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Journal of Chromatography B, 743 (2000) 349–356

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Extraction in aqueous two-phase systems of alkaline xylanase produced by *Bacillus pumilus* and its application in kraft pulp bleaching

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Abstract

The aim of this work was to extract and to purify xylanase, produced by *Bacillus pumilus* from the crude fermentation broth, using aqueous two-phase systems (ATPS). The xylanase was extracted by partitioning in ATPS composed of phosphate and polyethylene glycol (PEG). The effect of tie-line length, PEG molecular mass and NaCl concentrations upon the purification factors and yields of xylanase were investigated by statistical design. The best system studied was that containing 22% PEG6000, 10% K_2HPO_4 and 12% NaCl with a purification factor of 33 and a 98% yield of enzyme activity. This system was also used for continuous extraction in a pulsed caps column. Subsequently, the xylanase from the crude fermentation broth was tested in hardwood kraft pulp bleaching.

Keywords: Aqueous two-phase systems; Protein partitioning; Enzyme purification; *Bacillus pumilus*; Alkaline xylanase

1. Introduction

Xylan, a group of heteropolysaccharides, is an abundant biopolymer, found in plant tissues as a major component of the cell wall, which is hydrolysed by xylanase. Several of the xylanases commercially available are produced by fungi and are active at neutral or acidic pH values and an optimum temperature below 45°C. A variety of applications in the bioconversion and food industries have been suggested for xylanases, and one of their major potential applications is in the pulp and paper industry. In this context, enzymes which are active under alkaline conditions will have great potential in the bleaching process since there is no need to change the pH and they offer the advantage of reducing the release of polluting organic chlorine compounds.

The large variety of potential applications of these enzymes is the main reason for investigating fungal and bacterial xylanase production. Currently, the most important application of xylanases is in the prebleaching of kraft pulp. Xylanase prebleaching technology is now in use at several mills, mainly in Scandinavia and Canada; the main motivating factors for this technology are the economic and environmental advantages xylanase offers to the bleach plant [1]. Enzymes active at high temperatures and alkaline pH values have great potential as they can be introduced at different stages of the bleaching process without requiring changes in pH or temperature [2]. We have isolated and studied 500 microrganisms,

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00223-1

and three strains of *Bacillus pumilus* able to produce alkaline xylanase with insignificant levels of cellulases were identified as potentially useful in prebleaching processes [3].

Characteristics of biotechnological systems make purification the most expensive part of biomaterials process production. Thus, the development of new and economically advantageous purification methods is a challenging area. Purification of target proteins requires their separation from the media or from the raw extract used for the maintenance of the biomolecules. Protein extraction in ATPS is a rapid procedure which avoids most of the problems in denaturing fragile molecules, providing a gentle environment for biologically active proteins, and may be employed on a large scale [4].

The aim of this work was to extract the alkaline xylanase from crude fermentation broth in ATPS using batch and continuous process, and to apply xylanase in hardwood kraft pulp bleaching.

2. Experimental

2.1. Materials

Polyethylene glycol (PEG 1500, 4000 and 6000), potassium phosphate dibasic and sodium chloride were purchased from Synth, São Paulo, SP, Brazil and birchwood xylan from Sigma, St Louis, MO, USA was used as the substrate. All other chemical and biochemical reagents used were of analytical grade.

2.2. Fermentation broth

The fermentation broth was the kind gift of C.V. Tagliari. Xylanase was produced by *Bacillus pumilus* at 40°C in a 2.0 l reactor (Bioflo III-New Brunswick Scientific, New York, NY, USA) with a initial medium volume of 1.5 l. The pH was initially adjusted to 9.5 before sterilisation. Dissolved oxygen concentration was monitored and kept at 50% saturation by automatic control of the air flow-rate and of the stirring state.

