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Extraction of Xylanases from Media Based on three Agricolous Co-products by An Aqueous Two-phase System

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Abstract

Jonesia denitrificans BN13 grows very well on a medium made out of agricultural byproducts like orange peel, esparto grass, and retam; the relative enzyme activity of these three substrates was 0.662, 0.711, and 0.835 U/ml, respectively First time xylanases were effectively extracted utilising aqueous two-phase system (ATPS) that had 3.5% PEG, 14% KH_2PO_4/K_2HPO_4, and 9% KI, The inclusion of cells resulted in much improved output from the system, which is based on the technology's ability to work on the basis of the assumption that proteins may be selectively partitioned between the two aqueous phases. In the culture medium that is based on orange peel, a yield of 72% of the enzyme activity is found in the upper phase with a partition coefficient of 2.75 and a purification factor of 1.38. In the medium that is based on alfa, 57.5% of the enzyme activity is located in the lower phase with a partition coefficient of 0.67 and a purification factor of 1.78. In the medium that is based on retam, a yield of 82% of the enzyme activity.

Keyswords: Aqueous two phases system, Xylanases, *Jonesia denitrificans* BN13, *Citrus sinencis, Stipa tenacissima, Retama Raetam.*

INTRODUCTION

The use of white biotechnology and renewable energy has made significant progress in recent decades as an alternative to industrial chemistry.

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Important advances in recent decades, as an alternative to industrial chemistry, it raises the intention of industrialists and actors of sustainable development.

Xylan is the primary component of hemicellulose, which is the second most prevalent polymer on earth after cellulose. Representing between 25 and 35 percent of the lignocellulosic biomass, hemicellulose is an essential and renewable source of biopolymer; xylan represents the major compound [1].

Because xylan has such a complicated structure, the process of breaking it down requires quite a few different enzymes. There are enzymes that are capable of hydrolyzing the main chain, such as (1–4) xylan hydrolases, whereas other enzymes are

capable of cleaving the bonds between the substituents and the xylose residues of the main chain. They are auxiliary enzymes, such as arabinofuranosidases and -D-glucuronosidases, that work on xylose residues in the main chain [2].

The most important application of xylanases is in the pre-bleaching of paper pulp, but they are also used in the textile industry as well as in the production of solvents and biofuels, especially bioethanol [3].

The use of enzymes on an industrial scale necessitates the development of cost-effective procedures for their separation, extraction, concentration, and purification. Enzymes are used in a variety of industrial processes [4].

The most basic method for separating proteins is to use an aqueous two-phase system (ATPS).

It is formed when low concentrations of two incompatible polymers (or a polymer and an inorganic salt) are mixed in such a way that two immiscible phases co such that two immiscible phases coexist. Selective partitioning of proteins between two aqueous phases is the principle of this technique [5], where the upper phase is rich in a polymer and the heavy phase (lower phase) is rich in a second polymer or a salt.

The ATPS described by ALBERTSSON-WIKLAND, WESTPHAL and WESTGREN [5], has been used for the recovery and purification of various bioproducts. These include proteins [6], viruses [7], virus-like particles [8], inclusion of organisms [6], and DNA [9, 10]. This method also creates an environment that is conducive to the activity of enzymes [11].

The most widely studied ATPSs are those formed by polyethylene glycol (PEG) and dextran. Due to the high cost of dextran, this system is not economically attractive compared to other purification methods, but other systems using salts instead of dextran have been considered [5]. Indeed, ATPS offers many advantages, particularly low material cost, low energy consumption, biocompatibility, better yields in a short period of time with no negative impact on the environment [12, 13]. Such a system includes fast mass transfer due to low interfacial tension,

In addition, this system is easily set up. This technique has proven to be an excellent system for the purification of xylanases in an operation that takes place in a single step [14].

Our present work focuses on the extraction of xylanases produced by the *Jonesia denitrificans* BN13 strain, by an aqueous two-phase system [15].

The high cost of pure xylan is a constraint at the industrial scale hence the need to use cheap substrates [16], especially agricultural waste, which significantly contributes to biomass valorization and environmental pollution mitigation.

