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Structural and functional analysis of *Aspergillus niger* xylanase to be employed in polyethylenglycol/salt aqueous two-phase extraction

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ABSTRACT

The structure and enzymatic activity of *Aspergillus niger* xylanase were evaluated in different media to establish an appropriate protocol for the extraction of the enzyme in polymer/salt aqueous two-phase systems. Different factors were studied: the concentration and molecular weight (1000, 2000, 4600 and 8000) of polyethyleneglycol, the concentration and type of salt (sodium citrate and potassium phosphate) and pH, time and temperature. Xylanase was stable for 5 h at pH between 2.7 and 9.0 and at temperatures up to 50 °C. Fluorescence spectroscopy and circular dichroism experiments showed that neither the secondary/tertiary structure of the enzyme nor its catalytic activity were significantly altered in the presence of either salt or PEG. Xylanase partitioned into the PEG-rich phase driven by the excluded volume effect. Partitioning was more favorable to the polymer phase in the PEG1000/NaCit system, where *Kp* was 12 times higher than in the others aqueous two-phase systems. These results demonstrate the potential application of the PEG1000/NaCit system as a first step for the extraction of *Aspergillus niger* xylanase.

1. Introduction

The biotechnological potential of xylanases in various industries, such as brewery and wine, animal feed, textile and laundry, pulp and paper, biofuel, agriculture, as well as in research and development has been shown in the last few years. The actual demand and use of xylanase in many industrial applications requires an efficient and cost-effective downstream operation to perform a high-yield enzyme separation (Polizeli et al., 2005).

Endo-1,4-β-xylanase (EC 3.2.1.8) randomly hydrolyzes the β-1,4-glycosidic bonds of xylan into xylooligomers with different lengths. Xylans, the most abundant hemicellulose, are heteropolysaccharides found in the cell walls of all land plants and in almost all plant tissues (Biely, 1985). Endo-1,4-β-xylanase is the major component of xylanolytic systems produced by biodegrading microorganisms such as fungi and bacteria. *Aspergillus niger* is one of the most potent organisms used in biotechnology for xylanase production and secretion (Khan et al., 2003), considered in the status of Generally Recognised As Safe (GRAS) by the United States Food and Drug Administration (FDA) (Schuster et al., 2002).

Traditional methods, such as ammonium sulfate precipitation, size exclusion or ion exchange chromatography, allow the purification of xylanase from a crude hydrolase mixture produced by fermentation broth.

Separation techniques based on aqueous two-phase extraction (ATPE) have been increasingly used as a first step of clarification, concentration and purification of important biomolecules from their natural source (Porfiri et al., 2011; Rocha and Nerli, 2013).

Aqueous two-phase systems (ATPSs) present several advantages such as, high biocompatibility (70–80% water content), low biomolecular degradation, high resolution low cost and easy scale-up (Albertsson, 1986). In order to form the two phases, aqueous solutions of either two polymers or a polymer and a salt are required. In ATPE, protein distribution between the two phases is influenced by system properties like type, size and concentrations of polymers and salts used, pH and temperature, and by partitioned-biomolecule properties such as: molecular weight, isoelectrical points, conformation and hydrophobicity.

In different works, polyethyleneglycol (PEG)/phosphate or PEG/sulfate ATPSs were found to be the most suitable for isolating fungal xylanase (Antov et al., 2006; Garai and Kumar, 2013). However, for industrial purposes, potassium phosphate represents a waste disposal problem. In the last few years sodium citrate has been used as a substitute for potassium phosphate since citrates are biodegradable and non-toxic and can be discharged into biological wastewater treatment plants (Tubio et al., 2009).

The aim of this article was to describe the effect of different polymers and salts on the structure and activity of *A. niger*

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xylanase with an emphasis on the effect of temperature and pH on its stability. In-depth characterization of the enzyme will be useful to evaluate xylanase partitioning in polymer/salt ATPS.

2. Materials and methods

2.1. Chemicals

Endo-1,4-β-xylanase from *A. niger* (Xyl) was obtained from Megazyme Int. (Wicklow, Ireland). According to the data sheet, the molecular weight is 25,000, the specific activity is 79.3 U/mg and the purity is 99.99%. Sodium citrate (NaCit), potassium phosphate (KPi), birchwood xylan, xylose, polyethyleneglycols of average molecular weight: 1000, 2000, 4600 and 8000 (PEG1000, PEG2000, PEG4600, PEG8000) and 1-anilino-8-naphthalene sulfonate (ANS), were purchased from Sigma Chem. Co. and used without further purification. All the other reagents were of analytical quality.

