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Partition and purification of a thermostable xylanase produced by *Paecilomyces thermophila* in solid-state fermentation using aqueous two-phase systems

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Abstract

In the present study, an aqueous two-phase system (ATPS) composed of polyethylene glycol (PEG) and ammonium sulphate ($(NH_4)_2SO_4$) was used to purify a thermostable xylanase produced by the thermophilic fungus *Paecilomyces thermophila* J18 in solid-state fermentation (SSF). The effects of PEG, $(NH_4)_2SO_4$, loading mass and pH on xylanase partition in ATPS were investigated. Xylanase partitioned in the top polymer-rich phase, while the remainder of proteins partitioned in the bottom salt-rich phase. The ATPS composition (w/w) of 12.5% PEG-4000, 25% $(NH_4)_2SO_4$ and 50% enzyme solution at pH 7.2 was proved to be an excellent system for purification of xylanase in a single-step operation. SDS-PAGE analysis revealed that the purified xylanase was near homogeneity. A purification factor of 5.54 and a 98.7% enzyme yield were achieved in the top phase of this system. However, the xylanase produced in SSF was purified to homogeneity with a recovery yield of 34.5% by three steps. Therefore, ATPS provides an efficient and attractive method for purifying the xylanase in SSF.

Keywords: Aqueous two-phase system; *Paecilomyces thermophila*; Partition; Purification; Thermostable; Xylanase

1. Introduction

An aqueous two-phase system (ATPS) is formed when low concentrations of two incompatible polymers (or of one polymer and an inorganic salt) are mixed such that two immiscible phases coexist [1]. ATPS offers many advantages including low-process time, low-material cost, low-energy consumption, good resolution, high yield and a relatively high capacity. In addition, this system is easily scaled-up [1–4]. Hence, ATPS has been successfully applied to the extraction, separations of proteins, nucleic acids, cells and cell particles [4]. The use of ATPS in downstream processing has also performed on the extraction, separation, concentration and primary purification of various enzymes that include xylanase, xylose reductase, β-glucosidase, potato polyphenol oxidase, etc. [5–8]. As ATPS can remove contaminants such as nucleic acids and undesirable proteins, it has been successfully used to purify β-xylosidase and lysozyme to near homogeneity [9,10].

Endo-1,4-β-xylanase (EC 3.2.1.8) randomly hydrolyzes the β-1,4-glycosidic bonds of xylan (the major constituent of hemicellulose) to produce xylooligomers with different lengths [11]. Recently, xylanases have attracted considerable research interest because of their potential industrial applications. The extraction, separation and primary purification of xylanases using a two-phase system has been performed from the crude enzymes [5,12–15]. It was reported that partitioning of xylanase in ATPS is suggested as powerful primary separation and purification step from crude enzyme obtained by solid-state cultivation of *Polyporus squamosus* [14]. Incorporation of Eudragit S-100 in the PEG phase of ATPS led to considerable selectivity in separation of microbial xylanases [16]. Fungal xylanases can be produced using solid state or submerged cultivation systems, the former of them gained renewed interest lately because of a number of economic and engineering advantages [17–20]. The newly isolated *Paecilomyces thermophila* J18 was proved to be a good producer (18,580 U/g) of

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xylanase in solid-state fermentation (SSF) [20]. The xylanase has shown its optimal temperature at 75–80 °C and exhibits potential applications in the future. However, the purification of the xylanase involved time-consuming ammonium sulphate precipitation and several column chromatographies [21]. In order to develop a simple, efficient, economical and industrially applicable purification method of enzymes from the crude culture filtrates of SSF, herein we investigate the possibility of using ATPS in a single-step for purifying the thermostable xylanase produced by *P. thermophila* J18 in SSF. ATPS was examined with regard to the effects of polyethylene glycol (PEG) molecular weight and concentration, ammonium sulphate ($(NH_4)_2SO_4$) concentration, loading mass and pH on xylanase partition and purification.

2. Materials and methods

2.1. Materials

PEG-600, 1000, 2000, 4000 and 6000 were obtained from Merck (Darmstadt, Germany). Birchwood xylan was purchased from Sigma Chemical Company (St. Louis, MO, USA). The Superdex 75 was from Pharmacia (Pharmacia, Uppsala, Sweden). DEAE 52 was from Whatman (Whatman Inc., Fairfield, NJ, USA). Other chemicals used were analytical grade reagents unless otherwise stated.

2.2. Xylanase production by SSF and enzyme extraction

Paecilomyces thermophila J18 was isolated by Yang et al. [20] and was deposited (under the number AS3.6885) at the Center for Culture Collection of Microorganisms of China. Stock cultures were maintained on PDA at 4 °C and were transferred every 6–7 weeks. PDA plates were incubated at 50 °C for 4–5 days and stored at 4 °C until use.

