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Production of a xylanase by *Trichoderma harzianum* (Hypocrea lixii) in solid-state fermentation and its recovery by an aqueous two-phase system

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Abstract

Production of xylanase enzyme by fungi strains has gained interest in the recent years due to its high productivity, high catalytic power, as well as its potential applications in different areas such as feed, food, textile, and biofuel industries. The conventional methodologies, to produce enzymes, involve complex apparatus and chemical solvents and are associated with high costs and low-yield recovery. To obtain the high-yield recovery of the enzymes, modern enzyme extraction methods are taken into account. Aqueous two-phase systems (ATPS) are an alternative separative methodology for the purification and recovery of the enzymes and other biomolecules. The advantages of ATPS are easy scale-up and extraction, volume reduction, and rapid separation. The objective of this study was to produce *Trichoderma harzianum* xylanase by solid-state fermentation (SSF) using corn cobs as a support/substrate and employing ATPS for its partial recovery. In this study, the results showed the ability of a microorganism to grow on the corn cobs and to produce the xylanase enzyme. Xylanolytic activity reached 7.85 U/g of corn cobs. The enzyme was efficiently concentrated by ATPS. In addition, a high purification factor (10-fold) and considerable enzyme recovery (%ER) (84%) percentage were obtained.

Keywords: Xylanase, *Trichoderma*, Solid-state fermentation, Corn cobs, Biphasic systems

Abbreviations: Solid-state fermentation: SSF, Aqueous two-phase systems: ATPS, Polyethylene glycol: PEG, Mineral medium: MM, Crude enzymatic extract: CEE, Ultrafiltered enzymatic extract: UEE, Tie-line length: TLL, Enzyme recovery: %ER, Enzyme partition coefficient: K*e*, Protein partition coefficient: K*r*, Purification factor: PF

Introduction

Due to a large number of agro-wastes generated worldwide and their interesting composition, several studies have been carried out with the objective of employing them to reduce the environmental pollution as well as toward attainment of high-added value products [1]. These studies have applied the microbial processes such as solid-state fermentation (SSF), which is considered as a viable alternative method to achieve the main goals. The characteristics of SSF like simple reproducibility, no requirement of a sophisticated equipment and an organic solvent, and cost-effective make it a suitable alternative method [2]. The SSF has been defined by the various authors as a bioprocess that is employed not only on the surface but also within a solid porous matrix with the absence or nearby absence of free water but with enough moisture for the microbial growth [3]. Agro-industrial residues are one of the most common organic matrices reported and are used as a substrate/ carbon source [4]. Similarly, the corn cobs have been implemented in solid processes for the production of microbial metabolites [5, 6]. In solid bioprocesses, fungi are

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the microorganisms that adapt better due to the high requirement of oxygen, lower aqueous activity than bacteria, and these biosystems mimic the natural habitat promoting their development [7]. The fungal genus of *Trichoderma* has been largely studied in the production of high-value products, principally hydrolase enzyme such as xylanase [8], which is responsible for the degradation of hemicellulose xylan, an important constituent in corn cobs [9]. Xylanases (EC 3.2.1.8) belong to the group of hemicellulolytic enzymes, which catalyze the hydrolysis reaction of the links β-1,4 -*D* bonds in xylans [10]. Several studies have reported the industrial applications of xylanase and the ability of different varieties of *Trichoderma* sp. to produce xylanase [11, 12, 13]. There are some significant factors of industrial interest, concerning recovery, purification, and separation process, in the production of enzymes [14, 15]. Although the accumulation of extracellular enzymes production during solid culture facilitates the recovery, most of the methodologies used for purification require long periods of time, are expensive, and recovery yields can be low [16]. The most commonly used methods for enzyme recovery (%ER) are by using solvents, salts, ion-exchange chromatography and/or gel filtration [17]. Conventionally, ammonium sulphate and acetone fractioning are used as an initial step in protein purification, however, these methodologies are laborious and time-consuming. Bioseparation techniques such as aqueous two-phase biosystems (ATPS) are a potential option for the initial protein purification step [18]. ATPS are well-studied methodologies and ideal for the recovery of enzymes due to high water content requirements and low interphase tension, which provides favourable conditions for the preservation of biomolecules [19, 20]. Several enzymes such as lipases, proteases, alpha-amylase, laccase, and xylanase [15, 21, 22], among others, have been partially purified through these systems obtaining high recovery yields and considerable levels of purification. However, there is little information on the implementation of the ATPS as a one-step process for the separation and purification of xylanase produced by SSF. The present study describes the partial purification of a fungi xylanase produced by SSF using agro-industrial wastes.