2.2. Methodologies

2.2.1. Determination of Xyl activity

Xyl activity was determined by the 3,5-dinitrosalicilic acid (DNS) method (Miller, 1959), measuring the amount of reducing sugars liberated when birchwood xylan (1% w/w) and Xyl were mixed in 50 mM citrate buffer pH 5.30, according to the Bailey et al. method (Bailey, 1992). Each sample was incubated at 50 °C for 10 min. Then 1 ml of DNS reagent was added to each tube, and the samples were boiled for 10 min. The absorbance of each sample was measured at 560 nm. A calibration curve of absorbance at 560 nm against the xylose concentration was used to calculate the μmoles of xylose liberated. Xyl activity was expressed as units (U) per milliliter of enzyme (U mL^{-1}). One unit is defined as the amount of enzyme required to release 1 μmol per minute of xylose reducing equivalent (μmol xylose min^{-1}). Solutions of substrate alone and enzyme alone were used as controls. All the experiments were performed in triplicate.

2.2.2. Effect of pH, time and temperature on the stability of Xyl

The pH stability assay was carried out by pre-incubating the enzyme (1.04 μM) in 50 mM citrate buffer at different pHs between 2.7 and 9.0 at 25 °C. Xyl activity was measured every 30 min for 5 h.

Thermal stability was evaluated by pre-incubating the enzyme (1.04 μM) in 50 mM citrate buffer pH 5.30 at different temperatures (from 20 to 60 °C) in a thermostatic bath. Incubation was carried out in sealed vials to prevent changes in the sample volume and, hence, in enzyme concentration due to evaporation. Aliquots of enzyme solution were taken every 30 min for 5 h and catalytic activity was measured. The residual Xyl activity was calculated as the percentage of activity remained after incubation respect to the activity of the enzyme without heat treatment.

2.2.3. Fluorescence and circular dichroism studies of Xyl in the presence of PEGs and salts

Fluorescence measurements were performed on an Aminco Bowman Serie 2 spectrofluorometer using a thermostatized quartz cuvette of 1 cm of optical pathway. Xyl (1.3 μM) was excited at 280 nm and the emission was recorded from 290 to 430 nm. The scanning rate was 3 nm min^{-1} and the bandwidth was 4 nm. Fluorescence quenching experiments were performed by using acrylamide. This is an efficient quencher of tryptophan fluorescence able to distinguish between buried and exposed side chains. Xyl was titrated with acrylamide (40 mM) in the absence and presence of PEG. After each addition, fluorescence was measured at 340 nm (exciting at 280 nm).

Circular dichroism spectra (CD) at 5.2 μM final concentration of Xyl were performed in a Jasco J-810 spectropolarimeter, using a thermostatized cuvette of 1 mm of pathlength, the scan rate was of 50 nm min^{-1} and the bandwidth was of 1 nm. Repetitive scanning of five cycles was used. All the experiments were performed in triplicate. Data were corrected by using the software provided by the instrument manufacturer.

CD data are presented in terms of mean residue ellipticity (MRE, expressed as deg cm^2 $dmol^{-1}$) as a function of wavelength, calculated by Eq. (1) according to the procedure described earlier (Kelly et al., 2005):

$$[\theta]_{MRE} = \frac{MRW \cdot \theta_{obs}}{10 \cdot d \cdot c} \quad (1)$$

where $[\theta]_{MRE}$ is the calculated mean residue ellipticity (deg cm^2 $dmol^{-1}$), θ_{obs} is the observed ellipticity (expressed in degrees), d is the pathlength (cm), and c is the protein concentration (g mL^{-1}). The Mean Residue Weight (MRW) for the peptide bond is calculated from $MRW = M/(N-1)$, where M is the molecular weight of the polypeptide chain (in Da), and N is the number of amino acids in the chain; the number of peptide bonds is $N-1$. All the CD spectra were corrected for polymer contributions. The secondary structure composition was estimated by using CDPro software package (http://lamar.colostate.edu/~sreeram/CDPro/main.html) developed by Sreerama and Woody, 2000.

2.2.4. Protein surface hydrophobicity (S_0)

Relative surface hydrophobicity of Xyl was determined by applying the optical method reported by Haskard and Li-Chan (1998). Stock solution (6 mM) of the anionic fluorescence probe 1-anililo-naphthalene-8-sulfonate (ANS) was prepared in 50 mM phosphate buffer pH 6.00. Aliquots of the protein were added to a sample containing 2551 μL of buffer solution with a final ANS concentration of 119.95 μM and the relative fluorescence intensity (I_f) was measured after each addition, the protein concentration ([protein]), varying from 0 to 10 μM. Excitation and emission wavelengths were 382 and 466 nm respectively with a bandwidth of 4 mm. Under the above-mentioned experimental conditions (with ANS excess), the surface hydrophobicity S_0 was determined from the initial slope of the linear regression analysis of the I_f vs. [protein] plot (Hatti-Kaul, 2001). The temperature of the sample was maintained at 25 °C by a HAAKE DC3 thermostatic bath and measured with a thermocouple immersed inside the cuvette. The heating rate was 0.01 °C/min.

