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Optimize purification of a cellulase from *Bacillus velezensis* A4 by aqueous two-phase system (ATPS) using response surface methodology

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ABSTRACT

This work aimed to study and optimize the parameters affecting partition of a cellulase from the strain *Bacillus velezensis* A4 by aqueous two-phase system (ATPS). Four factors, molecular weight of polyethylene glycol (PEG), PEG concentration, phosphate salt concentration and pH, were investigated. A $L_9(3^4)$ orthogonal experiment was initially carried out to screen important factors affecting partitioning of the enzyme in ATPS. The detailed study of the parameters on enzyme partitioning in ATPS was carried out using Box-Behnken (Box-B) design of response surface methodology (RSM). The statistical analysis indicated the molecular weight of PEG, PEG concentration, and pH affect the recovery rate and purification factor of cellulase partitioning significantly. The model was built and analyzed through RSM analysis and validated by our experiment. The maximum recovery rate of enzyme (67.8%) and purification factor (1.14) could be achieved under the conditions of PEG 4000 (20.75%, w/w), K_2HPO_4 (8.5%, w/w), pH 8.5 in ATPS. The cellulase molecular weight was 35 kDa exhibited in zymogram and has an optimum temperature and pH at 60 °C and 5.0.

1. Introduction

Cellulases are a group of enzymes which hydrolyze the β-1, 4-glycosidic linkage of cellulose, including endoglucanase (EC 3.2.1.4)), exocellobiohydrolase (EC 3.2.1.74), and endoglucanase (EC 3.2.1.21) [1]. These enzymes work together to hydrolyze cellulosic materials to produce reducing sugars [2]. Cellulases were widely applied in industries, such as bioconversion, pulp and paper, textile, and detergent industry, especially in bioenergy production [3]. Cellulase could be produced from different microorganisms (such as fungi, actinomyces and bacteria). The characteristics of cellulase differ significantly from various microorganisms [2–4]. Although many different types of cellulases have been found, there is still strong interest in finding the cellulases with specific properties. *Bacillus velezensis* A4 was one of the strains isolated from forest soils by our lab previously [5] which exhibited a strong ability in hydrolyzing different cellulosic biomasses. However, it is remain to be known what kind of cellulases was produced from this strain and what specific enzyme characteristics does the enzyme have. So it is necessary to purify the enzyme and to investigate the enzyme properties.

Aqueous two-phase system (ATPS) was formed by mixing polymers (usually polyethylene glycol, PEG), buffers (e.g. phosphate, citrate or phosphate), salts (e.g. NaCl, $(NH_4)_2SO_4$) and water to separate proteins [6]. ATPS has many advantages in purification of enzyme and proteins, such as simple, low cost, and easy to scale up [6,7]. It has been extensively used in the purification of protein, enzyme, and other bioproducts [8–10]. The partitioning of enzyme in ATPS was often affected by many factors such as type of polymers, concentration of polymer, salts, pH and temperature [7,11]. The factors affecting enzyme partitioning could be studied by single factor experiment design. However, it is time consuming and costly. The study of several independent variables affecting responses could be achieved by using design of experiment methods (DOE), e.g. orthogonal experiment, uniform design experiment, and response surface design method, and so on [12–14]. Orthogonal experiment could save experimental times, screen the main factors and levels of factors affecting experimental results [13]. Response surface method could be employed in modeling the factors and response, predicted and optimized the parameters affecting the response [14]. Recently, RSM has been often applied in the optimization of parameters of enzymes purification in ATPS [15–17]. However, different parameters are required in purification of various enzymes by ATPS.

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In this study, the parameters affecting the cellulase from *B. velezensis* A4 partitioning in the ATPS were investigated by using orthogonal experiment design method and RSM method. The model was built and employed for studying the interactions between parameters and predicting the optimum conditions for enzyme partitioning in ATPS. The enzyme characteristics were also investigated.

2. Materials and methods

2.1. Chemicals

The different molecular weights (MW) of polyethylene glycol (PEG 1500, PEG 4000, PEG 6000) were purchased from the Thermo Fisher Scientific Inc, Canada. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride and the other chemicals were obtained from Biobasic Inc, Candada. All the chemicals were analytical grade.

2.2. Crude enzyme preparation

The bacterum *Bacillus velezensis* A4 was used in this study, which was isolated in our lab previously [5]. It was cultured in 50 mL of minimal salt medium (MSM) in 250 mL flask containing carboxymethyl cellulose (CMC) (0.5%, w/v) at 37 °C and 200 rpm for 4 d. The MSM medium consisted of (g/L): KH_2PO_4 1.0 g, KCl 1.0 g, $NaNO_3$ 1.0 g, $MnSO_4$ 0.5 g, yeast extract 0.5 g, peptone 3 g. The pH was adjusted to 7.0 before the cultural medium was sterilized at 121 °C for 20 min. The cultural broth was centrifuged at 8000 rpm for 10 min in room temperature and freeze-dried. The freeze-dried sample was stored in −20 °C before use.