To achieve this work, three co-products available in Algeria were used to orange peel (Citrus sinensis), esparto grass (Stipa tenacissima) and retam (Retama reatam).

MATERIALS AND METHODS

Preparation of the Substrates

The orange peel was obtained from oranges bought from shops; the oranges are washed, and then the peel is air-dried. Oranges were washed, then the peel was air-dried. The esparto grass and the retam were provided by the director of the laboratory of microbial ecology. microbial ecology laboratory, the esparto powder was obtained after manual grinding followed by sieving (RetschR Test siere 1500 micron), while the cam powder was recovered after crushing of the leaves followed by sieving through a sieve (RetschR Test siere, 1500 micron).

Placing Cultural Media

Four tiny colonies from a Petri dish containing solid MM7 media were subcultured into liquid MM7 pre-culture medium and incubated at 37°C for 24 hours. This preculture inoculates three liquid culture mediums based on orange peel, esparto, and retam at 2% (v/v) in 500 mL of erlens with 100 mL of usable volume. Two series of cultures (based on orange peel, esparto grass, and retam) were established, the first of which was centrifuged before applying the ATPS and the second of which was directly applied to the system. All media were cultured at 37°C, but the incubation period varied. Three, two, and five days, respectively, are spent incubating orange peel, esparto, and retam.

Aqueous two-phase System

Two parallel studies were undertaken. In the first study, the culture media were first centrifuged for 10 minutes at 4000 g and then underwent the ATPS treatment. In the second, the system is applied directly: orange-peel-based, esparto-based, and retam-based cultures were filtered through Whatman No. 01 paper in order to get rid of solid residues suspended in the media. The filtrate was added to 3.5% (m/v) PEG 4000 (Prolabo), 14% (m/v) KH_2PO_4/K_2HPO_4, and 9% (m/v) KI, stirred and adjusted to pH 7, and then left under sedimentation in a separatory funnel.

Biomass Measurement

The measurement of the biomass is carried out at a wavelength of 600 nm in a spectrophotometer (Shimadzu UVmin-1240 V). A control is provided each time; it is the culture medium without seeding.

Quantification of Proteins and Enzymatic Assay

Birchwood xylan, 0.5% (w/v), was used as the substrate for the xylanase activity test and was suspended in a 50 mM sodium phosphate buffer (pH 7.0). 0.1 ml enzyme and 0.9 ml substrate made up the reaction mixture. For 10 minutes, the mixture was incubated at 50°C [17].

The 3,5-dinitrosalicylic acid was used to determine the amount of released reducing sugar [18]. Using a standard curve created using the absorbance of xylose as the reference, the absorbance was measured at 540 nm and translated into moles of reduced sugar produced. The quantity of enzyme that releases 1 mol xylose per min per ml under the aforementioned circumstances is referred to as one unit (U) of xylanase activity.

Using the Bio-Rad protein assay, the protein concentrations of the samples were calculated in accordance with the Bradford technique [19]. As a reference, 1 mg/ml of bovine serum albumin (BSA) was employed as the standard.

Yield Measurement (%)

The yield represents the enzymatic activity, after application of the system (STPA), in the considered phase, compared to the enzymatic activity in the sample before the application of the system [20] (eq. 1):

$$\mathrm{R}(\%) = \frac{\text{enzymatic activity of the considered phase}}{enzymatic\ activity\ of\ the\ initial\ pjhhyyhase} \times 100 \quad (1)$$

Partition Coefficient (K)

The partition coefficient indicates the distribution of the enzymatic activity in the upper and lower phase [20] (eq. 2).

$$K = \frac{\text{enzymatic activity of the upper phase}}{enzymatic\ activity\ of\ the\ lower\ phase} \quad (2)$$

Purification Factor (PF)

This is the ratio of the specific activity after STPA to this value before the application of this system [20].

$$\text{PF} = \frac{\text{enzymatic activity of the considered phase}}{\textit{enzymatic activity of the initial phase}}$$

RESULTS AND DISCUSSIONS

Measurement of biomass and xylanase activities

After seeding the culture media based on orange peel, esparto, and retam and their incubation at 37°C for 3, 2, and 5 days, respectively, we observe a cloudiness, indicating the growth of the *Jonesia denitrificans* BN13 strain on these media.