2.2.5. Aqueous two-phase diagrams

The cloud point and turbidimetric titration methods (Hatti-Kaul, 2001) were used to determine the binodal curve of the PEG/salt systems at 22 °C. In the cloud point method, either PEG or salt solutions were added drop-wise and mixed until the resulting mixture became cloudy. In the turbidimetric titration method, water was added drop-wise to several biphasic systems with different compositions, until a biphasic system was formed, after mixing in a vortex mixer. Tie Line Length (TLL) was calculated using the Eq. (2):

$$TLL = \sqrt{[\Delta PEG]^2 + [\Delta Salt]^2} \quad (2)$$

where [ΔPEG] and [ΔSalt] are the differences between the concentrations of PEG and Salt in the top and bottom phases, respectively expressed in % (w/w).

2.2.6. Preparation of the aqueous biphasic systems

To prepare the ATPSs, stock solutions of the phase components

Table 1
Composition of aqueous two-phase systems formed by PEGs of different molecular weight and sodium citrate and potassium phosphate Temperature 25 °C.

PEG molecular weight (Da)	Salt	TLL (% w/w)	Total system		Top phase		Bottom phase	
			PEG (%w/w)	Salt (%w/w)	PEG (%w/w)	Salt (%w/w)	PEG (%w/w)	Salt (%w/w)
1000	KPi	32.63	15.37	14.28	29.34	5.85	1.40	22.71
	NaCit	26.37	17.29	13.34	29.21	7.70	5.37	18.97
2000	KPi	23.80	13.31	10.53	21.36	5.89	0.74	17.77
	NaCit	21.29	13.31	10.53	24.18	5.97	4.62	14.17
4600	KPi	22.29	11.69	9.55	19.83	5.14	0.23	15.76
	NaCit	24.33	11.69	9.55	23.53	4.66	1.02	13.96
8000	KPi	23.59	11.69	9.55	22.51	4.74	0.86	14.35
	NaCit	22.64	11.69	9.55	22.08	5.02	1.30	14.01

Systems of polyethylenglycol (of different molecular weight) – potassium phosphate pH 6.00 and soduim citrate pH 5.20 were used at 25 ºC.

– PEG of different molecular weight 40% (w/w) and sodium citrate or potassium phosphate 20% (w/w) of a given pH – were mixed according to the binodal diagram obtained in our laboratory and described in Section 2.2.5. The suitable pH of salt solutions was adjusted by the addition of sodium hydroxide and potassium hydroxide respectively. Low-speed centrifugation (2000 rpm for 5 min) was used to speed up phase separation after a thorough gentle mixing of the system components. Then 1mL of each phase was mixed to reconstitute several two-phase systems in which the protein partition was assayed. The total system compositions and the tie line lengths selected for this work are shown in Table 1.

2.2.7. Determination of the partition coefficient (Kp)

Partitioning behavior of Xyl was analyzed by dissolving a given amount of protein (10.4 μM total system concentration) in the two-phase systems containing 1 mL of each equilibrated phase. Small aliquots (40 μL) of the protein stock solution were added to the systems in order to make the change of the total volume of each phase negligible.

After mixing by inversion for 1 min and leaving it to settle for at least 120 min, the system was centrifuged at 2000 rpm for 5 min for the two-phase separation. Samples were withdrawn from the separated phases and after the appropriate dilution (with the equilibrated phase free from protein), protein activity in each phase was determined. The partition coefficient was calculated using the Eq. (3):

$$Kp = \frac{[Act]_T}{[Act]_B} \times F \quad (3)$$

where $[Act]_T$ and $[Act]_B$ are the enzyme activities of the partitioned protein in the PEG-rich and salt-rich phases, respectively. The effect of phase composition on the enzyme activity was considered. A correction factor (F) was calculated as the ratio between the activities of reference solutions (of known enzyme concentration) in each phase. Temperature was maintained constant at 22 °C and controlled within ± 0.1 °C.

3. Results

All the results reported are the average of three independent experiments. The standard error was calculated as the standard deviation divided by the square root of the number of replicas.

3.1. Effect of pH on enzymatic activity and stability of Xyl

Fig. 1 compares the activity of Xyl at five different pHs (2.70, 4.07, 5.36, 7.02 and 9.00). The enzyme was active in the range assayed at 25 °C. The optimum pH was 5.50, similar to that observed by Sardar et al. (2000). The pH stability study showed that a pre-incubation of Xyl at pHs between 2.70 and 9.00 had no effect on the enzymatic activity. Moreover, it slightly increased with time as previously reported (Hmida-Sayari et al., 2012).