2.3. Aqueous two-phase system

2.3.1. Binodal curves building

The bimodal curves were conducted with turbidity method without NaCl. PEG solution 50% (w/w), K_2HPO_4/NaH_2PO_4 solution 40% (w/w) with different pH (6.0, 7.0, 8.0) were prepared in advance. 2 g of PEG solution was added into a 15 mL tube. Then, the K_2HPO_4/NaH_2PO_4 buffer solution, pH 7.0 was added drop by drop into the PEG solution, until it became cloudy. After that, distilled water was added to the system and made them clear until one-phase system was formed. The weight of PEG solution and K_2HPO_4/NaH_2PO_4 buffer, and total weight were recorded to calculate the concentration of PEG solution and phosphate buffer solution in the ATPS. The binodal curves were built by using 7~9 group of the data obtained.

2.3.2. Preparation of the aqueous two-phase system

Aqueous two-phase systems were prepared at room temperature by mixing 50% (w/w) PEG solution, 40% (w/w) of K_2HPO_4/NaH_2PO_4 buffer, 2 g crude enzyme (100 mg/mL in 50 mM citrate buffer pH 6.0) and H_2O in 15 ml centrifuge tubes with conical cap. The pH was pre-adjusted by phosphate buffer. The total weight of ATPS was adjusted to 10 g with distilled water. After vortexed for 3 min, the tube was centrifuged at 2000 rpm for 5 min in room temperature to make phase separation.

Three parameters, partition coefficient (K), recovery rate (R), purification factor (PF), were used to evaluate the efficiency of enzyme partitioning in ATPS. Partition coefficient (K) was defined as the protein concentration in the top phase as to that in the bottom phase. Recovery rate (R) was calculated as the total enzyme activity in the bottom phase as to that of crude enzyme. Purification factor (PF) was defined as the specific enzyme activity of bottom phase as to that of crude enzyme.

2.4. Experimental design

2.4.1. Orthogonal experiment design

The orthogonal experiment design was employed to investigate the factors affecting enzyme partitioning in aqueous two-phase system. Four factors three levels experimental design $L_9(3^4)$ were employed. The four factors are PEG molecular weight, PEG concentration, phosphate salt concentration and pH. Each of the factors has three-level as shown in Table 1. The experiment was performed according to the Table 2. All possible combinations of the levels of the factors are investigated. Three responses (i.e. partition coefficient (K), recovery rate (R) and purification factor (PF)), were calculated to evaluate the efficiency of enzyme partitioning in ATPS. The most important three factors are identified and employed to further investigate in subsequent experiment.

Table 1
Coded values of factors in orthogonal experiment design.

Level	A:PEG MW (kDa)	B:PEG concentration %(w/w)	C:Phosphate concentration % (w/w)	D:pH
1	1500	15	6.5	6
2	4000	20	7	7
3	6000	25	7.5	8

Table 2
Orthogonal experiment design and results of enzymes purification by ATPS.

Run	A:PEG MW (kDa)	B:PEG concentration %(w/w)	C:Phosphate concentration % (w/w)	D:pH	K[a]	R[b]	PF[c]
1	4000 (2)	20 (2)	6.5 (1)	8 (3)	0.541	62.487	0.851
2	1500 (1)	25 (3)	7.5 (3)	8 (3)	0.584	26.562	0.485
3	6000 (3)	15 (1)	7 (2)	8 (3)	0.507	70.133	0.813
4	4000 (2)	15 (1)	7.5 (3)	7 (2)	0.591	40.154	0.492
5	4000 (2)	25 (3)	7 (2)	6 (1)	0.493	0.731	0.031
6	6000 (3)	25 (3)	6.5 (1)	7 (2)	0.605	6.525	0.143
7	1500 (1)	15 (1)	6.5 (1)	6 (1)	0.706	5.766	0.587
8	1500 (1)	20 (2)	7 (2)	7 (2)	0.491	17.957	0.292
9	6000 (3)	20 (2)	7.5 (3)	6 (1)	0.021	0.273	0.368
y1	16.762	38.684	24.926	2.257			
y2	34.457	26.904	29.607	13.530			
y3	25.644	11.273	22.330	53.060			
R	17.696	27.412	7.277	50.803			

$y_i = \Sigma(A_i \text{ or } B_i \text{ or } C_i \text{ or } D_i)/3$.
$R = \text{Max } (y_{i)\text{-Min } (y_{i)}}$
K[a] Partition coefficient: The protein concentration in the top phase as to that in the bottom phase.
R[b] Recovery rate (%): The total enzyme activity in the bottom phase as to that of crude enzyme.
PF[c] Purification factor in bottom phase: The specific enzyme activity of bottom phase as to that of crude enzyme.

2.4.2. Response surface method design

A three-factor experimental design was employed to optimize the enzyme partitioning in ATPS using Box-Behnken (Box-B) experiment design of RSM. The most significant variables, PEG 4000 concentration (A), phosphate buffer concentration (B), and buffer pH (C), selected from orthogonal experimental test were used to further optimize the enzyme partitioning in ATPS. For each of the variables, three points, high value (+1), middle value (0) and low value (-1), were chosen on the basis of bimodal curves and orthogonal test analysis. The Box-B design is one of the response surface design methods, which utilize the combination of midpoints of the edges of a surface at a center. The Box-B designs are rotatable and require 3 levels for each factor and could be used to estimate a quadratic model for every response. All the 17 experiments and results are shown in Table 4. The experimental results were analyzed using the following equation:

$$Yi = b0 + \sum biXi + \sum biiXi^2 + \sum bijXiXj \quad (1)$$

Where Yi represent the response; $b0$, bi, bii and bij represent the regression coefficients, first-order model coefficients and the linear coefficient for the interaction between variable i and j, respectively. Analysis of variance (ANOVA) was employed to evaluate the statistical significance of the model. Fisher's statistical test (F test) was used to test the significance between the sources. F value, calculated by dividing mean square of regression by that of error, was used to evaluate the significance of each variable in the model. p value of the parameters ($p < 0.05$) was shown as significant difference.