Materials and Methods

Materials

Polyethylene glycol (PEG) of different molecular weights (1000, 3350, and 8000 $gmol^{-1}$), 3,5-dinitrosalicilic acid, *D*-glucose, and xylan (beechwood) were purchased from Sigma Aldrich chemicals (St-Louis, MO, USA). Dibasic potassium phosphate (K_2PO_4) was purchase from J.T Baker (Center Valley, PA, USA). All the other reagents used were of the analytical grade.

Microorganism and plant material

A strain of *Trichoderma harzianum* was obtained from the culture collection of the DIA-UAdeC. The fungal strain was cryopreserved at -20°C until propagation. The corn cobs were kindly provided by the Universidad Autónoma Agraria Antonio Narro. The plant material was dehydrated and milled to a particle size of approximately 0.5 cm. After milling, the corn cobs were stored in the dark without humidity until used.

Culture media and inoculum preparation

Cryopreserved spores were added to sterile potato dextrose agar (PDA) in the Erlenmeyer flasks at 30°C for 7 days. After fungal propagation, the spores were harvested by stirring with Tween 80 (0.1% v/v) solution until spores were suspended. A Neubauer chamber was used to establish the number of *Trichoderma harzianum* spores in the suspension. The composition of the Mineral medium (MM) was as follows: 1.4 gL^{-1} $(NH_4)_2SO_4$, 2.03 gL^{-1} KH_2PO_4, 0.3 gL^{-1} $CaCl_2$, 1.0 gL^{-1} peptone, 0.005 gL^{-1} $FeSO_4$, 0.0016 gL^{-1} $MnSO_4•4H_2O$, 0.0014 gL^{-1} $ZnSO_4•7H2O$, 0.02 gL^{-1} $CoCl_2•6H_2O$, 0.001 gL^{-1} $CuSO4•5H_2O$, 0.3 gL^{-1} urea, and 0.25 gL^{-1} yeast extract. The salts were dissolved in water and the pH was adjusted to 4.6 using 1 M NaOH.

Small SSF

Enzymes production in the SSF system (Fig. 1) was performed utilizing the 15 g of milled corn cobs placed in aluminum bioreactors (trays). The dry material was impregnated with MM (45 ml) to reach 75% of the moisture content. Then, *Trichoderma harzianum* inoculum was added ($2x10^6$ spores/g substrate) to the wet material and homogenized. Finally, the bioreactors were sited at 30°C for 144 h. Every 12 h, one sample was taken to determine the highest activity of xylanase. Crude enzymatic extract (CEE) was obtained by adding 20 ml of citrate buffer (0.05 M, pH 5.2) to the fermented material and filtered with Whatman No. 40 filters using a vacuum pump. CEE was stored at 0ºC until the measurement of the xylanolytic activity and protein content. Each sample was made in triplicate. It is pertinent to mention that none of the work materials were sterilized and under this condition no other microorganism grew in the bioreactors due to the high level of inoculation.

Large SSF

According to the results of the highest enzymatic activity by the small fermentation, xylanase production was carried out in a different large stainless bioreactor (diameter: 46 cm, height: 22 cm) during 96 h. In this SSF, 5 kg of milled corn cobs were placed along with 15 L of MM. The wet material was carefully homogenized and incubated at 30°C. Then, 6.7 L of citrate buffer (0.05 M, pH 5.2) was added to the fermented material for the recovery of CEE, which was stored until analysis. In this process, none of the materials were sterilized.