2.3. Determination of xylanase activity

Xylanase activity was determined according to Bailey et al. [5] with Birchwood xylan 1% solution in 100 m*M* glycine–NaOH buffer at pH 10.0 and 45°C. The amount of reducing sugars was determined according to Miller [6]. One unit of xylanase activity was defined as 1 μmol of xylose produced per minute under the given conditions.

2.4. Experimental design

The experimental design was applied to determine the best ATPS to extract the xylanase (Table 1), according to Haaland [7].

2.5. Aqueous two-phase systems preparation

Ten-gram samples of the ATPS, whose compositions are shown in Table 1, were prepared in duplicate in test tubes, according to Franco et al. [8]. Phase separation was achieved by centrifugation for 5 min at 3000×*g*. The pH of both phases was approximately 8.5.

2.6. Partitioning of total proteins

Five hundred microlitres of the top phase was transferred from each prepared system to a cuvette containing 1000 μl of water and 1500 μl of Coomassie blue solution, according to Sedmak and Grossberg [9]. It was mixed well and the A_{595} was read with a spectrophotometer (UV/VIS 911A, GBC Scientific Equipment, Victoria, Australia). A solution containing 500 μl of a top phase of a system in the same manner prepared was used as the reference.

Table 1
Factors studied in ATPS using a mixed fractionated experimental design

Factor	Real values of levels		
	−1	0	1
PEG MW	1500	4000	6000
PEG concentration (%)	16	19	22
Phosphate concentration (%)	10	11.5	13
NaCl concentration (%)	0	6	12

The procedure was repeated for the bottom phase of each system, and a BSA standard curve was used to calculate protein concentration. The partition coefficient, $K_{protein}$, was calculated as the ratio of protein concentration in the top phase to that in the bottom phase at room temperature.

$$K_P = \frac{[\text{protein}]_{\text{top phase}}}{[\text{protein}]_{\text{bottom phase}}}$$

2.7. Xylanase partitioning

A known volume of each phase of the ATPS was transferred for the measurement of enzyme activity. The ratio of xylanase activity in the top phase to the enzyme activity in the bottom phase was calculated (K_{enzyme}).

$$K_E = \frac{[\text{enzyme}]_{\text{top phase}}}{[\text{enzyme}]_{\text{bottom phase}}}$$

Other parameters were calculated in order to evaluate the purification process: specific activity (SA_{perox}, expressed in U/mg protein), the purification factor (PF) and the enzyme yield recovered in the top phase ($R_{top\ phase}$).

$$SA_{perox} = \frac{\text{Enzyme activity}}{\text{Protein concentration}}$$

$$PF = \frac{SA_{perox} \text{ in the collected phase}}{\text{Initial } SA_{perox}}$$

$$R_{\text{top phase}} = \frac{100}{1 + \frac{1}{R_V \cdot K}} \quad \text{where} \quad R_V = \frac{V_{\text{top phase}}}{V_{\text{bottom phase}}}$$

2.8. Electrophoresis

SDS electrophoresis (SDS–PAGE) was carried out in 12% homogeneous gel [10]. The gels were stained with Bio-Rad silver (Hercules, CA, USA). The molecular mass (M_r) markers were phosphorylase b (94 000), BSA (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400), available as a standard kit (Amershan-Pharmacia Biotech, Uppsala, Sweden).

2.9. Continuous extraction

The continuous experiments of the best ATPS system were performed in the pulsed caps column shown in Fig. 1. The column was hand made of a glass tube (190×25.4 mm). Located at the center of the column was a stainless steel shaft in which three stainless steel caps were soldered. The base caps had a diameter of 24.9 mm and the free area of the caps was around 38% of the total area. Pulses were provided by a device and controlled by a dimmer. Continuous (salt-rich) and dispersed (PEG-rich) phases were added by a peristaltic pump; fermentation broth was added only in the salt-rich phase. Samples of both phases were collected at 10, 15, 20, 40, 50, 60 and 70 min. Extraction was performed at flow-rates of 2.8 and 2.6 ml/min in the PEG-rich phase and the salt-rich phase, respectively, and at two pulse frequencies of the caps: 1/1 (1 s at the top of the column and 1 s at the bottom) and 1/5 (5 s at the top of the column and 1 s at the bottom) were used.

