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Direct recovery of *Bacillus subtilis* xylanase from fermentation broth with an alcohol/salt aqueous biphasic system

Hui Suan Ng,[1] Cindy Xin Yi Chai,[1] Yin Hui Chow,[2] Wai Leng Carmen Loh,[1] Hip Seng Yim,[1] Joo Shun Tan,[3] and John Chi-Wei Lan[4,*]

Faculty of Applied Sciences, UCSI University, UCSI Heights, 56000 Cheras, Kuala Lumpur, Malaysia,[1] School of Engineering, Taylor's University Lakeside Campus, 47500 Subang Jaya, Selangor, Malaysia,[2] Bioprocess Technology, School of Industrial Technology, Universiti Sains Malaysia, 11800 Gelugor, Pulau Pinang, Malaysia,[3] and Biorefinery and Bioprocess Engineering Laboratory, Department of Chemical Engineering and Materials Science, Yuan Ze University, Chungli, Taoyuan, 320, Taiwan[4]

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Xylanase enzyme degrades linear polysaccharide β-1,4 xylan and the hemicellulose of the plant cell wall. There is a growing demand in finding a cost-effective alternative for industrial scale production of xylanase with high purity for pharmaceutical applications. In this study, an alcohol/salt aqueous biphasic system (ABS) was adopted to recover xylanase from the *Bacillus subtilis* fermentation broth. The effects of several ABS parameters such as types and concentrations of alcohols and salts (i.e., sulphate, phosphate, and citrate), amount of crude loading and pH of the system on the recovery of xylanase were investigated. Partition coefficient of xylanase (K_E), selectivity (S) and yield (Y_T) of xylanase in top phase of the ABS were measured. Highest K_E (6.58 ± 0.05) and selectivity (4.84 ± 0.33) were recorded in an ABS of pH 8 composed of 26% (w/w) 1-propanol, 18% (w/w) ammonium sulphate. High Y_T of 71.88% ± 0.15 and a purification fold (P_{FT}) of 5.74 ± 0.33 were recorded with this optimum recovery of xylanase using alcohol/salt ABS. The purity of xylanase recovered was then qualitatively verified with sodium dodecyl sulphate (SDS) gel electrophoresis. The SDS profile revealed the purified xylanase was successfully obtained in the top phase of the one-step 1-propanol/sulphate ABS with a distinct single band.

[**Key words:** Aqueous biphasic system; Recovery; Xylanase; *Bacillus subtilis*; Fermentation]

Enzyme xylanase degrades the linear polysaccharide β-1,4 xylan to xylose, a monosaccharide and thus is able to breakdown the hemicellulose of plant cell wall. Xylanase can be applied in a wide array of industries such as bleaching of craft pulp in the pulp and paper industry; food additives and clarifying agents of juices in food industry; and antimicrobial agents for the production of pharmacologically active polysaccharides in pharmaceutical industry (1). In recent study, xylanase was applied to improve the rumen fermentation and reduced the production of greenhouse gases (2). In view of the great potential of xylanase, considerable research interest has drawn towards the production of xylanase over the past two decades. The organisms that are responsible for the production of xylanase can range from prokaryotes to eukaryotes (e.g., bacteria, algae, fungi, snail, insect, protozoa, anthropods, gastropods). However, bacterial fermentation of xylanase is generally more feasible for large-scale production. *Bacillus subtilis* which is naturally found in the soil and vegetation is among the most industrially important xylanase producer because of the inherent higher enzyme activity. Thus, the xylanase was fermented using the *B. subtilis* strains in this study for the optimum production of xylanase.

The economic feasibility of the large scale enzyme production relies on the advances applied in both the upstream and the downstream processing. Aqueous biphasic system (ABS) is a simple liquid–liquid separation method for purification of various biological molecules and particles (3). ABS consists of two immiscible aqueous phases which can be formed by dissolving two mutually incompatible liquid components above a critical concentration (4). ABS has successfully purified various enzymes, bioactive compounds, proteins, and nucleic acids in previous literature (5,6). As compared to conventional enzyme separation and purification methods, ABS offers several advantages such as biocompatibility, low energy consumption, easy to scale-up and feasibility of process integration (3).