2.5. Analytical methods

The enzyme activity was measured using method according to Ghose, 1987 [18] with minor modification. Briefly, the enzyme was assayed at 55 °C for 30 min using 0.05% (w/v) CMC in citrate buffer (pH 4.8) as substrate. The reaction was carried out with 50 µL of enzyme solution and 50 µL substrate 0.05% (w/v) CMC in citrate buffer (pH 4.8) at 55 °C for 30 min. Then 50 µL of the reaction mixture was used to measure reducing sugars using DNS method [19]. Glucose was used as standard. One U of enzyme activity was defined as the enzyme reacted with the substrate at the optimum conditions and produce 1 mg of reducing sugar per minute. The protein concentration was determined according to the instruction of protein assay kit (Bio Basic Inc., Canada) using bovine serum albumin (BSA) as standard. The samples were analyzed against control containing the same phase composition to avoid interference from PEG and phosphate.

2.6. Polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram

SDS-PAGE was carried out in a 12% (w/v) polyacrylamide gel according to the published method [20]. The samples from aqueous two-phase system or crude enzyme were precipitated with 10% (w/v) of trichloroacetic acid (TCA) and incubated at 4 °C for 30 min. The pellet was collected by centrifuged at 12,000 rpm for 5 min and washed with 500 µL 95% (v/v) of ethanol and dried in the air at room temperature. The precipitate was dissolved with 30 µL PBS buffer (pH 8.0), mixed with 10 µL 4X loading buffer (Biobasic Inc, Canada), boiled at 100 °C for 3 min, centrifuged at 12,000 rpm for 3 min in room temperature before it was used for electrophoresis. The gel was run at constant voltage of 120 V at the beginning and the voltage was changed to 160 V after half hour. Gel was stained with Comassie Brilliant Blue R-250 (0.25% (w/v) in the solution (methanol: acetic acid: water (v/v), 45:10:45) and decolored in the same solution without dye. Zymogram was run at same conditions as SDS-PAGE, with 0.1% (w/v) CMC addition in the gel. After electrophoresis the gel was soaked with 1.0% (v/v) Triton X-100 solution and incubated in a shaker at room temperature with speed 120 rpm for 30 min to renature. Then the gel was taken out and stained with 0.1% (w/v) Congo Red solution at room temperature for half hour. After that, the gel was dipped into 1 M sodium chloride at the same condition to visualize.

2.7. Enzyme characterizations

The effect of temperature on enzyme activities was carried out in the standard reaction system with temperature range from 30 to 70 °C. The effect of pH on enzyme activities was determined in the pH range of 3.0-8.0. Different buffers were used to maintain the pH, pH 3.0–5.0 (citrate buffer), pH 6.0–8.0 (phosphate buffer) and pH 9.0–10.0 (glycine buffer), respectively. Results were shown as relative activity (%), calculated by using the enzyme activity assayed at certain conditions compared to the highest enzyme activity in same conditions. The thermo-stability and pH stability were assayed at the temperature range of 40–75 °C for 60 min or pH range of 3.0–10.0 for 5 h. Relative activity (%) was shown as the enzyme activity, assayed at certain temperature and pH conditions, compared to the enzyme activity under standard conditions.

2.8. Data analysis

The experiment design and data were analyzed using the software Design-expert V 8.06 (Stat-Ease, Inc. Minneapolis. MN. USA).

3. Results and discussion

3.1. Binodal curves

The binodal curve indicates the borderline of two-phase formation, while the tie-line shows the ratio of the two-phases in equilibrium. When the concentration of PEG and phosphate is above the borderline, two-phase will be formed. On the contrary, only one phase will be formed. It can be shown from the Fig. 1, higher PEG concentration

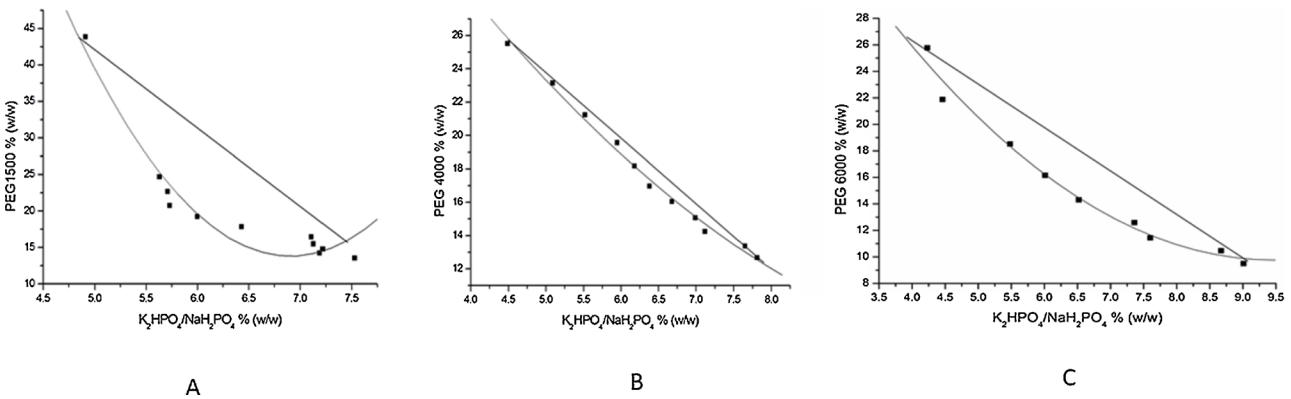


Fig. 1. The Binodal curves for PEG/phosphate aqueous two-phase system (ATPS). A: PEG1500; B: PEG4000; C: PEG6000.