2.10. Pulp bleaching

Commercial oxygen-bleached hardwood kraft pulps (*Eucalyptus*) were obtained from a pulp mill (Champion Papel & Celulose SA, Mogi-Guaçu, São

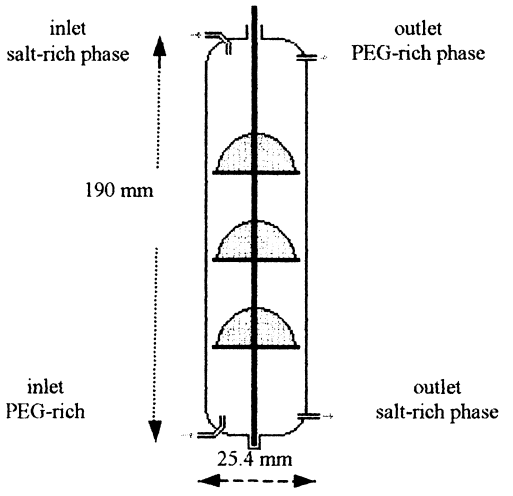

Fig. 1. Pulsed caps column.

Paulo, Brazil). The enzyme treatment was carried out at a pulp consistency of 10% at pH 9.0 and 50°C for 120 min. The pulp was treated with crude fermentation broth with a xylanase concentration ranging between 5 and 50 U/g dry pulp. The control was given exactly the same treatment as the pulp samples, with the sole exception that no enzyme was added. After treatment, the pulps were washed with water. The chemical and physical properties of the pulps, kappa number, viscosity and brightness were analyzed in accordance with the TAPPI Test Methods [11]. The kappa number (kappa n^o) indicates the delignification level of the pulp with lower values showing higher delignification levels and greater delignification efficiency (DE), as determined according to the following equation:

$$DE = \frac{\text{kappa } n^o_{\text{Initial}} - \text{kappa } n^o_{\text{Final}}}{\text{kappa } n^o_{\text{Initial}}} \times 100$$

Another pulp characteristic evaluated was viscosity, which is a good indication of pulp quality because low viscosity can indicate pulp fiber degradation.

3. Results and discussion

3.1. Aqueous two-phase systems

Partitioning of biomaterials in aqueous two-phase systems (ATPS) is a selective method for purification and for analytical studies of cellular components of several sizes, including proteins, nucleic acids, membranes and cellular organelles. ATPS is formed by addition of aqueous solutions of two different polymers, such as PEG and dextran, or of a polymer and a lyotropic salt, such as PEG and potassium phosphate. The extraction and separation process in ATPS can substitute the initial steps of purification such as preparative chromatography, can be scaled up, without a significant loss of efficiency and can be accomplished in the absence of sophisticated equipment [12].

The optimization of ATPS aims at a high enzyme recovery without affecting biochemical properties. Therefore, the xylanase was partitioned in the 12 systems composed of three concentrations of PEG of different molecular masses (1500, 4000 and 6000) and of three phosphate concentrations (Table 2).

The experimental design showed that the increase of molecular mass of PEG (from 1500 to 6000) raised the partition coefficient of the xylanase. In the same way, an increase in the concentration of NaCl from 0 to 12% contributed to the increase of K_{enzyme}.

Another important point to be observed in order to have an effective purification factor is that the contaminant proteins should not be extracted in the same phase as the target enzyme. Therefore, for a high value of K_{enzyme} a small K_{protein} is necessary, and vice-versa.