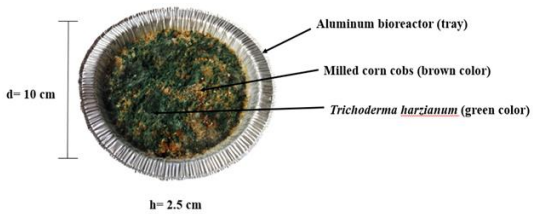

Fig. 1: SSF system in aluminum tray at 96 h employing corn cobs, a sole carbon source, for the microorganism's growth and xylanase production.
*The systems contained 15 g of corn cobs and were incubated at 30°C. All the systems were carried out in triplicate. d: internal diameter; h: height.

Enzymatic assay

Xylanase activity was analyzed using xylan as a substrate. The reaction contained 0.050 ml of CEE and 0.2 ml of xylan (0.1% w/v) prepared in a citrate buffer (0.05 M, pH 5.2). Then, the mixture was incubated at 50°C for 10 min. Afterward, 0.375 ml of dinitrosalisylic acid (DNS) was added to the mixture, and the reducing sugars were determined using *D*-glucose as standard [23]. Also, the blanks of enzyme and substrate were prepared to obtain the xylanase activity for later calculations. One xylanase activity unit was defined as the amount of enzyme that releases 1 µmol of xylose from xylan per min under the assay conditions.

CEE processing

The initial volume of CEE (1 L) was concentrated using an ultrafiltration system with 5 kDa membrane until the achievement of 0.1 L of ultrafiltered enzymatic extract (UEE). The total protein content was measured before and after the ultrafiltration according to Bradford's method [24].

ATPS preparation and xylanase partitioning

ATPS were used in this study for the partial recovery and purification of xylanase produced by SSF. The systems were carried out in Eppendorf tubes with the following characteristics: volume ratio (VR) equal to 1, the weight of 1.8 g, constant pH 7, and four tie-line length (TLL)- 15, 25, 35, and 45 %w/w. TLL is defined as the mass concentration of the chemicals forming PEG-phosphate in the top and bottom phase [20]. Three different PEG molecular masses were employed (1000, 3350, and 8000 KDa). These polymers were mixed with the stock solution of K_2PO_4 (50% w/w, pH 7) and distilled water. ATPS were stirred to complete the dissolution of each component and stored during 24 h until two-phase formation:

Table 1: Equations used to calculate the parameters of partition in the ATPS under the evaluated conditions and a summary of most relevant results.

Name	Symbol	Definition	Units	PEG	%TLL	Value
CEE specific activity	CEE SA	$\frac{[\text{Act (U/mL)}]}{[\text{Protein (mg/mL)}]}$	U/mg	-	-	0.028
Specific activity (top)	SA	$\frac{[\text{Act (U/mL)}]}{[\text{Protein (mg/mL)}]}$	U/mg	8000	25	0.290
Purification factor	PF	$\frac{[\text{Act. esp}]\text{ATPS}}{[\text{Act. esp}]\text{ initial}}$	-	8000	45	10.29
Enzyme recovery yield (top)	ER	$\frac{100}{1+\left(\frac{1}{R*Ke}\right)}$	%	1000	35	84.049
Protein recovery percentage	PR	$\frac{[\text{Act (U)}]\text{ATPS}}{[\text{Act. (U)}]\text{initial}} * 100$	%	8000	45	81.023
Enzyme partition coefficient	Ke	$\frac{[\text{Enzyme}]\text{Top phase}}{[\text{Enzyme}]\text{Bottom phase}}$	-	1000	35	6.174
Protein partition coefficient	Kr	$\frac{[\text{Protein}]\text{Top}}{[\text{Protein}]\text{bottom}}$	-	1000	15	2.691
Volumetric activity	EA	$\left(\frac{g}{mL}\right)\left(\frac{Vol rxn}{Vol\ EE}\right)\left(\frac{1\text{ mol}}{MW\ Prod}\right)\left(\frac{1x10^6\text{ umol}}{1\text{ mol}}\right)\left(\frac{1}{t}\right)$	U/mL	8000	45	6.39