(15%, w/w) is required to form two-phase in the PEG 1500 ATPS than that of in PEG 4000 and PEG 6000 ATPS (Fig.1 A, B, C). The higher molecular weight of PEG, the less concentration of PEG is needed in formation of aqueous two-phase system. This result is consistent with previous report [21,22]. The phase gram could be employed as an important guideline to study the parameters affecting enzyme partitioning in ATPS.

3.2. Factor affecting enzyme partition in ATPS

The orthogonal experiment design was employed to screen the main factors affecting partition of the enzyme in ATPS. Through this method, we can estimate the contribution of each parameter to the enzyme partitioning in ATPS and determinate and predict the optimal parameters affecting enzyme purification in ATPS. The experiment design and results are shown in Table 2. Results showed that the enzyme were likely to partition in bottom phase. The effect of molecular weight of PEG, PEG concentration, phosphate concentration and pH on the enzyme partitioning in ATPS was calculated, it indicated that the importance of the factors affecting recovery rate of enzyme extraction in ATPS is pH > PEG concentration > molecular weight of PEG > phosphate concentration. The best conditions for enzyme participation were PEG 4000 (15% w/w), K_2HPO_4/NaH_2PO_4 (7.0%, w/w), pH 8.0 system in ATPS. The intuitive analyses were performed and shown in Fig. 2. The recovery rate of the enzyme increased by increasing pH in ATPS, while the recovery rate of enzyme decreased with increase of PEG concentration (Fig. 2). The Plackett–Burman experiment design was often been used to screen the main factors among independent variables. It could be applied to screen more than 5 factors at one time [14]. In this study, the orthogonal experiment design was employed, because it is more convenient to be used to screen less than 5 factors and it could also be used to screen the main factors and the best level of the variables at one time.

A statistical analysis of variance (ANOVA) is conducted to evaluate the significant parameters affecting enzyme partitioning in ATPS. The three parameters, pH, PEG concentration and the molecular weight of PEG, were the main factors affecting the recovery rate of enzyme confirmed by ANOVA (Table 3). These results were consistent with the previous report that pH has significant influence on electrochemical

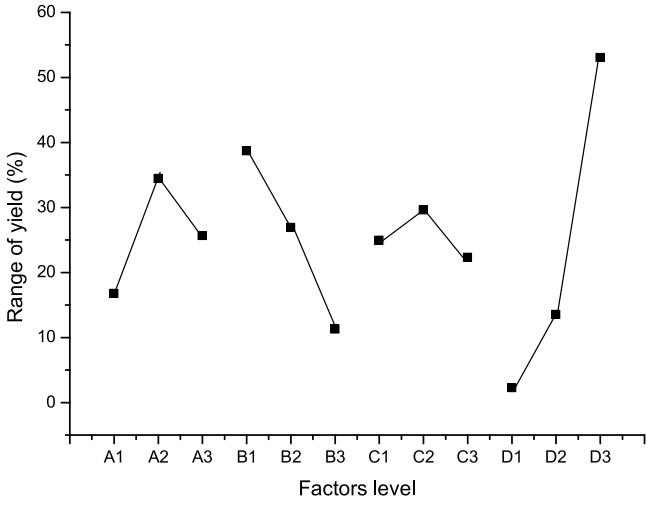


Fig. 2. The range analysis results of orthogonal test. A1-A3: PEG (1500, 4000, 6000); B1-B3: PEG concentration (15, 20, 25%, w/w); C1-C3: Phosphate concentration (6.5, 7.0, 7.5%, w/w); D1-D3 pH (6, 7, 8).

Table 3
ANOVA analysis for the orthogonal experiment test.

Source	Squares	df[a]	*F* value	Significance
Total	4869.930	8.0	608.741	
A-PEG	469.799	2.0	11.511	*
B-PEG concentration	1134.323	2.0	27.794	**
C-Phosphate concentration	81.624	2.0	2.000	
D-pH	3102.560	2.0	76.021	**
Residue error	81.624	4.0		

[a] df: degree of freedom.
* *F* value > $F_{0.05}(2, 4) = 6.94$.
** *F* value > $F_{0.01}(2, 4) = 18$.