The contaminant proteins were partitioned into the bottom salt-rich phase ($K_p = 0.1$) and xylanase was

Table 2
Compositions of the investigated ATPS and partition coefficients of xylanase (K_{enzyme}) and of contaminant proteins (K_{protein})

Assay	ATPS composition					
	PEG MW	% PEG	% Phosphate	% NaCl	K_{enzyme}	K_{protein}
1	1500	16	10	0	24.1	1.9
2	1500	16	13	12	9.2	1.4
3	1500	22	10	12	1.1	2.6
4	1500	22	13	0	27.5	0.8
5	4000	16	10	12	36.6	0.3
6	4000	16	13	0	2.0	4.3
7	4000	22	10	0	2.2	2.6
8	4000	22	13	12	0.5	0.3
9	6000	16	10	0	5.1	0.3
10	6000	16	13	12	15.2	3.5
11	**6000**	**22**	**10**	**12**	**46.9**	**0.1**
12	6000	22	13	0	23.8	1.0

selectively partitioned into the top phase (K_E value was 46.9), with the highest PEG molecular mass, and with the lowest phosphate concentration (system 11). The effect of the increase of NaCl concentration (from 0 to 12%) is also well represented in this system, where the $K_{protein}$ decreased from 2.6 to 0.1.

Therefore, the best ATPS to purify xylanase was composed of 22% PEG 6000, 12% NaCl and 10% phosphate and had a 98% enzyme recovery and a purification factor of 33. The reproducibility of the extraction in system 11 was studied by repeating the process several times in systems of the same composition. It was verified that the purification factor in the top PEG-rich phase varied from 40 to 50 and xylanase recovery was always higher than 95%

Gaikaiwari et al. [13] used ATPS to purify xylanase, although they reached only a purification factor of about 2.6 with a 98% enzyme recovery. The partitioning of xylanase produced by *Penicillium janthinellum* was studied in ATPS by Costa [14]. The optimum conditions found were pH 7.0, 8.8% PEG 4000, 10% phosphate and 6.0% NaCl. The xylanase had a partitioning coefficient (K_{enzyme}) of 2.21, a purification factor of 1.34 and an 80% recovery.

Fig. 2 illustrates the SDS–PAGE electrophoresis of the main proteins in the fermentation broth of *B. pumilus* and the protein bands of the purified material in the system composed of 22% PEG 6000, 12% NaCl and 10% phosphate.

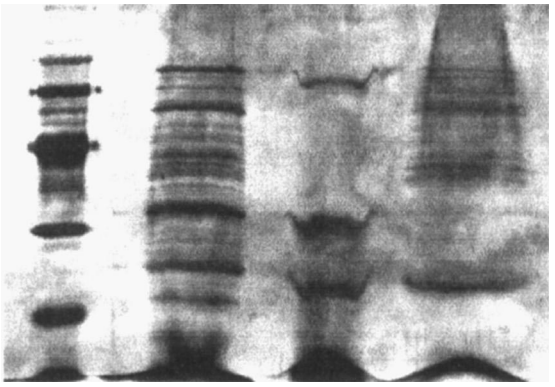

Fig. 2. Electrophoresis SDS–PAGE of purification step in ATPS. Column (1) molecular markers, M_r (phosphorylase b 94 000, bovin serum albumim 67 000, ovalbumin 43 000, carbonic anhydrase 30 000, trypsin inhibitor 20 200 and lactalbumin 14 400); (2) fermentation broth; (3) top phase of system 11 and (4) bottom phase of system 11.

Most of the purified xylanase activity was collected in the top phase, where three main bands with M_r of 22 600, 32 600 and 74 200 are observed. Lumba and Penninckx [15] purified three xylanases produced using *Streptomyces* sp. with M_r f 21 000, 22 000 and 32 000. Nakamura et al. [16] obtained a xylanase with 40 000; Blanco et al. [17] a xylanase with a M_r of 32 000 using *Bacillus* sp. and Panbangred et al. [18] a xylanase with a M_r 24 000 using *B. pumilus*. In pulp bleaching tests, xylanases with low molecular masses spread throughout the pulp fibers more effectively.

