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Research paper

Optimization of Aqueous Two-Phase System (ATPS) of Recombinant Bromelain by Response Surface Methodology

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Abstract

Recombinant bromelain is a protease that was partially purified using aqueous two-phase system (ATPS). The process variables (pH, PEG 6000 and potassium phosphate concentration) were optimized on enzyme activity and partition coefficient using response surface methodology (RSM) based on a face-centered central composite design (FCCCD) model. The optimum conditions for purification were at 18.47% [w/w] PEG6000 and 13% [w/w] potassium phosphate, pH 7.0 with enzyme activity was obtained as 0.272±0.0036 unit m/L, and partition coefficient as 1.394±0.093. The recombinant bromelain was preferentially partitioned into the top phase and the band was reduced in contrast to crude sample on SDS-PAGE gel.

Keywords: *Aqueous Two-Phase System (ATPS); Face-Centered Central Composite Design (FCCCD); Optimization; Response Surface Methodology (RSM); Recombinant Bromelain;.*

1. Introduction

Bromelain is the major protease enzyme present prominently in pineapple (*Ananas comosus*) fruit and wastes (peel, core, stem and crown). [1] The increase demand of bromelain in various industrial applications such as food, beverage, tenderization, cosmetic, pharmaceutical and textile [2] has drawn attention among researchers to clone the bromelain gene in various hosts including *E.coli* BL21-A1,[3] *E.coli* BL21 DE3pLysS, [4] *Pichia pastoris*, [5] and *Brassica rapa*. [6] However, the recovery of the recombinant bromelain released to the medium is not simple. The downstream process remains to be one of the most costly and challenging parts in a production of recombinant protein. Hence, it is vital to develop a reliable, efficient and cost-effective process for development of high purity and recovery of recombinant bromelain. Following expression of recombinant bromelain in a cytoplasm of *E. coli*, the bacterial cell needs to be disrupted to liberate the protein.

Current finding by Arshad et al. [7] demonstrated a mechanical disruption technique using ultrasonication with an extractant buffer containing sodium phosphate, cysteine and EDTA is found to be effective to release the recombinant bromelain. While such disruption method effectively liberates the recombinant bromelain to the medium, the purification step becomes more challenging because the host cell proteins and nucleic acids are also released, thereby contaminating the recombinant bromelain with undesired cellular proteins. Affinity chromatography using an instrument such as Akta Prime Plus (GE Healthcare, USA) is one of the working horses in downstream processing of recombinant bromelain under native condition. [3, 8] It is because the plasmid vector of recombinant bromelain has a unique polyhistidine (His6) tag which has a high affinity towards nickel ions compared to other proteins that bind nonspecifically to the resin. Despite the robustness and striking features of affinity chromatography in the His-tag interaction between recombinant bromelain with nickel ligand, there are still research limitations that have to be alleviated to avoid disadvantages in the downstream processing. Proteins from the *E. coli* host sometimes naturally binds to the nickel ligand and are co-eluted with the recombinant bromelain during the purification process. Proteins that were observed to be co-purified with the His-tagged protein can be divided into four groups: i) proteins with natural metal-binding motifs, ii) proteins with histidines clusters on their surfaces, iii) proteins that bind to heterologously expresses His-tagged proteins, and iv) proteins with affinity to agarose-based supports.[9] Re-engineering an *E. coli* strain by mutation or alternative tag addition can be a realistic solution to improve the purity of recombinant bromelain and reduce the number of purification steps. [10] Additional steps such as centrifugation, ultrafiltration or precipitation can remove the remaining host cell proteins, polish, and intensify the desired protein. However, multiple steps in the chromatographic method associates with the huge amount of manufacturing cost for the large-scale process. [11]

Aqueous Two-Phase Systems (ATPS) could be promising in the bromelain research. This purification method is low cost and easy to scale up as it only comprises of water, salt, and a polymer that is biocompatible with the environment. Besides, the phase separation only needs centrifugation step, resulting in only one step separation. Extensive literature has been reviewed by Arshad et al. [2] on the purification of bromelain using aqueous two-phase systems. Bromelain had been extracted from pineapple fruit by ATPS system containing PEG-potassium, [12, 13] pineapple peels using a PEG/magnesium sulfate system, [14] and pineapple wastes (stems, barks and leaves) using a PEG/ammonium sulfate system. [15] From all of these studies, it can be concluded that the recovery of

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bromelain is influenced by the pH, and concentration of PEG and salts applied.

An optimization of partition condition using response surface methodology (RSM) would be desirable as a statistical technique to approximate and predict the actual relationship between response and independent variables in ATPS study. [16] Besides, the RSM technique have been successfully employed to enzyme extraction by ATPS system.[16, 17] At present, there is no report focusing on the optimization of recombinant bromelain purification using ATPS system. Thus, the aim of this study was to optimize three ATPS process parameters (pH, salt and PEG concentration) as independent variables using face-centered central composite design (FCCCD) under response surface methodology (RSM) as a tool of experimental design and statistical analysis. The enzyme activity and partition coefficient (KE) were selected as responses to be optimized.

2. Materials and Methods

2.1. Materials

PEG with a molecular weight of 6000, sodium dipotassium phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium hydroxide, and hydrochloric acid were purchased from Merck (USA). All chemicals were of analytical grade.