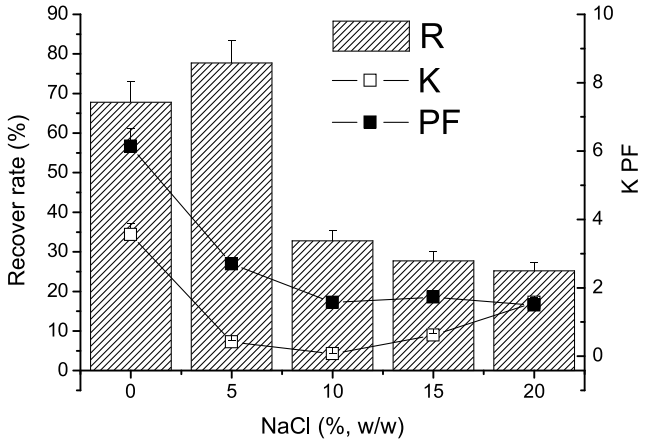


Fig. 3. The effect of NaCl concentration on enzyme partitioning in aqueous two-phase system (ATPS). Recovery rate (R); Purification factor (PF); Partition coefficient (K). NaCl were added into the PEG4000/phosphate ATPS in different concentration. The experiments were carried out three times. The data shows the mean ± deviation.

Table 4
Experimental design and results for cellulase extraction by PEG/phosphate using Box-B method.

RUN	PEG4000 (% w/w)	K_2HPO_4/NaH_2PO_4 (% w/w)	pH	K[a]	R[b]	PF[c]
1	18 (0)	7.5 (0)	8 (0)	0.644	66.634	0.879
2	22 (+1)	7.5 (0)	7.5 (-1)	0.378	28.951	0.446
3	22 (+1)	8.5 (1)	8 (0)	0.478	43.085	0.682
4	18 (0)	7.5 (0)	8 (0)	0.530	55.805	0.808
5	18 (0)	6.5 (-1)	8.5 (+1)	0.438	58.929	0.778
6	18 (0)	8.5 (+1)	7.5 (-1)	0.510	34.229	0.434
7	22 (+1)	6.5 (-1)	8 (0)	0.462	51.702	0.796
8	18 (0)	7.5 (0)	8 (0)	0.631	51.463	0.734
9	14 (−1)	7.5 (0)	8.5 (+1)	0.679	56.079	0.703
10	14 (−1)	6.5 (−1)	8 (0)	0.587	46.922	0.568
11	14 (−1)	8.5 (+1)	8 (0)	0.589	70.441	0.805
12	18 (+1)	7.5 (0)	8 (0)	0.330	61.824	0.763
13	22 (+1)	7.5 (0)	8.5 (+1)	0.389	63.693	0.940
14	18 (0)	6.5 (−1)	7.5 (-1)	0.595	46.277	0.814
15	18 (0)	8.5 (+1)	8.5 (+1)	0.741	74.316	1.170
16	18 (0)	7.5 (0)	8 (0)	0.807	59.635	0.991
17	14 (-1)	7.5 (0)	7.5 (−1)	0.484	52.736	0.593

K[a] partition coefficient: The protein concentration in the top phase as to that in the bottom phase.
R[b] recovery rate: The total enzyme activity in the bottom phase as to that of crude enzyme.
PF[c] purification factor: The specific enzyme activity of bottom phase as to that of crude enzyme.

Table 5
Results of ANOVA for quadratic model regression.

Source	Squares	df[a]	Mean Square	*F* Value	Prob[a] > *F*	
Model	2138.36	9	237.6	9.12	0.0041	significant
A	187.67	1	187.67	7.2	0.0313	
B	41.59	1	41.59	1.6	0.2468	
C	1031.12	1	1031.12	39.59	0.0004	
AB	258.18	1	258.18	9.91	0.0162	
AC	246.47	1	246.47	9.46	0.0179	
BC	188.17	1	188.17	7.22	0.0312	
A^2	87.32	1	87.32	3.35	0.1098	
B^2	9.23	1	9.23	0.35	0.5703	
C^2	72.64	1	72.64	2.79	0.1388	
Residual	182.33	7	26.05			
Lack of Fit	48.68	3	16.23	0.49	0.7103	not significant
Pure Error	133.64	4	33.41			
Cor Total	2320.69	16				

where A represents: PEG4000, %(w/w); B K_2HPO4/NaH_2PO4, %(w/w); C pH; AB interaction of PEG4000 and K_2HPO4/NaH_2PO4; AC interaction of PEG4000 and pH; BC interaction of K_2HPO4/NaH_2PO4 and pH; A^2 quadratic term PEG4000 ; B^2 quadratic term K_2HPO4/NaH_2PO4 ; C^2 quadratic term pH.
[a] df: degree of freedom; Prob: probability.

characteristic of ATPS and protein [23]. The electrochemical interactions play an important role in protein partition in ATPS [6]. As a result, it will affect the enzyme partition significantly. Hydrophobicity is also a very important factor affecting the partitioning of proteins in ATPS. PEG concentration and the molecular weight of PEG affect the hydrophobic of the enzyme [7,22].

The hydrophobic of proteins will affected by NaCl concentration, which also has great influence on the enzyme partitioning in ATPS [11]. The effect of NaCl concentration on participation of enzyme in ATPS was investigated (Fig. 3). Results showed that the enzyme was partitioned in the bottom phase, when NaCl concentration was in 0–5% (w/w) in ATPS. When NaCl concentration was increased higher than 10% (w/w), the recovery rate of enzyme was decreased rapidly to 32.8%. The addition of 5% (w/w) NaCl in PEG4000/phosphate ATPS achieved the highest enzyme recovery rate (77.7%). However, the purification factors decrease rapidly with addition of NaCl (5%, w/w) in ATPS compared with no addition of NaCl in ATPS, which showed that more non target protein was partitioned bottom phase. Our results indicated that NaCl concentration affected the enzyme partitioning significantly. It was consistent with the previous report [21,24]. To obtain high recovery rate and purity of the enzyme, we choose no addition of NaCl in PEG4000/phosphate ATPS for further optimization of the parameters in enzyme extraction using response surface experiment design.