Elucidation of the exact mechanisms of the xylanase-aided bleaching method is a key question. One hypothesis says xylan hydrolysed by xylanase makes the fibre structure more porous and thus more permeable to the extraction of lignin by chemicals. Another possible explanation involves the role of redeposited xylan, which may physically shield the residual lignin from bleaching chemicals. Xylanase hydrolyses part of the redeposited xylan, allowing better access of bleaching chemicals to the residual lignin [19].

3.2. Continuous extraction in ATPS

Purification in ATPS can be conducted in continuous operation and countercurrent flow, using classic liquid–liquid extraction equipment. Among other advantages, this technique reduces the processing time. Perforated plate columns have proven very effective with respect to liquid-handling capacity and efficiency of extraction, particularly for systems of low interfacial tension [20].

The continuous extraction process was carried out in the pulsed caps column, using the top and bottom phases of the ATPS initially composed of 22% PEG 6000, 12% NaCl and 10% phosphate. Fermentation broth was added only to the bottom phase. Figs. 3 and 4 show the xylanase activity in both phases and at both pulse frequencies (1/1 and 1/5).

Figs. 3 and 4 do not show large differences between the two pulse frequencies studied. These experiments did not present advantages over the batch experiment using 22% PEG 6000, 10% phos-

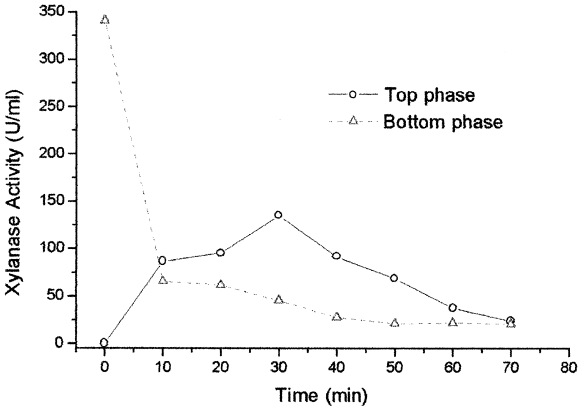

Fig. 3. Xylanase activity at pulse frequency 1/1.

phate and 10% NaCl because xylanase was equally partitioned in both phases. Shaking in liquid–liquid extraction breaks liquid drops inside the column; this promotes a decrease in drop size and an increase in the mixing of the phases. In this way, the contact area between the phases is increased and mass transfer also increases. However, this behaviour was not found in this work, probably because a stable emulsion was formed in the system interrupting mass transfer. Carneiro da Cunha et al. [21] also observed the problem of mass transfer with this sort of equipment. Currently we are improving the extraction system in order to separate the emulsion into two phases.

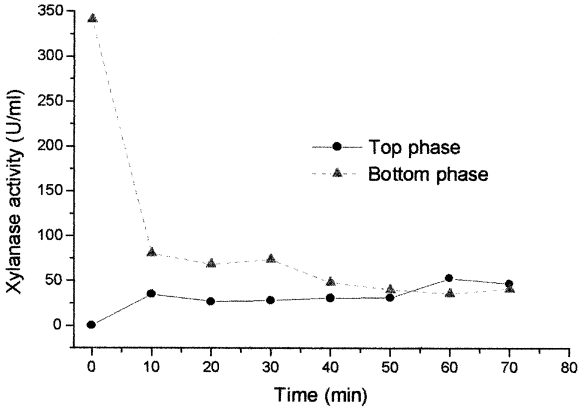

Fig. 4. Xylanase activity at pulse frequency 1/5.

3.3. Pulp bleaching

Commercially, kraft pulp is submitted to a chemical bleaching sequence (Fig. 5). Xylanase treatment makes the pulp more permeable and the lignin more accessible to chemicals, for this reason, by using xylanase in the prebleaching treatment it is possible to save up to 20% of the chemicals.

Results of the xylanase bleaching of commercial hardwood kraft pulp are shown in Table 3. As can be observed in Table 3 and Fig. 6, the kappa number decreased 2.5 units from 10.3 (control without enzyme) to 7.8 and xylanase treatment had no effect on the viscosity of the pulp, indicating that xylanase did not affect the pulp fibers.