All these equations have been employed according to *IPROBYQ-CONICET*.

top phase rich in PEG and bottom phase rich in K_2PO_4. Enzyme partitioning was performed at room temperature with the addition of 0.2 g of UEE to each previously formed biphasic systems. ATPS were lightly vortexed for 15 min. Complete phase separation was achieved by centrifugation during 15 min at 10,000 rpm and 25°C, using Galaxy 16 centrifuge (VWR, Philadelphia, PA, USA). Then, xylanase activity and total protein content were measured in both the phases. Also, the parameters such as purification factor (PF) and %ER along others, were calculated (Table 1). All the samples were analyzed in triplicate.

Results and Discussion

As shown in Fig. 2, xylanase was successfully produced by *Trichoderma harzianum*. The production was detected at 36 h with constant detection until 144 h with the highest xylanolytic activity at 96 h with 7.85 U/g of corn cobs (2968.69 UL^{-1}). The xylanolytic activity decreased during the fermentation process. This could be probably attributed to enzyme degradation by protease activity and enzyme inactivation. The xylanolytic activity obtained is lower than that reported by Simões et al. [25] and Yasinok et al. [22]. Simões et al. produced the xylanase by *Trichoderma viride* using wheat bran supplemented with sorbitol (1%) and reported the xylanolytic activity to be 3366 UL^{-1}. Yasinok et al. utilized the submerged fermentation with 2% corn cobs and reported 1,84,000 UL^{-1} to be the activity of the xylanase produced by Bac*illus pumilus*. But our xylanolytic activity is higher than that reported by Chapla et al. [26] and Grujic et al. [27] who showed 1560 UL^{-1} and 2310 UL^{-1} as the activity of the xylanase produced by *Aspergillus foetidus* and Trichoderma atroviride, respectively, using SSF. In this study, the xylanase was anticipated to be produced from the corn cobs as a sole carbon source due to the high xylan content in the hemicellulose. The cellulase activity was also measured which revealed the β-glucosidase, but the endoglucanase and exoglucanase were not detected (data not shown). Fungi can produce several hydrolytic enzymes, including xylanases and β-glucosidases, in the presence of xylose by *Trichoderma* strain [28]. The facts regarding the usage of corn cobs in SSF make it a promising inducer for producing xylanase enzyme by fungi strains without involving extra steps such as pretreatment hydrolysis or sterilization process. With the aim of finding new applications and solving contamination issues caused by agro-industrial residues, SSF was carried out at a major scale with 5 Kg of corn cobs. The value of enzymatic activity (Fig. 3) in CEE was 5.28 U/g of corn cobs (1980 UL^{-1}) whereas, in UEE, the activity was 7.35 U/g of corn cobs (2500 UL^{-1}). This rise was due to the usage of 5 kDa membrane which holds sugars as low molecular weight residuals, which were removed due to the interference with the DNS method, and the high molecular weight proteins. Also, the total protein content was concentrated from 208.40 mg/g of corn cobs (70 mgL^{-1}) to 601 mg/g of corn cobs (200 mgL^{-1}) in CEE and UEE, respectively. The separation efficiency of ATPS was studied on the addition of PEG of different molecular weight. This parameter affects the purification and recovery of the enzymes. Xylanase partitioning was carried out using the methodology previously described. Fig. 4 shows the effect of the different molecular weight of PEG along with the TLL on the xylanase activity. Some studies about protein bioseparation using ATPS have reported that PEG does not interfere during the enzymatic assay [18]. Xylanolytic activity was detected in both the phases, however top phase rich in PEG showed higher activity than the bottom phase, this could be possible due to the better solubility that represents major enzyme affinity and active sites