3.3. Response surface methodology

According to the preliminary test results of binodal curve and orthogonal experiment, the range of variables for purification of endoglucanase was set as follows: PEG 4000 (14–22%, w/w), K_2HPO_4/NaH_2PO_4 concentration (6.5–8.5%, w/w), pH (7.5–8.5). The experiment design and results were shown in Table 4. The ANOVA analysis was employed to determine the significant variables on the response. *p* value of the variables less than 0.05 was shown as having significant impact on response. Values of "Prob > *F*" less than 0.050 indicate model variables are significant. As shown in Table 5, the model *F*-value (9.12) implies the model is significant. There is only a 0.41% chance that a "Model *F*-Value" this large could occur due to noise. The *F*-value of "Lack of Fit" equals to 0.49 which implies the Lack of Fit is not significant, the effects of variables are not relative to the pure error. In addition, PEG concentration and pH exhibited significant influence on enzyme participation (Table 4). Each combination of the variables, PEG concentration, K_2HPO_4/NaH_2PO_4 concentration and pH, has significant impact on enzyme purification (Table 4). The evaluation of main effects of factors as well as the interactions was plotted on residuals. As shown from the normal plot of residuals (Fig. 4), the residuals were normal distributed and no deviation of the variance was exhibited.

The interaction of factors and the optimum level of each factor to the maximum response value can be exhibited clearly from the response surface plot. The effect of three parameters on participation of the enzyme could be seen from the three-dimensional graph (Fig. 5). The figure shows the response value (R) against different PEG concentration, phosphate buffer concentration and pH as one of these variables is fixed. As shown from the figure Fig. 5A, the response surface value (R) exhibited a significant increase when the K_2HPO_4/NaH_2PO_4 concentration was increased, and a slight increase when PEG concentration was increased. The response value (R) was decreased with the increasing of PEG concentration, while it was increase with the increasing

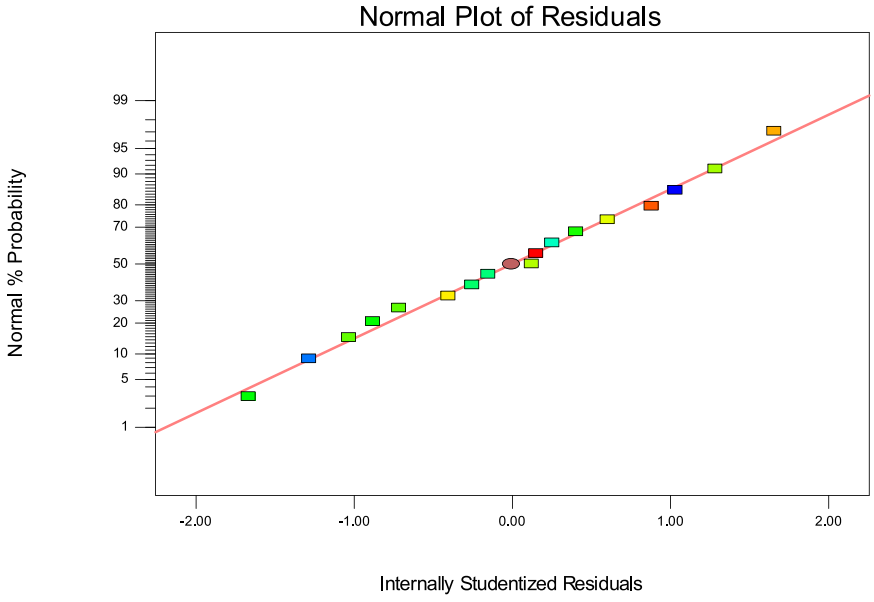
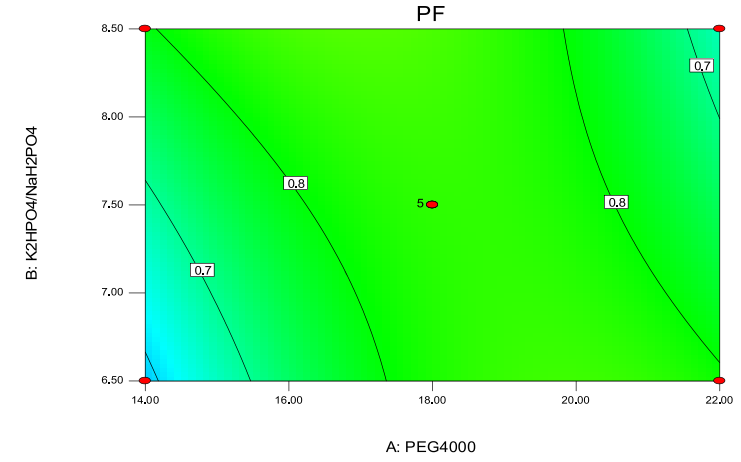


Fig. 4. Normal plot of residual for enzyme partition in aqueous two-phase system (ATPS).