Tavares et al. [22] obtained only a one-unit decrease in kappa number using enzymatic treatment. They continued chemical bleaching with ClO_2 and alkaline extraction (NaOH), thereby achieving reductions of 23 and 37% in ClO_2 and NaOH, respectively.

Munk [23] used xylanase treatment and further ECF (elementary chlorine free) achieving a reduction of 1.4 units in kappa number after xylanase treatment, and by the end of bleaching they had saved 25 and 16% of the active chlorine and NaOH, respectively.

In general, xylanase treatment has been shown to reduce the chlorine requirement for bleaching while still achieving a high degree of brightness and good pulp properties. Results from laboratory studies and mill trials showed about a 20–25% reduction in total active chlorine for hardwoods and 10–15% for softwoods [24–27]. Therefore, xylanase treatment of pulps reduces chemical requirements of bleaching and permits attainment of higher degrees of brightness. The reduction in the use of chemicals can be translated into significant cost savings when high

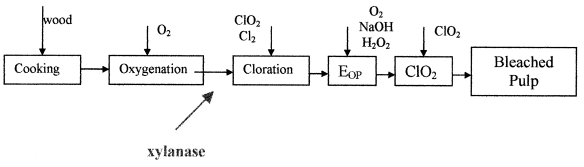

Fig. 5. Scheme of kraft pulp production.

Table 3
Xylanase bleaching of hardwood kraft pulp[a]

Assay	Xylanase concentration (U/g dry pulp)	Time (h)	Kappa number	Brightness	Viscosity	Delignification efficiency (%)
E_0	–	–	10.5	49.2	41.0	–
E	5	2	9.0	50.9	42.8	14
E	20	2	8.8	52.0	42.4	16
E	50	2	7.8	52.6	42.0	26
C	–	2	10.3	48.8	42.0	–

[a] E_0, kraft pulp before xylanase treatment; E, kraft pulp after xylanase treatment; C, control without xylanase.

levels of chlorine dioxide and hydrogen peroxide are used. Decreasing the use of chlorine chemicals reduces the formation and release of chlorinated organic compounds in the effluents and in the pulps [1].

4. Conclusion

The ATPS composition of 22% PEG 6000, 10% K_2HPO_4 and 12% NaCl has proved to be an excellent system for the concentration and purification of xylanase in a single-step operation. A purification factor of 33 and a 98% enzyme yield were achieved in the top phase of this system.

Although the effect of xylanase treatment on hardwood pulp was significant, it is necessary to study a full bleaching sequence in order to discover how much chlorine can be saved.

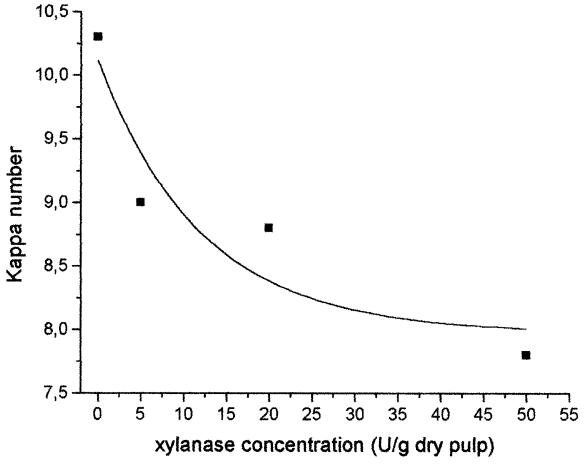

Fig. 6. Kappa number vs. xylanase concentration.

Acknowledgements

The authors would like to thank Dr. Elias B. Tambourgi (FEQ, Unicamp, Brazil), Dr. Marta C.T. Duarte (CPQBA, Unicamp, Brazil) and M. Flávio Tessa (Champion Papel e Celulose do Brazil).

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