Several polymer/salt ABSs have been applied for the recovery of bacterial xylanase from fermentation broth of different strains in previous studies (7). Xylanase sourced from *Bacillus pumilus* fermentation can be purified up to 33-fold with a yield of 98% with ABS composed of 22% (w/w) polyethylene glycol (PEG) 6000 and 10% (w/w) phosphate with an addition of 12% (w/w) sodium chloride (NaCl) (7). A yield of more than 92% of recombinant *Bacillus halodurans* xylanase was recovered with ABS composed of 18.3% (w/w) PEG 1000 and 14.4% (w/w) phosphate at pH 8.5 (8). Polymer/salt ABS exhibits high viscosity, slow phase separation, and complication in the recycling of the phase-forming components despite the fact that it is simple to conduct. In view of this, alcohol/salt ABS is applied in this study for recovery of *B. subtilis*

* Corresponding author at: Biorefinery and Bioprocess Engineering Laboratory, Department of Chemical Engineering and Materials Science, Yuan Ze University, No. 135, Yuan Tung Road, Chungli, Taoyuan, Taiwan. Tel.: +886 3 4638800x3550; fax: +886 3 4559373.
E-mail address: lanchiwei@saturn.yzu.edu.tw (J.C.-W. Lan).

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xylanase from crude feedstock to overcome the above-mentioned limitations of polymer/salt ABS.

Alcohol/salt ABS on the contrary is of lower cost and viscosity as compared to the conventional polymer/salt ABS (9). Moreover, the enzyme extracted to the alcohol-rich phase can be easily recovered by evaporation, and thus enabling the recycling and reuse of the phase-forming component (10). In this study, the effects of types and concentrations of alcohol and salt, crude load, and pH of the system were examined for optimum recovery of *B. subtilis* xylanase.

MATERIALS AND METHODS

Materials Bicinchoninic acid (BCA) protein assay kit was purchased from Bio Basic (Markham, Canada). Ethanol, 1-propanol, 2-propanol, ammonium sulphate, potassium phosphate and sodium citrate were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) and 30% acrylamide/bris solution were obtained from Bio-Rad (Hercules, CA, USA). Glacial acetic acid, sodium chloride (NaCl), 3,5-dinitrosalicylic acid (DNS), and sodium potassium tartrate were obtained from Friendemann Schimidt (Australia). All the chemicals and reagents used in this study were of analytical grade.

Bacterial cultivation of *B. subtilis* for xylanase production *B. subtilis* was obtained from Microbiological Laboratory, UCSI University, Malaysia. Inoculation was performed by transferring a loopful of *B. subtilis* culture into 10 mL of nutrient broth and incubated in 37°C for 24 h with an agitation speed of 150 rpm (11). Fermentation media was prepared by mixing 2 g of sucrose; 0.05 g of K_2HPO_4; 0.02 g of NaCl; 0.016 g of $MgSO_4 \cdot 7H_2O$; 0.05 g of yeast extract; and 2 g of sugarcane bagasse (11). After the 10% (v/v) inoculation, the fermentation broth was incubated in 37°C for 48 h with the agitation speed of 150 rpm. Crude xylanase (supernatant) was collected after centrifuged with a centrifugal force of 8000 $\times g$ for 10 min.

Aqueous biphasic system Alcohol/salt ABS was prepared as described in previous literature (10). ABS of 5 g was prepared with 15 mL centrifuge tube. The aqueous system consists of appropriate amount of alcohol/salt solutions and crude xylanase was then centrifuged at 8000 $\times g$ for 5 min for complete phase formation. The volumes of the top and bottom phases were noted. The two separated phase samples were collected for enzyme activity assay and BCA protein assay analysis. The effects of the concentrations of alcohol and salt, crude load and pH of the system on the ABS recovery of xylanase were investigated.

Determination of xylanase enzyme activity The xylanase enzyme activities exhibited by different phase samples were determined using the xylanase activity assay (11). Substrate solution of 1% (w/w) birchwood xylan solution was prepared in citrate buffer. A total of 0.5 mL of diluted enzyme sample was added to 0.5 mL of 1% (w/w) birchwood xylan solution. The resulted mixture was incubated for 15 min at 50°C. Next, the mixture was added with 7.5 mL of 3,5-dinitrosalicylic acid and left in the boiling water bath for 10 min to stop the enzyme reaction. The mixture was then left to cool to room temperature before the absorbance was measured at 550 nm. Triplicate measurements were taken and the results were expressed as mean ± standard error. One unit of xylanase activity was defined as the amount of enzyme needed to release 1 μmol of reducing sugar equivalent to xylose per min under the assay condition as above-mentioned.

Determination of total protein concentration The protein concentration in the phase samples was determined using the Bio Basic Bicinchoninic Acid Assay (BCA) protein assay kit (Lot: SK30210-K1045R0J). A total of 20 μL of the phase samples were pipetted into the 96 wells microtiter plate, followed by the addition of 200 μL of BCA working reagent. The resulted mixture were mixed gently and incubated at 37°C for 30 min. After the incubation, the absorbance of the mixture was measured at 562 nm. Bovine serum albumin (BSA) standard curve was constructed to determine the protein concentration in the phase samples. All the measurements were quantified in triplicates and expressed as mean ± standard error.