Xylanase production by *P. themophila* J18 was carried out in SSF using wheat straw as substrate [20]. The strain was grown in 300 ml Erlenmeyer flasks containing 5 g of wheat straw, 0.2 g yeast extract and 23 ml deionized water. To each flask 2.0 ml of spore suspension (1×10^6) was inoculated. The cultures were incubated statically at 50 °C for 7 days. The xylanases were extracted from the fermented carbon source with 10-fold (v/w) 50 mM citrate buffer (pH 6.2) by shaking (200 rpm) at room temperature for 2 h. The suspended materials and fungal biomass were separated by centrifugation (12,000 × *g* for 10 min) and the clarified supernatant was used as the source of crude enzyme. This crude extract was used for further purification by the aqueous two-phase system.

2.3. Aqueous two-phase systems

Ten-gram systems were prepared by weighing the phase-forming components to reach the final concentrations in flasks. The systems were mixed with a Vortex for 1 min and after thorough homogenization, transferred to graduated centrifuge tubes. The phases were then separated by centrifugation at 3000 × *g* for 3 min. The pH of the phase systems was adjusted with NaOH or HCl. All partition experiments were done at room temperature. Volumes of the separated phases were measured. Aliquots of the top and bottom phases were taken for enzyme assay and determination of protein concentration. The results given are averages of three separate experiments.

2.4. Enzyme assay and protein determination

Xylanase activity was assayed according to the method of Bailey et al. [22]. The reaction mixture containing 0.9 ml of 1.0% (w/v) birchwood xylan and a suitably diluted enzyme solution was incubated in 50 mM citrate–phosphate buffer (pH 6.0) at 50 °C for 10 min. The reaction was stopped by adding 1 ml DNS (dinitrosalicylic acid). The amount of reducing sugar liberated was determined by DNS method using xylose as the standard. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute. Protein concentrations were measured by the Lowry method [23] with BSA (bovine serum albumin) as the standard. Specific activity is expressed as units per mg of protein. Specific activity and protein concentration of the enzyme solution are 201 U/mg and 1.0 mg/ml, respectively.

2.5. Determination of phase volume ratio, partition coefficient, purification factor and yield

The phase volume ratio (R) was defined as: $R = V_t/V_b$, where V_t and V_b are the top and bottom phase volume, respectively. The xylanase activity partition coefficient (K) was calculated by the ratio of the xylanase activity in the top phase (X_t) to that in the bottom phase (X_b): $K = X_t/X_b$. Xylanase yield (Y) in the top phase was determined from the equation $Y\ (\%) = 100 \times K/(K + 1/R)$ [2]. The purification factor (PF) was calculated by the ratio of the specific activity in the top phase to the specific activity in the crude extract.

2.6. Purification of the xylanase produced by P. thermophila J18 in SSF

All purification steps were performed at 4 °C unless stated otherwise. The crude enzyme was subjected to 20–60% ammonium sulphate saturation. The precipitated protein collected by centrifugation (10,000 × *g*) was dissolved in 20 mM phosphate buffer (pH 7.2). Further purification was done by gel filtration and ion-exchange chromatographies. A 0.5 ml sample from ammonium sulphate precipitation was loaded on a Superdex 75 column (40 cm × 1.0 cm) and equilibrated with 20 mM phosphate buffer (pH 7.2), and the protein was eluted at a flow rate of 0.50 ml/min. All active fractions were combined and were applied to a DEAE 52 column (20 cm × 1.0 cm) previously equilibrated with 20 mM phosphate buffer (pH 7.2) at a flow rate of 0.8 ml/min. The bound proteins were eluted with a NaCl gradient (0.3–1 M) in the same buffer. The flow rate was 0.6 ml/min. The highly active fractions were pooled and dialyzed against 20 mM phosphate buffer (pH 7.2). The homogeneity of dialyzate was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12.5% (w/v) acrylamide in gels as described by Laemmli [24]. Protein bands were visualized by Coomassie brilliant blue R-250 staining. The molecular weight standard used was the low-molecular weight calibration kit (Amersham): phophorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

3. Results

3.1. Effects of PEG molecular weight on the xylanase partition

The addition of PEGs with different degree of polymerization affected the extraction efficiency. Table 1 shows the effects

Table 1
Effects of PEG molecular weight on the xylanase partition[a]

PEG molecular mass	K	R	Y	PF
PEG-600	88.9 ± 3.2	0.21 ± 0.01	95.0 ± 3.5	0.98 ± 0.045
PEG-1000	67.4 ± 2.8	0.33 ± 0.015	95.7 ± 3.4	1.32 ± 0.064
PEG-2000	67.5 ± 2.7	0.30 ± 0.015	95.3 ± 3.8	1.68 ± 0.013
PEG-4000	93.5 ± 3.3	0.27 ± 0.012	96.2 ± 3.1	2.99 ± 0.12
PEG-6000	100.5 ± 3.8	0.25 ± 0.013	96.2 ± 3.3	2.81 ± 0.1

[a] Phase compositions (w/w) (10 g) contain 10% various PEGs, 20% $(NH_4)_2SO_4$ and 20% enzyme solution.