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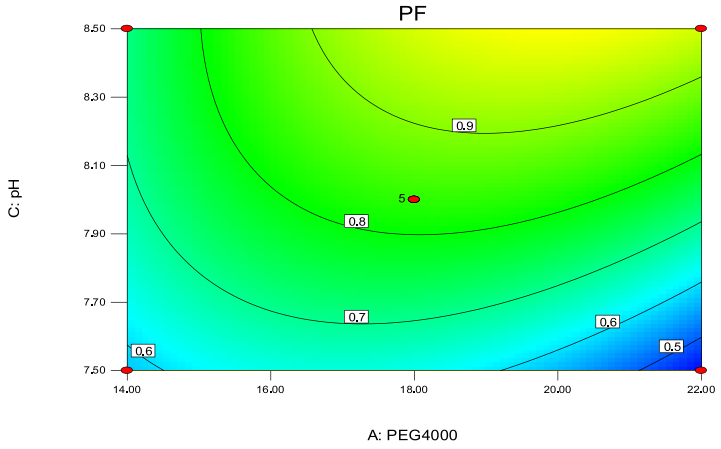

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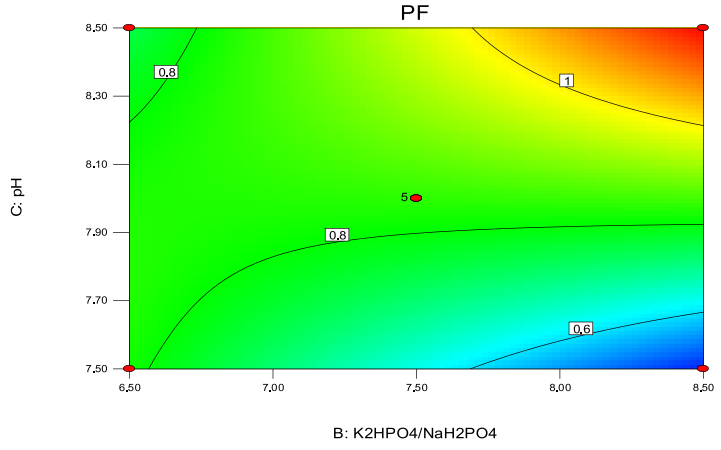

C

Fig. 5. Contour plot shows the effects on the yield of enzyme. (A) Effects of PEG4000 and K_2HPO_4/NaH_2PO_4 concentration on the yield of enzyme; (B) Effects of pH and PEG4000 concentration on the yield of enzyme; (C) Effects of pH and K_2HPO_4 /NaH_2PO_4 concentration on the yield of enzyme.

Table 6
Validation of model with experiment in ATPS.

	R[a]	PF[b]
Model	0.681	1.130
Experimental	0.678	1.140

R[a]: Recovery rate.
PF[b]: Purification factor.

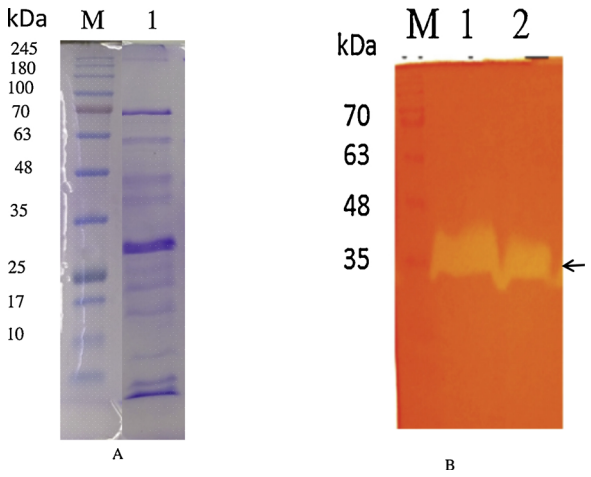

Fig. 6. SDS-PAGE and zymogram analysis crude and purified enzyme. 10 μL of 100 μg protein sample was loaded into each well. A: SDS-PAGE analysis of crude endoglucanase from *B. velezensis* A4. M: protein ladder; Lane 1: Crude enzyme B: Zymogram analysis of the purified endoglucanase. M: protein ladder; Lane 1: Crude enzyme; Lane 2: enzymes purified by aqueous two-phase system (ATPS).

of pH value, when K_2HPO_4/NaH_2PO_4 concentration was fixed (Fig. 5B). When PEG concentration was fixed, the response value (R) was decreased with the increasing of pH value, while it was changed slightly when K_2HPO_4/NaH_2PO_4 was increased (Fig. 5C). The response surface graph could provide an intuitive way to visualize the relationship between response and variables and the interactions between variables [17,25].

3.4. Model analysis and application

The Box-B experimental results were employed for regression analysis. The model is expressed by eq. (1) and eq. (2), which takes the actual value of each variable. The R, PF, A, B, C represents the recovery rate, purification factor, PEG concentration, K_2HPO_4/NaH_2PO_4 concentration and pH. This equation could be used to fit the response value and predict the recovery rate, purification factors with the value of variables.

$$R = +239.632–7.300A–49.094B + 115.007C - 2.009AB + 3.925AC- + 13.718BC–0.285A^2–1.481B^2–16.614C^2 \quad (1)$$

$$PF = +12.722 + 0.0687A - 2.72B - 0.929C–7.836E–003A^2 + 3.125-E–003B^2\text{-}0.157C^2–0.0219AB + 0.0480AC + 0.386BC \quad (2)$$

The coefficient R^2 for the models are 0.9214 (92.14%) and 0.9189 (91.89%), respectively, which means the model is fit with the actual response value very well. Only 7. 86% and 8.11% of the total variability was not fit with the response value of the model. The model could be used to predict the response of the system in the range of the variables.