Selectivity (S), purification factor (P_{FT}) and yield (Y_T) The partition coefficient of xylanase (K_E) is defined as the ratio of xylanase activity in top phase (A_T) to the xylanase activity in bottom phase (A_B) of the alcohol/salt ABS (Eq. 1) and the partition coefficient of total protein (K_P) is determined as the ratio of protein concentration in the top phase (P_T) to the protein concentration in the bottom phase (P_B) of the alcohol/salt ABS (Eq. 2):

$$K_E = \frac{A_T}{A_B} \tag{1}$$

$$K_P = \frac{P_T}{P_B} \tag{2}$$

whereas, selectivity (S) was calculated as the ratio of K_E to K_P as indicated in Eq. 3:

$$S = \frac{K_E}{K_P} \tag{3}$$

Purification fold (P_{FT}) was determined as the ratio of the xylanase specific enzyme activity in the top phase (SA_T) to the xylanase specific enzyme activity of the crude feedstock (SA_F) as shown in Eq. 4:

$$P_{FT} = \frac{SA_T}{SA_F} \tag{4}$$

The percentage yield of xylanase in the top phase of the ABS (Y_T, %) was calculated according to Eq. 5:

$$Y_T(\%) = \frac{100}{1 + \left(\frac{1}{V_R} \times K_E\right)} \tag{5}$$

where V_R is the ratio of volume of the top phase to the volume of bottom phase of the ABS.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with Bio-Rad Mini-PROTEAN electrophoresis system. The 12% resolving gel and the 4% stacking gel solutions were prepared as described (12). The casting frame was set on the casting stands. An appropriate amount of resolving gel solution was added into the gap between the glass plates. The gel was left for 20–30 min for the gelation process. The stacking gel solution was then added into the gap between the glass plates after the resolving gel gelated. The resultant ABS purified xylanase sample was then subjected to the SDS-PAGE analysis. Electrophoresis was conducted with a voltage of 120 V for 1.5 h.

RESULTS AND DISCUSSION

Effect of different types of alcohol/salt ABS on recovery of xylanase ABS with high water content is extremely suitable for recovery of enzyme due to the high biocompatibility (6). Whereas, organic solvents such as alcohols can maintain the enzyme's open conformation and enhance the activity of an enzyme (10,13). Thus, ethanol, 1-propanol and 2-propanol are suitable to be applied as the phase-forming components for the ABS to recover target enzyme from crude feedstock. To identify the type of alcohol/salt ABS for better recovery efficiency of *B. subtilis* xylanase, crude xylanase was added to ABS with different types and concentrations of alcohol and salt as referred (10). The amount of crude xylanase and the pH of the ABS were fixed at 20% (w/w) crude xylanase and pH 7, respectively in this ABS partition experiment.

The effect of different types of alcohol/salt ABS on the K_E and Y_T of *B. subtilis* xylanase was evaluated (Table 1). Results showed that the partition of xylanase to the alcohol-rich top phase of the alcohol/salt ABS was considerably enhanced by the sulphate salt, but to a lesser extent, by phosphate salt and citrate salt. Sulphate ions which exhibit higher salting-out effect as compared to phosphate ions will repel more xylanase molecules to the alcohol-rich top phase and therefore further strengthen the interaction between the xylanase and the alcohol molecules (14). This resulted in high K_E values ($K_E > 2.77 \pm 0.04$) for all alcohol/sulphate ABSs investigated (Table 1), and thus ammonium sulphate is the most suitable phase-forming salt for the ABS recovery of *B. subtilis*

TABLE 1. Partition coefficient and yields of *B. subtilis* xylanase with different types of alcohol/salt ABS.

Types of ABS	Phase composition, % (w/w)	K_E	Y_T (%)
Ethanol/sulphate	24/26	2.77 ± 0.04	40.91 ± 0.35
Ethanol/phosphate	20/20	1.31 ± 0.08	37.07 ± 1.26
Ethanol/citrate	24/26	1.22 ± 0.06	34.36 ± 1.17
2-Propanol/sulphate	18/20	3.39 ± 0.14	12.37 ± 0.46
2-Propanol/phosphate	16/16	1.32 ± 0.35	35.74 ± 0.30
2-Propanol/citrate	18/20	2.32 ± 0.01	8.80 ± 0.02
1-Propanol/sulphate	18/20	4.83 ± 0.26	39.68 ± 0.27
1-Propanol/phosphate	16/16	1.32 ± 0.16	24.80 ± 0.29
1-Propanol/citrate	18/20	2.51 ± 0.25	21.80 ± 1.72

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