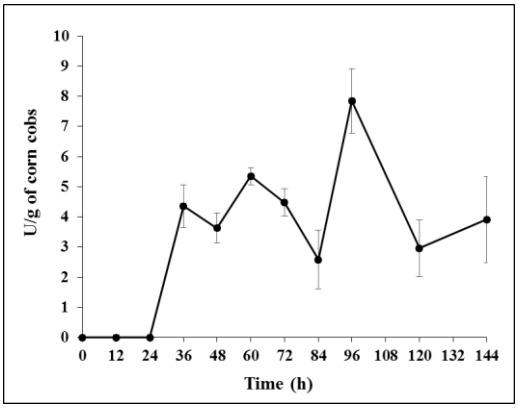

Fig. 2: Xylanase production by *Trichoderma harzianum* on solid bioprocess using 15 g of corn cobs as a support/substrate during 144 h.

*Milled corn cobs were not sterilized. All the determinations were carried out in triplicate and are presented as mean value ± standard deviation.

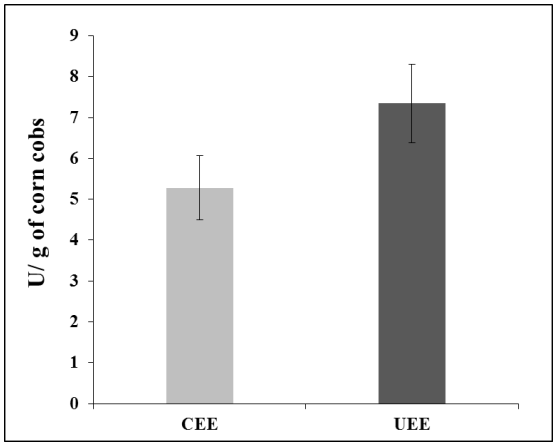

Fig. 3: Xylanase activity quantification before and after ultrafiltration process using 5 kDa membrane.

*The measurement was carried out in triplicate. All the results are presented as mean value ± standard deviation.

preservation. By using TLL 45, the enzymatic activity was favoured, preserved, and increased progressively with an increasing molecular weight of PEG (15.24 U/g of corn cobs (PEG 1000), 16.24 U/g of corn cobs (PEG 3350) to the highest 19.19 U/g of corn cobs (PEG 8000)). This phenomenon can be attributed to the interaction and preservation of the actives sites of lateral amino acids of protein with terminal hydroxyl and ether groups of the polymer which are mildly acidic and basic, respectively, promoting safe interaction sites and stability. Moreover, these characteristics of interactions have not been studied yet in order to understand the xylanase purification produced by fungi agents. Results about the %ER during ATPS partitioning are shown in Table 2. In this study, the highest %ER was obtained in the top PEG-rich phase as indicated by the enzyme partition coefficient (*K*e) and protein partition coefficient (*K*r), values greater than 1. The highest %ER of 84% was in the PEG 1000 ATPS using TLL 35. This percentage is a little lower than that reported (88%) by Garai and Kumar [15] using PEG 4000, and the lowest %ER of 52% was observed in PEG 8000 using TLL 25. Protein recovery of 81% in the PEG 1000 ATPS was the highest recovery, and PEG 3350 ATPS protein recovery was the lowest ranging from 34 to 50%. Regarding these results, PEG 1000 and PEG 3350 protein partitioning was equilibrated in top PEG-rich phase for the steric interaction process (PEG/protein). In the case of PEG 8000, PEG concentration was higher in this phase, and water

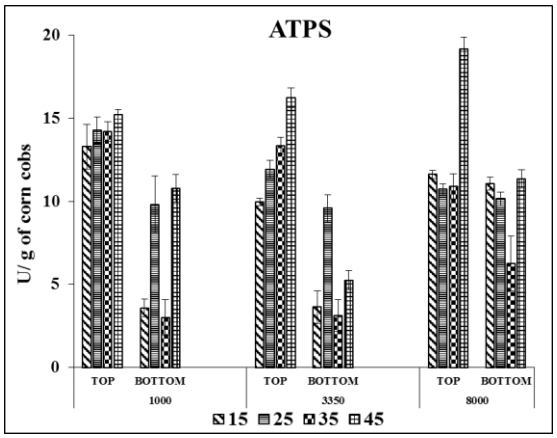

Fig. 4: PEG-phosphate ATPS partitioning of xylanase from solid culture (5 Kg) at PEG of different molecular weight and TLL. *pH of the systems used was kept at 7. The weight of UEE from SSF was kept constant at 0.2 g. All the determinations were carried out in triplicate and the results are presented as mean value ± standard deviation.