Biomass was measured at 600 nm for each media, and the results obtained are represented in the Table 1.

Table 1: Measurement of biomass at 600 nm.

OD / Medium based on co-products	Before seeding	After seeding	After substraction
Orange peel	0.265 ± 0.02	1.730 ± 0.32	1.465
Esparto	0.526 ± 0.09	2.011 ± 0.3	1.485
Retam	0.600 ± 0.07	1.835 ± 0.09	1.235

The results show that there is indeed growth in the three media; however, the strain grew well on the medium based on esparto, which means that the strain *Jonesia denitrificans* BN13 has the enzymatic machinery necessary for the use of this co-product, and knowing that it is woody (18.76%), this suggests that the strain has at least a laccase.

Two series of inoculation of each culture medium based on each of the three coproducts were carried out, after fermentation, the culture supernatant of each medium was measured for xylanolytic activity according to the method of Bailey, Biely and Poutanen [18]; the results obtained are represented in Table 2:

Table 2. Summary of xylanolytic activities.

Media based on coproducts	Activity (U/ml)
Orange peel (series 1)	0,662 ± 0.16
Orange peel (series 2)	0,336 ± 0.001
Esparto (series 1)	0,711 ± 0.01
Esparto (series 2)	0,44 ± 0.04
Retam (series 1)	0,853 ± 0.02
Retam (series 2)	0,45 ± 0.08

The activities obtained are considered low compared to the activities described in the majority of actinomycete strains [17].

Several studies have tested the production of xylanases on pure xylan-based media such as birch xylan, spelt xylan, and oat xylan, Nascimento et al. reported an activity of 10.3 U/ml in a culture of Streptomyces sp. AMT-3 incubated for 10 days (Table III); Techapun, *et al.* [21] reported an activity of 8 U/ml in a culture of Streptomyces sp. Ab 106 (55°C, pH 7.5) containing 1% (w/v) xylan.

Nevertheless, an activity of 0.39 U/ml has been reported in the mold Aspergillus terrus grown in 1% birch xylan [22].

The comparison of our results with other works must take into account the nature of the substrate, its purity, and its concentration, in addition to the contaminants, which could partly explain the low activity obtained.

Xylan preparations can differ from each other depending on the type and degree of substitutions that influence the measured xylan activity. The activity test, especially the determination of reducing sugars by the DNS method, gives low apparent activities compared to the Somogyi-Nelson method [23].

The production of xylanases by various microorganisms in liquid media was tested (Table 3).

Table 3. Xylanase production in liquid fermentations.

Micro-organismes	Substrates	Growing conditions	Activity (U/ml)	References
Jonesia denitrificans BN13	Xylan of birch, 7 g/l	4l fermentation, 37°C, pH 7, 2 days	10,80	[15]
Streptomyces sp. Ab106	xylane, 10 g/l	4l fermentation, 55°C, pH 7.5, 5 days	8	[21]
Fusarium solani F7	Wheat straw 30 g/l	Erlen under stirring, pH 5.5, 30°C, 10 days	78,32	[24]
Coriolus versicolor	Tomato pulp	Erlen under shaking, 25°C, 14 days	2,56	[25]

Xylanase production by Jonesia denitrificans BN13 was tested by fermentation in liquid orange peel media; 8.4 U/ml were measured after the 5th day of incubation. The enzymatic activity that we obtained in the orange peel-based medium still remains low compared to the previously cited study, the orange variety, the concentration of orange peel used (1% instead of 5%), the absence of agitation (shaker), the probable presence of pesticide residues, but also because the orange peel used in previous studies was powdered and therefore the substrate was more accessible to the cells [15].

A number of studies on the use of agricultural and industrial wastes have been published. Seyis and Aksoz [26] tested xylanase production in a culture of *Trichoderma harzanum* 1073 D3 on orange and lemon peel substrates; results showed better activity with lemon peel. However, Knob and Carmona [27] showed that the activity was not significant with a *Penicillium scleroticum* culture with orange peel as substrate. Relatively low xylan activity was detected in *Aspergillus carneus, Cochliobolus sativus, Bacillus subtilis*, and *Sclerotinia sclerotiorum* S2M34 using orange peel as substrate [28–30]. Discrepancies in activity also remain between two culture media based on the same substrate (series 1 and series 2), such as the variety of orange peel, and the quality of esparto, which was really of inferior quality, as well as for the quality of the esparto, which was really of inferior quality, as well as for the camels, which had a higher moisture content compared to the first cultures.

