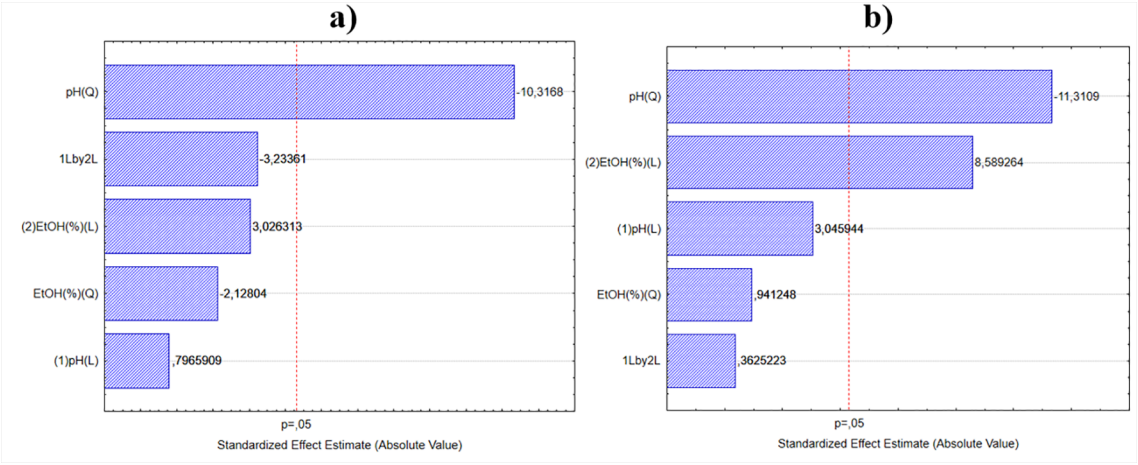

Figure 2. Pareto chart for the effects of the variables ethanol concentration and pH on the recovery of xylanase activity (A) and total protein (B) for the enzymatic complex from *A. niger* cultivated under SmF.

Table 4. Coefficient values and statistical analysis for total protein concentration and xylanase activity.

	SSF				SmF			
	Total protein concentration		Xylanase activity		Total protein concentration		Xylanase activity	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Mean	83.43*	0.000	66.30*	0.000	74.13*	0.000	66.97*	0.000
pH	8.99*	0.009	2.58*	0.036	2.67	0.093	0.43	0.509
pH^2	-12.84*	0.007	-8.59*	0.005	-11.82*	0.008	-6.65*	0.009
EtOH	0.81	0.454	0.15	0.791	7.54*	0.013	1.64	0.094
$EtOH^2$	-0.44	0.714	-2.44	0.055	0.98	0.446	-1.37	0.167
pHxEtOH	-2.55	0.176	-0.88	0.344	0.45	0.752	-2.48	0.084
R	0.98		0.79		0.98		0.60	
F-value	50.77		3.83		30.72		1.48	

R: coefficient of determination;*Significant at $p<0.05$.

Overall, the values for the conditions at the central point (85% (v/v) ethanol; pH 5.5) resulted in the best recoveries of xylanase from both the SSF and the SmF preparations, with average values of 63% and 67%, respectively. These precipitation conditions were therefore selected in the subsequent kinetic study. The recoveries were superior to those previously reported in studies of xylanase precipitation. For instance, Farinas et al. (2011) reported that the use of 80% (v/v) ethanol to precipitate xylanases from *A. niger* cultivated under SSF resulted in up to 23% recovery. This difference could have been due to the different precipitation operational conditions employed, especially the temperature, which must be controlled in order to avoid protein refolding and denaturation.

Kinetic study of xylanase precipitation with ethanol

The kinetic curves for xylanase precipitation with ethanol using the SSF and SmF crude enzyme preparations (pH 5.5) are shown in Figures 5 A and B, respectively. These correspond to the temporal profiles for the xylanase activity and protein concentration recoveries obtained using precipitation at 15 ºC with 85% (v/v) of ethanol. The recovery of total protein from the SSF medium showed a rapid initial increase, reaching 90% after 75 min, and then remained stable. The recovery of xylanase activity from the SSF medium also remained stable (at around 60%) from the start of the period up to 360 min, after which there was a slight decrease (Figure 5A). The decrease could have been due to enzyme denaturation following exposure to a high concentration of ethanol for such a long period. For the precipitation of proteins from the SmF broth (Figure 5B), the recovery of total protein increased in