3.5. Validate the model

The model could be exploited to optimize enzyme extraction by ATPS. The optimized conditions for enzyme extraction were calculated by finding optimum value of variables to obtain the maximum response value for the equation. The predicted maximum recovery rate of the enzyme (68.1%) and PF (1.130) could be achieved at PEG 4000 (20.75%, w/w), K_2HPO_4/NaH_2PO_4 (8.5%, w/w), pH 8.5 in the ATPS. The experimental results of endoglucanase purification under predicted conditions were compared with the predictions (Table 6). It shows that the model fits well with the experimental data. The response surface methodology is an effective tool in the optimization of the response with multivariable and interpretation the relationship between the variables and response. This method has also been employed to modeling the amylase, lipase and xylanase participation in other ATPS

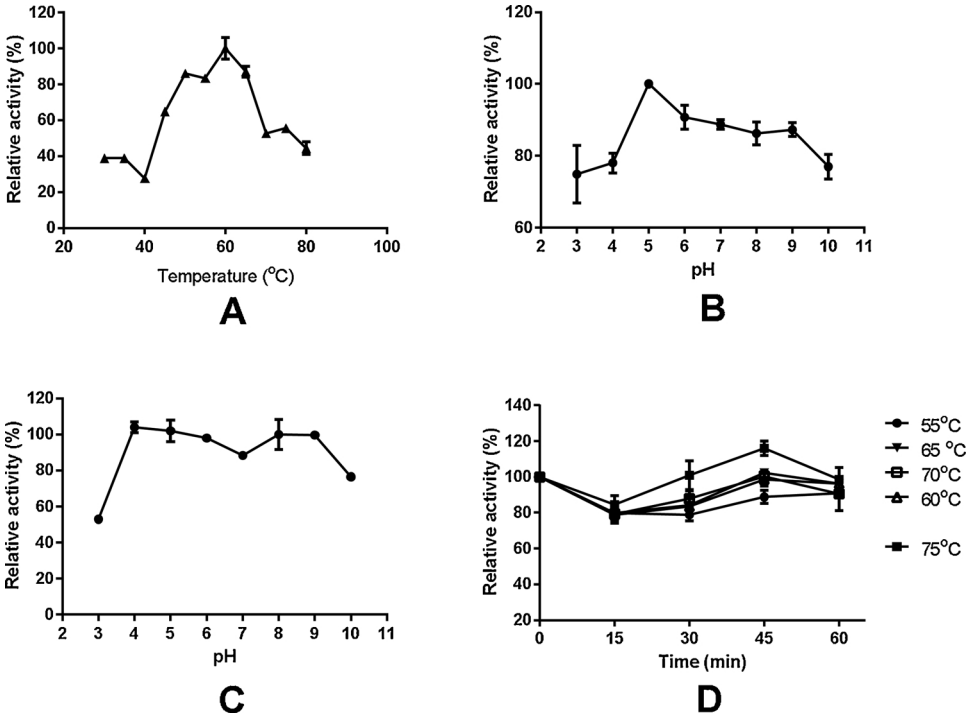

Fig. 7. The enzyme characterizations of purified endoglucanase by aqueous two-phase system (ATPS). A: The effect of temperature on enzyme activity. B: The effect of pH on enzyme activity. C: Thermostability. D: pH stability. The highest enzyme activity was set as 100%. The results were shown as relative activity (%) comparing the enzyme activity with the highest enzyme activity. The data shows the mean ± deviation. All the experiments were carried out three times.

[9,15,26].

This study provides a simple, rapid, reproducible and low-cost method for the recovery and the purification of cellulase from *Bacillus velezensis* A4. Furthermore, ATPS can be employed in industry scale by using consecutive multistage operations and remove the phase-forming components of PEG by back extraction [23,24,27].

3.6. Enzyme characterizations

The partitioning of enzyme in PEG/K_2HPO_4-NaH_2PO_4 system was analyzed with SDS-PAGE and zymogram (Fig. 6A, B). The molecular weight of the crude enzyme and purified enzyme was around 35 kDa exhibited in zymogram. The enzyme showed an optimum temperature at 60 °C and pH at 5.0 (Fig. 7A, B). The enzyme was stable at the pH range 4.0–9.0 and unstable when pH below 4.0 (Fig. 7C). 75% of enzyme activity was lost at pH 3.0. The enzyme was stable at 55–70 °C for 60 min, more than 80% of enzyme activity was remained (Fig. 7D).

4. Conclusions

This work shows the cellulase from *B. velezensis* A4 could be highly effectively purified with single step purification with ATPS. The study indicated PEG concentration, pH were the most significant factors affecting recovery rate and purification factor in the purification of enzyme in ATPS. The maximum recovery rate (67.8%) and purification factor (6.14) could be achieved under the conditions of PEG 4000 (20.75%, w/w), K_2HPO_4 (8.5%, w/w), pH 8.5 in ATPS. The interactions between the variables could be clearly explained by the model analysis. The model could be used in predict the optimized conditions for the enzyme partitioning in ATPS.

Declaration of Competing Interest

None.

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