Fig. 2 shows that Xyl was stable up to 50 °C during the first hour of incubation, then, it started to decrease and finally remained constant along the time. On the other hand, at 60 °C the

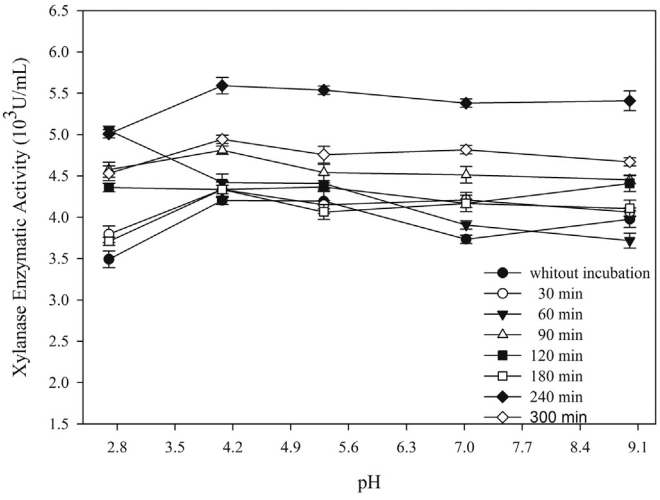

Fig. 1. Effect of pH on Xyl stability at 25 °C in citrate buffer 50 mM.

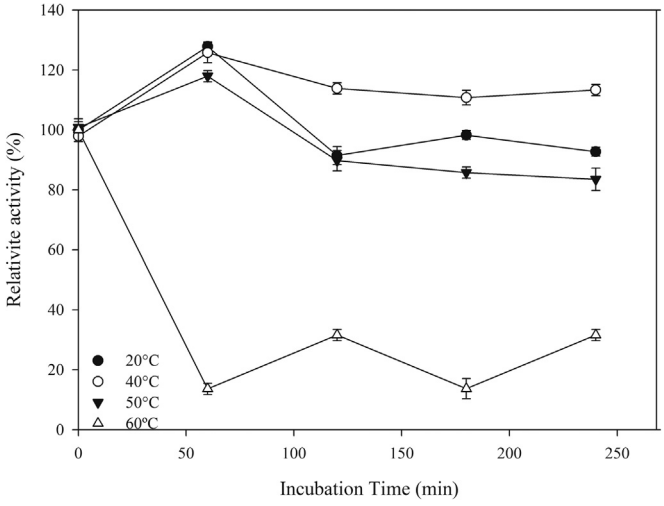

Fig. 2. Effect of temperature on Xyl stability at pH 5.30.

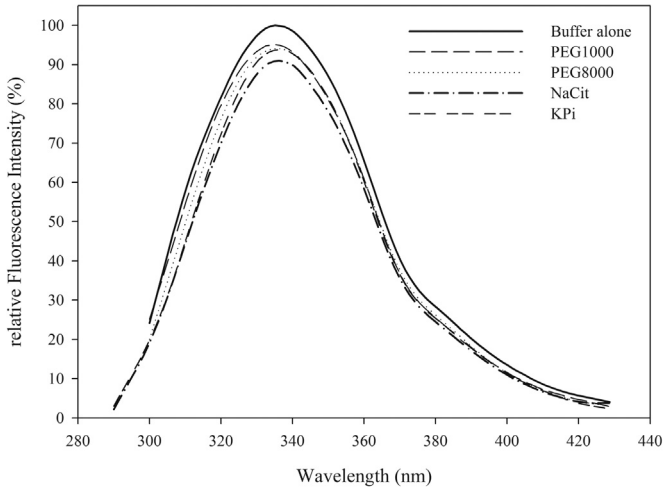

Fig. 3. Fluorescence intensity spectra for Xyl in absence and presence of PEG1000 and 8000, NaCit and KPi at 5% w/w at 25 °C.

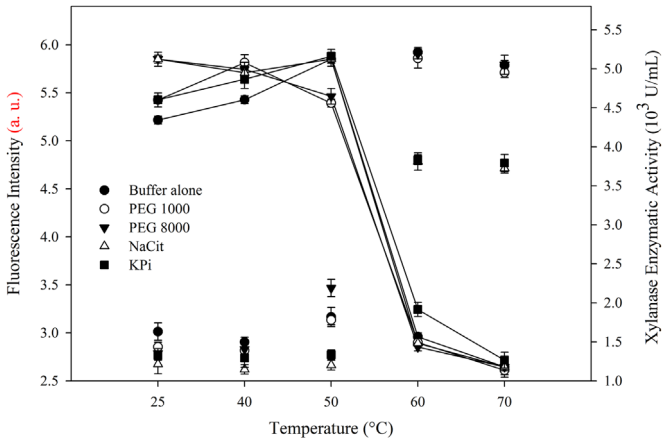

Fig. 4. Temperature dependence of Xyl (1.3 μM) activity (lines) and fluorescence intensity (points) in the absence and presence of NaCit, KPi, PEG1000 and PEG8000 5% w/w. Citrate buffer 50 mM, pH 5.30.

activity started to decrease from the beginning of the incubation. These results indicate that at temperatures over 50 °C Xyl suffers conformational changes that affect the active site of the enzyme.