Preparation of the ATPS

In the above-mentioned culture media, a system containing 3.5% (w/v) PEG 4000 (Prolabo), 14% KH_2PO_4/K_2HPO_4, and 9% KI (w/v) is prepared, and two phases are formed after shaking and decanting in a separatory funnel. This result is consistent with the results obtained by Ersayin Yasinok, Biran, Kocabas and Bakir [11]. The ATPS was applied as well as a second system which consists in centrifuging the cultures to eliminate the cells before the application of the ATPS.

Centrifuged Cultures before ADPS

The fermentation media is centrifuged at 4000 g for 10 minutes to remove cells before being introduced into the two-phase system.

The different parameters measured are summarized in Table 4

In the orange peel-based medium, the xylanasic activity is distributed mainly in the upper phase, in the medium based on esparto, the activity is distributed almost equally between the two aqueous phases, whereas in the retam-based medium, 69.51% of the activity is found in the upper phase.

The partition coefficients are consistent with the work reported by Kulkarni, *et al.* [31], Jain and Johri [32] who achieved the separation of xylanases in the upper phase with a maximum partition coefficient of 1.2; 3.6. However, Bim and Franco [33] reported a partition coefficient of 46.9, the cells were separated before the application of ATPS.

Table 4. Summary of the parameters measured after ATPS for the culture media centrifuged media.

	Parameters / Samples	Activity (U/ml)	Specific activity (U/mg)	Yield (%)	Partition coefficient (K)	Purification factor (PF)
orange peel-based medium	supernatant	0,662 ± 0.16	0,171 ± 0.04	100		1
	upper phase	0,45 ± 0.08	0,3 ± 0.02	67,97	2,52	1,75
	lower phase	0,178 ± 0.12	0,178 ± 0.16	26,88		
Esparto-based medium	supernatant	0,711 ± 0.01	0,11 ± 0.01	100		1
	upper phase	0,393 ± 0.21	0,32 ± 0.02	55,27	1,25	2,87
	lower phase	0,313 ± 0.09	0,08 ± 0.06	44,73		
Retams-based medium	supernatant	0,853 ± 0.02	0,114 ± 0.09	100		1
	upper phase	0,593 ± 0.14	0,059 ± 0.005	69,51	2,23	1,93
	lower phase	0,266 ± 0.1	0,032 ± 0.003	30,49		

Aqueous two-phase System

Catalytic activities, specific activities, the yields of enzymatic activity, the partition coefficient, and the purification factor were calculated for the upper and lower phases in relation to the culture supernatant after the application of ATPS; the results obtained are summarized in Table 5:

Table 5. Summary of the results obtained after application of the ATPS.

	Parameters / Samples	Activity (U/ml)	Specific activity (U/mg)	Yield (%)	Partition coefficient (K)	Purification factor (PF)
Orange peel-based medium	supernatant	0,336 ± 0.001	0,24 ± 0.03	100		1
	upper phase	0,242 ± 0.05	0,334 ± 0.02	72,18	2,75	1,38
	lower phase	0,088 ± 0.02	0,1 ± 0.06	26,2		
Esparto-based medium	supernatant	0,44 ± 0.14	0,327 ± 0.09	100		1
	upper phase	0,169 ± 0.03	0,046 ± 0.03	38,40	0,67	1,78
	lower phase	0,253 ± 0.22	0,077±0.008	57,5		
Retam-based medium	supernatant	0,45 ± 0.08	0,121 ± 0.06	100		1
	upper phase	0,1 ± 0.04	0,1 ± 0.05	17,8	0,27	1,63
	lower phase	0,37 ± 0.04	0,195 ± 0.09	82,22		