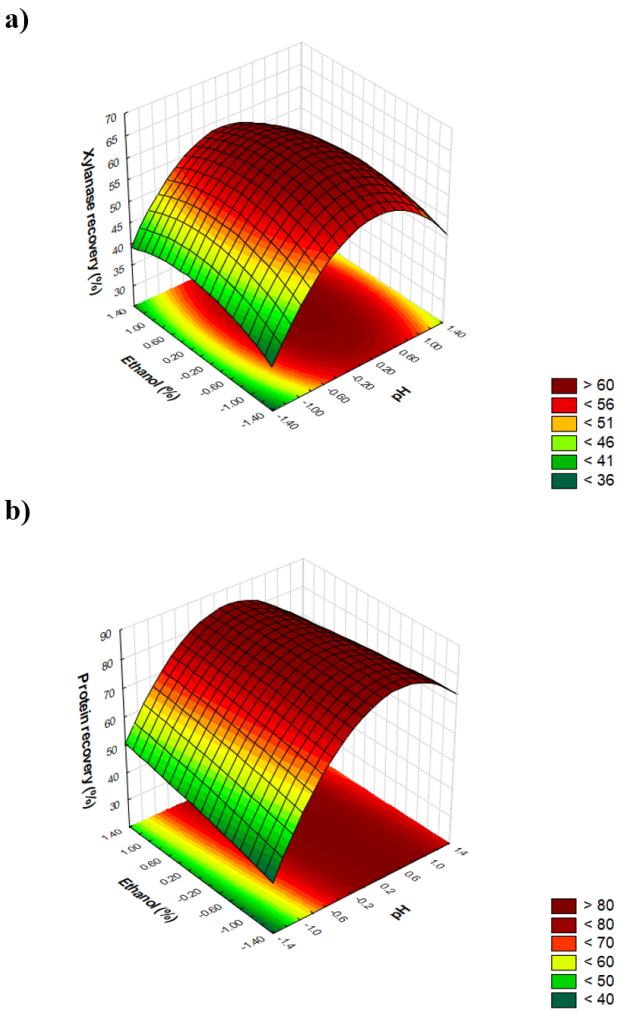

Figure 3. Response surface plots for ethanol precipitation of the enzymatic complex from *A. niger* cultivated under SSF, varying the ethanol concentration and pH values. Recovery of xylanase activity (A) and total protein (B).

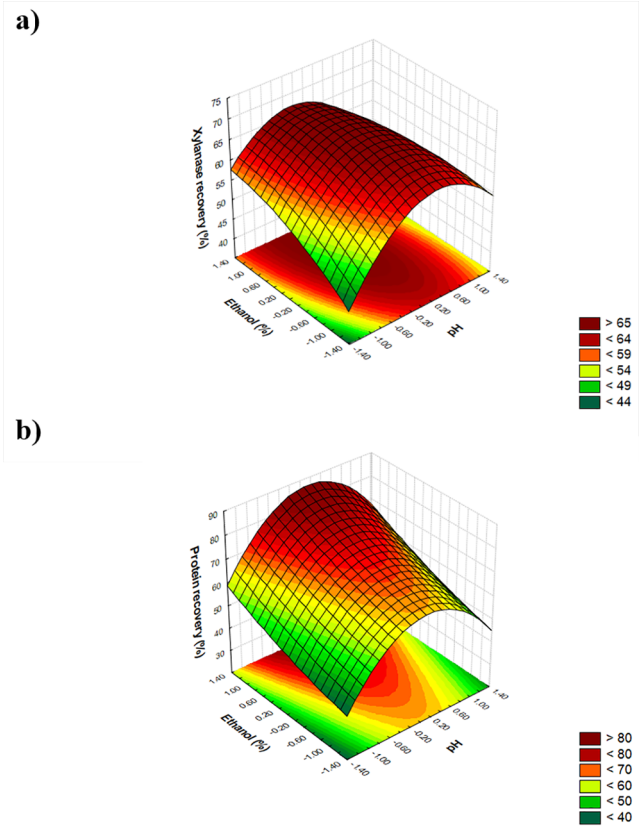

Figure 4. Response surface plots for ethanol precipitation of the enzymatic complex from *A. niger* cultivated under SmF, varying the ethanol concentration and pH values. Recovery of xylanase activity (A) and total protein (B).

the first 45 min and then remained quite stable. For the SmF broth, the xylanase activity recovery was stable (at around 80%) from the beginning of the period up to 360 min.