3.1.1. *Effect of the presence of PEG and salts on protein fluorescence*

The position and intensity of the protein emission peak of fluorescence is sensitive to the surroundings of chromophores, such as tryptophan (Weber and Teale, 1966). Changes in the non-polar environment of chromophores lead to characteristic shifts of the peak position, this being used for investigation of structural transformations in proteins. Since polymer-protein or salt-protein interactions may induce changes in the tertiary and secondary structure of the protein, the fluorescence emission spectra of native Xyl (1.3 mM) were analyzed in the presence of PEG1000 and 8000 at 5% and 10% w/w, NaCit and KPi at 5% and 15% (w/w) at 25 °C.

Fig. 3 shows that no significant shifts in the position of the emission peak (335 nm) were observed in the different media, which suggests the absence of a conformational change in the environment of the tryptophan residues on the protein. This indicates that no modification in the tertiary structure of the protein was induced. However, a small decrease in the relative fluorescence intensity of Xyl was observed in PEG solutions. Moreover, changes in PEG molecular weight at low polymer concentration (5%P/P) did not induce changes in the fluorescence spectra of Xyl. However, when PEG concentration increased, there was a decrease in the relative fluorescence intensity of Xyl, this being more pronounced in PEG8000 than in PEG1000 (data not shown). The presence of salt also induced a decrease in the relative fluorescence intensity of the enzyme, this effect in NaCit resulted to be higher than in KPi solution. Fluorescence quenching experiment showed that acrylamide did not quench the native fluorescence of Xyl, thus indicating that the tryptophan residues were not solvent-exposed. Therefore, the presence of PEGs or salts induced structural changes in the enzyme that affect the environment of buried tryptophan.

3.1.2. *Thermal and kinetic stability of Xyl in the presence of salts and polymers*

The thermal stability of Xyl was analyzed by measuring enzymatic activity and native fluorescence, as explained above. All experiments were performed at different temperatures (pre-incubation for 10 min) in the absence and presence of NaCit and KPi at 5% and 15% (w/w), PEG1000 and PEG8000 at 5% and 10% (w/w) at 25 °C.

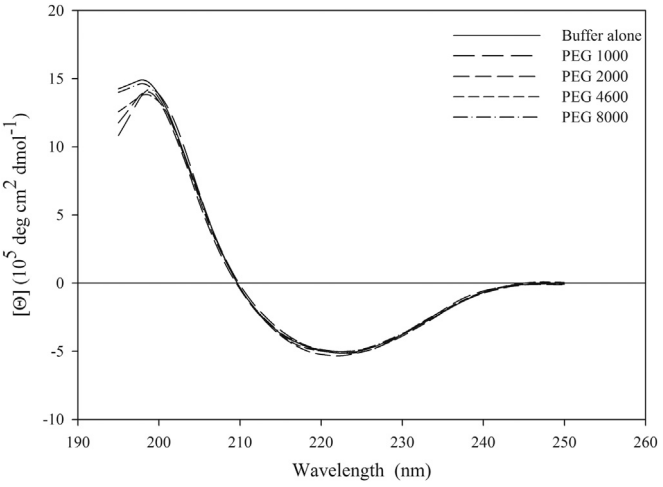

Fig. 5. CD spectra (190-250 nm) of Xyl in citrate buffer 50 mM pH 5.30 (solid line) and in different PEG molecular weight 5% (w/w) (dotted line) at 25 °C.

Fig. 4 shows the experiments performed in the absence and presence of salts and PEGs at 5% (w/w). It can be seen that the fluorescence intensity and the enzymatic activity did not considerably change between 20 °C and 50 °C. Both results indicate that the tertiary structure of Xyl was not altered in this range of temperature. When the temperature was higher than 50 °C, an increase in the fluorescence intensity and a loss of enzymatic activity was observed. This may be due to a gradual conformational change of the protein that leads to an exposure of chromophores that affect the active site. The change in the maximum fluorescence intensity observed between 50 and 60 °C indicates that a conformational transition takes place between these two temperatures, irrespective the salt/PEG presence. No appreciable modification either in the presence of 10% (w/w) salt or 10% (w/w) PEG solutions were observed (data not shown).