Concerning orange peel, the enzymatic activity is found mostly in the upper phase (72%), in contrast, the enzymatic activity of the other two media based on esparto and retam are found in the lower phase with a rather high yield of 82% for the medium based on retam and a lower yield for the medium based on esparto (57, 5%), the purification factors are relatively low, xylanase produced by *Bacillus pumilus* was extracted from the fermentation medium using ATPS, the system contained 22% PEG-6000, 10% K2HPO4 and 12% NaCl, the results show a purification factor of 33 and 98% yield of enzyme activity. Most of the purified xylanase activity was collected in the upper phase, where three major isoenzymes are detected with PM of 22.6, 32.6, and 74.2 kDa on denaturing electrophoresis gel (SDS-AGE) [33].

Paecilomyce thermophila J18, isolated by Yang, Yan, Jiang, Li, Tian and Wang [14], produces large amounts of thermostable xylanase, when grown in solid fermentation, Xylanase was purified to homogeneity using one-step ADPS, the optimal system for xylanase extraction was performed at pH 7.2 containing 12.5% (w/w) PEG-4000 and 25% (w/w) $(NH_4)_2SO_4$, with a partition coefficient of 231.7, a purification factor of 5.54 and a yield of 98.7% in the upper phase.

Bacillus Pumilus alkaline xylanases were extracted by an ATPS composed of 16% PEG-6000 and the phosphate salt of 8%. A purification factor of 57 and a yield of 41% was obtained in the lower phase of this system, which contained only two major only two major protein bands [34].

The extraction of xylanases produced by Bacillus pumilus directly from the culture medium medium containing the microbial cells is performed by a system composed of 3.5% PEG PEG, 14% KH_2PO_4/K_2HPO_4 and 9% KI; the results indicate that 70% of the xylanases were found in the upper phase, 7 times more purified and 4 times more concentrated; no activity was found in the lower phase and the microbial cells on the other hand are mainly collected at the interface or in the lower the interface or in the lower phase [4].

The partition coefficients for esparto and retam-based culture media are low due to the distribution of xylanases in the lower phase [35], so the purification protocol may contain only a centrifugation step to remove the cells and a dialysis step to get rid of the salts.

Li, *et al.* [36] reported a partition coefficient of 47.3 and a yield of 97.03% in the upper phase of a system composed of 12.99% PEG and 12.09% sodium citrate sodium citrate applied to *Trichderma viride* species.

The pI of our xylanases is unknown, but it is an essential parameter for separation; positively charged functions trap proteins in the lower phase, while negatively charged proteins are directed to the upper phase [37], so if our xylanase is negatively charged relative to the pH of the system, it will tend to be distributed in the upper phase and vice versa.

The purification factors and the yields obtained are below the expected results because we result because we directly applied a model that has already been optimized for another strain and in a strain and in a completely different medium, in order to evaluate its efficiency in the extraction of in the extraction of xylanases produced by *Jonesia denitrificans* BN13, moreover the construction of the binodial curve is a crucial step that determines a good separation.

The results for ATPS after cell recovery are lower than those found for cultures that underwent ATPS in the presence of cells, particularly for recovery efficiencies of enzyme activity, which confirms that the presence of the cells facilitates and promotes the separation.

In the purification protocol performed on *Jonesia denitrificans* BN 13 xylanase [15], a purification factor of 19 was achieved in two steps, whereas the one we performed with ATPS is almost equivalent to that of ultrafiltration (1.8).

CONCLUSION

Our work consisted in applying ATPA for the extraction of xylanases, and as a first purification step. The system composed of 3.5% PEG 4000, 14% KH_2PO_4/K_2HPO_4-9% KI, provided better results without any cell recovery step; in the orange peel medium, 72.18% of the enzyme activity was found in the upper phase with a purification factor of 1.38; For the esparto-based medium, the partitioning was almost equivalent between the two phases with a yield of 57.5% in the lower phase and a purification factor of 1.78. on the other hand, in the retam-based medium, a yield of 82% was achieved in the lower phase and a purification factor of 1.63 was measured.

This study is a draft for the purification of xylanases produced by Jonesia denitrificans BN13 grown on agaricles co-products, indeed, it would be interesting to repeat the same work but optimizing an aqueous two-phase system.

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