Table 2: Purification and recovery values of xylanase produced by *Trichoderma harzianum* in SSF through PEG-phosphate systems.

PEG ($gmol^{-1}$)	PEG (%p/p)	Phosphate (%p/p)	TLL (%w/w)	PF		%ER	
				top	Bottom	top	bottom
1000	12	14.6	15	5.2	3.03	79.52	20.48
	13.3	15	25	4.93	8.47	57.19	42.81
	16	16	35	5.29	2.65	84.05	15.95
	19.2	17.1	45	5.55	9.68	64	36
3350	10	10.8	15	7.16	2.79	76.45	23.55
	11.9	11.8	25	8.89	6.87	53.68	46.32
	15	13.2	35	7.46	2.96	81.55	18.45
	18.8	15	45	9.05	4.37	77.67	22.33
8000	10.2	8.8	15	6.30	8.84	68.85	31.15
	13	10.4	25	5.74	7.54	52.29	47.71
	16.1	12.3	35	4.98	4.18	67.12	32.88
	20.2	14.8	45	10.29	7.72	62.86	37.14

The systems were made at room temperature and the volume ratio was kept constant at 1.

content is not available enough, this could cause the proteins to move to the bottom salt-rich phase. Also, using a PEG of high molecular weight creates an exclusion effect, stronger than the salting-out effect, affecting the protein partitioning to the salt-rich phase where the polymer is in less proportion. There are reports about this steric effect as a determinant factor between PEG/protein interaction due to the increased molecular weight of the polymer, increased exclusion volume, and the displacement of protein to salt-rich phase [29]. Also, the protein molecular weight is another factor that can affect protein partitioning in ATPS. Table 1 shows the most relevant parameters and results using ATPS such as specific activity (SA) which, in this study, was very low at an initial value of 0.028 U/mg of protein (CEE) and increased with the highest value of 0.290 U/mg of protein. PEG 8000 obtained the high values of PF (Table 2) during xylanase partitioning. The maximum value of xylanase purification was more than ten-fold which is higher than the three-fold of enzyme produced by the solid culture of *Aspergillus candidus* [15]. High SA obtained at the end of protein separation can be attributed to differential partitioning in the bottom phase, which can contain impurities, and could affect xylanase activity and protein quantification [30]. In this case, the enzyme can act effortlessly due to great substrate availability without any interference and could have a better reproducibility of the enzymatic activity. The results of PEG-phosphate ATPS were favourable for the recovery and partial separation of xylanase obtained in SSF by *Trichoderma harzianum*. Furthermore, this preliminary report could be the beginning of new further research studies including parameters of optimization to improve enzyme production and purification through the environmental methodologies.

Conclusion

Fungal xylanase was successfully produced using corn cobs as an inducer. The partial purification of the xylanase resulted in the achievement of an enhanced enzymatic activity. *Trichoderma harzianum* grew rapidly under SSF conditions, showed great affinity to the agro-industrial residue which is used as a sole carbon source, and produced a high level of xylanase enzyme. ATPS is demonstrated to be a very useful methodology for xylanase partitioning. This PEG-phosphate system is considered as an integrated and large scale-up process for the %ER, with industrial interest, in a cost-effective manner compared to the conventional methodologies for purification. The ATPS conditions with the highest xylanolytic activity were: 20.2% PEG 8000, 14.8% K_2PO_4, and TLL of 45, obtaining 19.19 U/g of corn cobs with 62.85% of %ER and 50% of protein recovery from the solid culture which resulted in ten-fold purification. In our study, the enzymatic activity was affected positively during the partitioning process. This activity increased progressively with an increase in the TLL and the molecular weight of PEG.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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