3.1.3. *Circular dichroism spectra*

According to previous reports, the CD spectrum of Xyl (Fig. 5) shows that the enzyme has predominantly β-sheet structure (Sardar et al., 2000). As shown, the Xyl spectrum presents a positive band at 197–201 nm and a negative band at 215–225 nm with its characteristic features. The far UV CD spectra of proteins are particularly sensitive to changes in the secondary structure.

Table 2
Effect of PEG presence on xylanase secondary structure content at 25 and 50 °C.

PEG molecular weight (Da)	Temperature (°C)	α-Helix (%)		β-Strand (%)		Turn (%)	Unordered (%)
		r	*d*	*r*	*d*		
Buffer alone	25	4.82	4.93	32.76	11.66	20.80	24.26
	50	1.00	4.20	30.30	12.80	22.00	30.56
1000	25	4.15	4.50	35.25	12.00	21.30	22.40
	50	0.33	3.80	30.56	12.56	22.56	30.46
2000	25	4.47	4.33	38.20	11.83	20.73	20.60
	50	1.30	4.30	31.00	12.83	21.56	28.97
4600	25	4.83	5.28	32.53	11.85	20.82	24.47
	50	1.30	4.00	30.27	12.83	22.37	29.17
8000	25	4.13	4.43	39.63	11.90	20.40	19.53
	50	1.20	4.70	29.80	12.47	22.03	29.40

r, regular; d, distorted.

The presence of PEG1000, 2000, 4600 and 8000 at 5% (w/w) (Fig. 5) induced both a blue-shift of the global peak and a slight change in the elipticities of Xyl around 198 and 222 nm. Table 2 shows the results obtained using the CDPro program to analyze CD spectra. This analysis showed a slight increase in Xyl β-strand content when molecular weight of PEG increased at 25 °C. However, at 50 °C the regular α-Helix content decreased, in association with an increase in the disordered structure in both presence and absence of PEG.

No effect was observed in the near UV region of the Xyl CD spectra (270–300 nm) in PEG presence. This suggests that the polymer does not induce conformational changes in the tryptophan residues that are accessible to the solvent (data not shown). This finding agrees with the behavior observed in the fluorescence quenching experiments.

3.2. Effect of PEGs and salts on surface hydrophobicity of Xyl

According to previous reports, the protein partitioning behavior is very sensitive to surface hydrophobicity properties in PEG/salt systems (Gulyaeva et al., 2000 and Malpiedi et al., 2011). Therefore, experiments using NaCit, KPi and PEGs with different molecular weight (1000, 2000, 4600, and 8000) were carried out. As can be seen in Fig. 6, the presence of NaCit and KPi produced an increase in the relative surface hydrophobicity (S_0) at 25 °C and 50 °C. Moreover, an increase in salt concentrations led to higher values of S_0 at both temperatures. A possible reason for this observation is that in the presence of salt (Andrews et al., 2005), the interactions among hydrophobic groups increased, resulting in an enhancement of ANS-protein binding, and therefore, in an increase in S_0. On the other hand, the S_0 measured in the presence of PEG was nearly three times lower than in the presence of salt (0.424 and 0.1228 μM^{-1}, respectively. It can also be seen that S_o values increased as the PEG molecular weight increased, which is in agreement with an enhancement of the exclusion effect.

Temperatures above xxx °C induced an enhancement in S_0 in all the cases, as we have seen in previous reports (Tubio et al., 2004). The binding of ANS to a protein requires a previous disruption of hydrogen bonds of structured water around the hydrophobic surface on the protein and ANS molecules. At Higher temperatures this disruption is facilitated and, the interaction between Xyl and ANS is favored, thus resulting in a S_o increase.

Xyl activity was assayed in each case. As can be seen in Fig. 6, at 25 °C catalytic activity slightly decreased only in the presence of PEG2000. On the other hand, at 50 °C the enzyme lost a 55% of its activity in the presence of PEG2000, 4600 and 8000, while in the presence of PEG1000 the enzyme only lost 8% of its activity. As

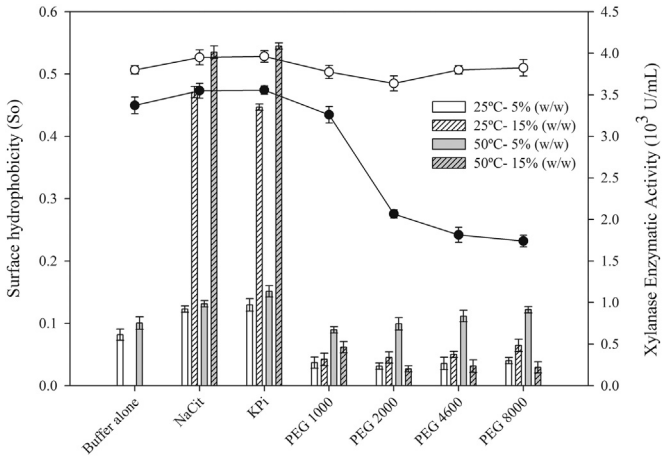

Fig. 6. relative surface hydrophobicity (S_0) of Xyl in medium of salts and PEGs (5% and 15% w/w). Medium citrate buffer 50 mM pH 5.30 at 25 °C. Each point is the mean of three independent measurements.

observed in the thermal stability assay, this trend indicates that it is the temperature, not PEG, which affects the active site of the enzyme.