The differences in the initial total protein concentrations and xylanase activities of the crude enzyme preparations obtained from SSF and SmF were reflected in the specific activity values and, consequently, in the purification factors achieved after precipitation. For example, the maximum purification factors obtained for SSF and SmF were 0.88 and 1.12, respectively. For both SSF and SmF, the highest purification factor values were found using short precipitation periods, when much of the total protein was still in the soluble phase. These results showed that relatively short periods of time (up to 15 min) were sufficient to recover most of the xylanase activity precipitated under the operational conditions employed (15 ºC; pH 5.5; 85% (v/v) ethanol). Overall, the results achieved here for xylanase precipitation with ethanol were very satisfactory, when compared to the literature (Table 5), indicating that the procedure can be considered as a potential DSP unit operation for the concentration of *A. niger* xylanases from different cultivation systems.

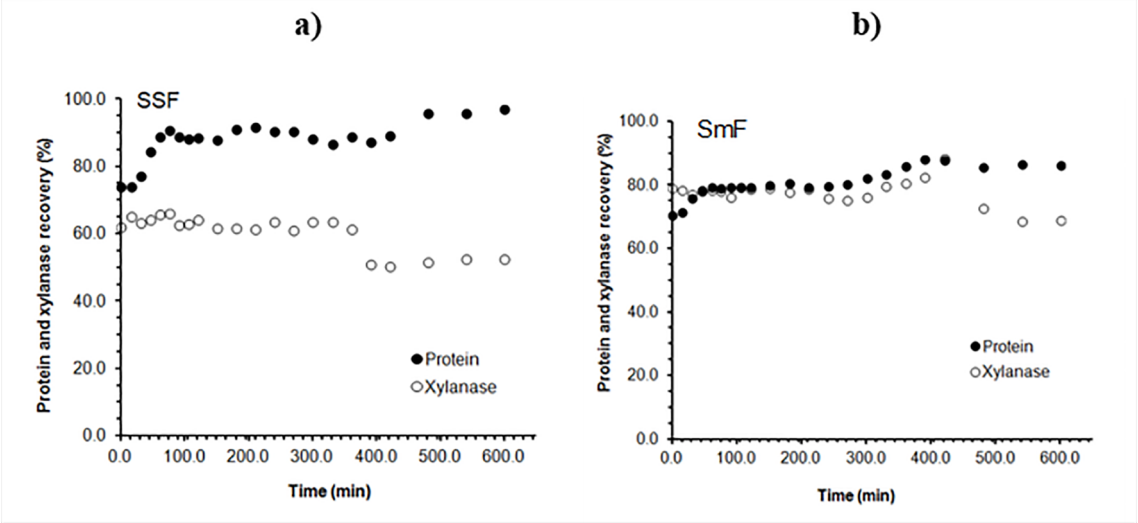

Figure 5. Kinetics of ethanol precipitation of the crude enzymatic SSF extract (a) and SmF broth (b) from *A. niger*. Initial xylanase activities: 4.15 IU/mL (a) and 7.68 UI/mL (b); initial protein concentrations: 0.23 mg/mL (a) and 0.12 mg/mL (b). Precipitation conditions: 15 °C, pH 5.5, 85% (v/v) ethanol.

Table 5. Comparative data from ethanol precipitation studies of xylanase enzymes produced by different fungal strains cultivated under SSF and SmF.

Cultivation System	Microorganism	Precipitant (v/v)	Time (min)	X_R (%)	P_F	Reference
SSF	*A. niger*	85% ethanol	15	65.0	0.74	This work
	A. niger	80% ethanol	180	23.0	-	(Farinas et al., 2011)
		80% ammonium sulfate		27.0	-	
	T. harzianum	80% ethanol	30	94.0	-	(Fadel, 2001)
SmF	*A. niger*	85% ethanol	15	79.0	1.03	This work
	T. harzianum	90% ethanol	0	100.0	-	(Marino et al., 2015)
	Chainia sp.	66% ethanol	overnight	78.5	13.70	(Varma et al., 1999)
		80% ethanol		99.0	15.97	

X_R: xylanase activity recovered; P_F: purification factor.

CONCLUSIONS

A systematic comparative study was undertaken of the ethanol precipitation of xylanases produced by *A. niger* cultivated under SSF and SmF. The process was evaluated using a central composite design, considering the effects of pH and ethanol concentration on recovery yields. There was a significant effect of pH on the total protein and xylanase activity recoveries, with the best results for an initial pH of 5.5. The ethanol concentration had no significant effect on the precipitation of xylanase from the SSF extract, within the range tested, but had a more pronounced effect in the case of the SmF extract, especially at acidic pH. The kinetic profiles showed that relatively short periods of time (15 min) were sufficient to recover most of the xylanase activity precipitated under the conditions employed, reaching xylanase recoveries of 65% and 79% for the SSF and SmF enzymatic complexes, respectively. In summary, our findings demonstrate that ethanol precipitation is a potential DSP unit operation for the concentration of xylanases from different cultivation systems.

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