3.3. Phase diagrams

Phase diagram data are required for the design of aqueous two-phase extraction processes and for the development of models that predict partitioning of proteins. Fig. 7 A compares the binodal curves, obtained for this work in our laboratory (Section 2.2.5): PEG2000-PEG4600/NaCit pH 5.20 and PEG1000-PEG2000-PEG4600-PEG8000/KPi pH 6.00 with those obtained previously: PEG1000-PEG8000/NaCit pH 5.20 (Tubio et al., 2006). It can be seen that the shapes of binodal curves were similar. As expected for lower molecular weight polymer, phase separation occurred at higher PEG and salt concentrations. However, the binodal curve of the PEG/KPi ATPS was shifted to the origin compared to that of PEG/NaCit ATPS. This means that a lower concentration of phosphate is required to form two-phase systems.

A four-parameter sigmoidal equation (Tubio et al., 2006) and other previously used non-linear expressions (Graber and Taboada, 2000; Mistry et al., 1996) were used in fitting the binodal data for PEG/salts systems. Better results were obtained with Mistry equation for all binodal curves. Fig. 7B shows the binodal diagram for PEG4600/NaCit systems. For all the tie lines assayed, the total

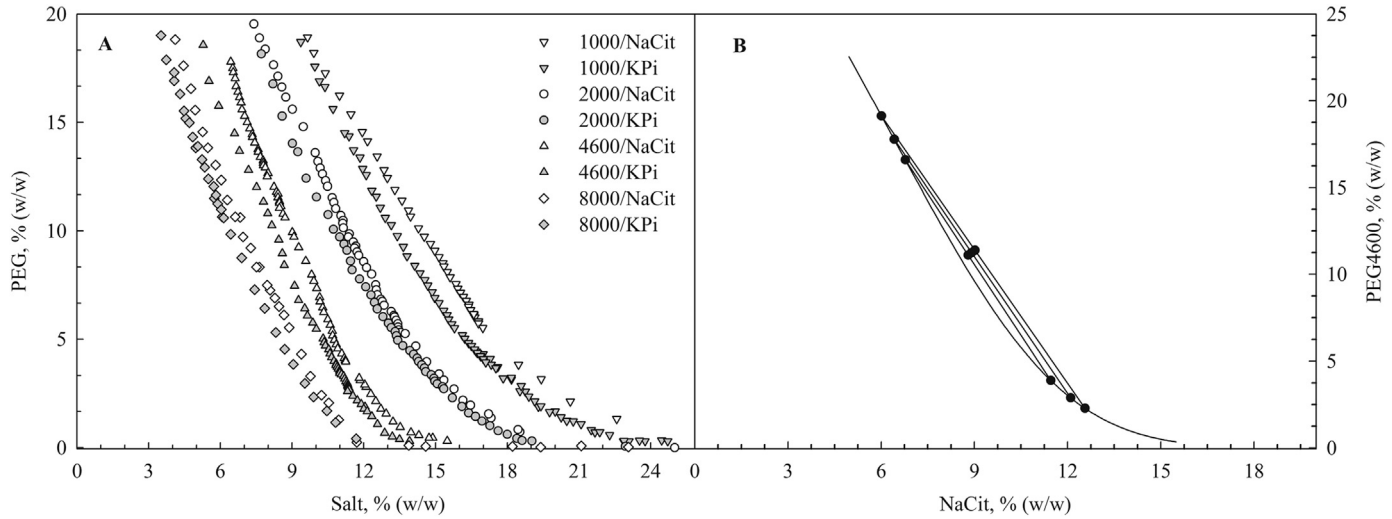

Fig. 7. Phase diagrams for PEG/NaCit and PEG/KPi systems at 22 °C: (A) binodal curves for PEG 1000, 2000, 4600, 8000 in the presence of NaCit and KPi; (B) tie-lines for PEG4600/NaCit.

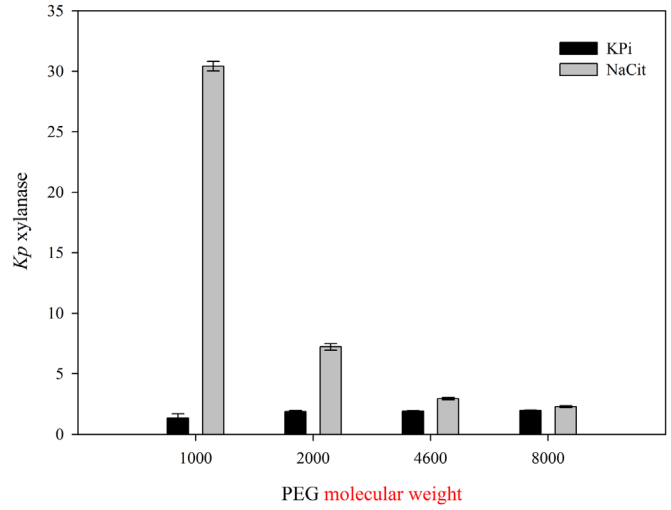

Fig. 8. Effect of PEG molecular weight (from 1000 to 8000) and salt (KPi and NaCit) aqueous two-phase systems formation on the Kp_{xyl} at 25 °C. Total system compositions are those of Table 1.

system composition had no significant effect upon their slopes, which implies that they were parallel to each other. A similar behavior was observed for all the other systems (data not shown).

3.4. Partition behavior of Xyl in PEG/sodium citrate and PEG/potassium phosphate ATPSs

The effect of PEG molecular weight and salt formation of ATPs on the Xyl partition coefficients (*K*p) was analyzed (Fig. 8). A linear behavior (data not shown) was observed when activities in the top phase were plotted against the activity in the bottom phase, demonstrating that the partition coefficient was independent of the protein concentration in the range 0–10.20 μM. In all cases, Xyl was partitioned in favor of the PEG-rich phase according to the observed *K*p values higher than one. This behavior was more pronounced in PEG/NaCit than in PEG/KPi ATPSs, since the *K*p was 12 in the PEG1000/NaCit system and 1.2 in the PEG8000/NaCit. Note that S_0 adopted similar values in both PEGs (1000 and 8000). This suggests that there is no correlation between S_0 and *K*p. Previous reports have demonstrated that the protein partition in ATPS is driven by different factors (Tubio et al., 2004): firstly, the interaction between the macromolecule and the medium and, secondly, the excluded volume effect. This theory shows that the increase in PEG molecular weight induces a decrease in the protein solubility in the phase where the polymer is situated. According to these results, the excluded volume effect drives the Xyl partition between the two phases.

In systems formed by KPi, the increase in the molecular weight of PEG induced a slight increase in *K*p values. On the other hand, in PEG/NaCit systems, the partition equilibrium resulted in a significant decrease. This has been shown to be a general rule for many proteins in PEG/salt systems (Tubio et al., 2006) and could be attributed to a reduction of the space available for proteins into the polymer-rich phase when the polymer chain length increased, causing a strong partition of the protein to the salt-rich phase. The major change in the *K*p value was observed when the molecular weight of PEG varied from 1000 to 2000. This behavior is predicted by the Flory Huggins' theory (Albertsson, 1986) which states a linear relationship between the ln *K*p and the reciprocal of the flexible chain polymer molecular weight.

4. Conclusions

In this work, the structure and stability of *A. niger* Xyl was studied at different pHs, temperatures and in the presence of PEG, NaCit and KPi in order to establish experimental conditions for purifying Xyl using ATPS.

Stability experiments showed that Xyl maintains its structure and enzymatic activity at pHs between 2.7 and 9.0 and temperatures below to 50 °C. At 60 °C, Xyl suffers conformational changes that drastically affect its catalytic activity. Fluorescence intensity remains almost constant between 20 °C and 50 °C and increases when temperature is over 50 °C, indicating that the tertiary structure of Xyl is affected. These results agree with those observed by CD measurements since at 50 °C, in the presence and absence of PEG, the α-helix content of Xyl decreases while the content of disordered structure increases.

The relative surface hydrophobicity of Xyl increased when the enzyme was incubated at 50 °C with a loss of 55% of its activity in the presence of PEG2000, 4600 and 8000. However, the presence of PEG1000 and salts did not change the enzymatic activity when Xyl was incubated at 25 °C and 50 °C.

Partition experiments demonstrated that as the molecular

weight of PEG increases, *K*p decreases, indicating that the excluded volume effect drives the partitioning of Xyl. All the *K*p values were higher than one, thus indicating the Xyl preferentially partitions into the PEG-rich phase. This behavior was more pronounced in PEG/NaCit than in PEG/KPi ATPSs, since the *K*p was 12 in the PEG1000/NaCit system. Finally, these results are very interesting for a first step in the primary recovery of Xyl. Although further work needs to be done to choose the most appropriate ATPS, these findings allow as to conclude that PEG1000/NaCit ATPS could be employed as potentially useful tool for the extraction of *A. niger* Xyl.

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