2.2. Media and culture condition

The cultivation of recombinant *E. coli* BL21-A1[3] harboring stem bromelain gene was carried out in a batch process using auto-induction method as described previously.[18] About 10 mL of culture medium supplemented with 100 µg m/L ampicillin was inoculated with single colony of recombinant *E. coli*. After 16 hrs incubation at 37°C and 300 rpm, 1 L of ZYM medium [19] supplemented with 100 µg m/L ampicillin was inoculated with 10 mL of starter culture and incubated for 12 hrs at 37°C and 250 rpm until the OD_{600nm} reached 0.6-1.0. After 12 hrs, the cells were harvested by centrifugation (4°C, 16000 × g, and 15 min) and the cell pellets were stored at -20°C until needed. The cell pellets were resuspended at a ratio of 1 g : 5 mL of lysis buffer (0.1 M sodium phosphate, 15 mM L-Cysteine, 2 mM EDTA, pH 7.0) before sonication process by applying 20% amplitude, 0.5s cycle, and 1 min bursting period with three times process.[7] The lysates were then centrifuged at 16000 × g for 45 min to obtain a clear supernatant for further studies.

2.3. Enzyme activity assay

The proteolytic activity of recombinant bromelain was measured using Nα-CBZ-L-Lysine ρ-nitrophenyl ester (LNPE) as a substrate at 44°C and pH 4.6. Initially, 2.60 mL of LNPE buffer consists of 30 mM acetate buffer, 100 mM potassium chloride and 1 mM L-cysteine, was mixed and incubated with 100 µL of enzyme solution for approximately 3 min. Later, 100 µL of 50 mM LNPE substrate was added and the solution was mixed by inversion, where the increment of absorbance reading at 340 nm was measured for 5 min using a microplate reader (Thermo Scientific, USA). One unit of enzyme activity corresponds to the release of 1.0 µL of the ρ-nitrophenyl ester from LNPE substrate per minute after the reaction with bromelain. [20] The enzyme activity was calculated using (1).

$$\frac{\left(\frac{\Delta A_{340nm}}{\min\ \text{Test}} - \frac{\Delta A_{340nm}}{\min\ \text{Blank}}\right) \times 2.8 \times DF}{6.32 \times 0.1} \quad (1)$$

where the value of 2.8, 6.32, 0.1 and DF denotes the volume of the assay reaction in milliliters, the millimolar extinction coefficient of ρ-Nitrophenol at 340 nm, the volume of enzyme used in milliliters, and dilution factor, respectively. All assay reactions were carried out in triplicates.

2.4. Protein assay

The protein content was measured based on Bradford method using a BioRad Protein Assay kit (USA) at an absorbance of 595 nm with a microplate reader (Thermo Scientific, USA). Bovine serum albumin was used as a protein standard. [21] All assay reactions were carried out in triplicate.

2.5. SDS PAGE

The purified samples from ATPS were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[22] The ATPS and crude samples were applying to 12% resolving gel and 4% stacking gel. After electrophoresis was performed at 200 V, 700 mA for 37 min, the gels were silver stained.

2.6. Purification of recombinant bromelain by ATPS

In this study, a stock solution of PEG 6000 and potassium phosphate was prepared at 50% [w/w]. A potassium solution was prepared using different ratios of K_2HPO_4 and KH_2PO_4 to achieve pH 7.0. The ATPS was prepared in a graduated tube with 20% [w/w] of lysate recombinant bromelain, known weight of the PEG stock solution, potassium phosphate stock solution at definite pH and deionized water to reach a total of 50% [w/w]. The mixture was mixed thoroughly for 3 min using a Vortex mixer. Phase separation was achieved by centrifugation at 1000 × g for 10 min at 25°C. Then, the top and bottom phases were collected for assay. In order to evaluate the purification process, enzyme activity, and partition coefficient (KE) recovered in the bottom and top phases were calculated. The partition coefficient (KE), was calculated using equation (2).

$$K_E = \frac{A_T}{A_B} \quad (2)$$

where AT and AB are the enzyme activity in the top and the bottom phase, respectively.

2.7. Response surface methodology (RSM)

Response surface methodology (RSM) with Design-Expert 6.0.8 software (State-Ease Inc., Minneapolis, MN, USA) was used to establish the optimum conditions for the purification of recombinant bromelain. A set of 17 experiments consists of three independent variables (pH, polyethylene glycol and potassium phosphate concentration) were coded according to face-centered central composite design (FCCCD) with three replications in the center point. In each run, the partition coefficient, and enzyme activity were chosen as responses. The results were expressed as the means of triplicate readings. In this study, the variables and its levels are presented in Table 1.

Table 1: Factors for face-centered central composite design (FCCCD) study

Symbols	Factor	Range and levels		
		-1	0	+1
A	pH	6	7	8
B	PEG6000 concentration (% w/w)	15	17	19
C	Potassium phosphate concentration (% w/w)	8	13	18

3. Results and Discussions

3.1. Optimization of ATPS experiment

For the development of successful ATPS purification of recombinant bromelain, the empirical approach using face-centered central composite design (FCCCD) under response surface methodology (RSM) has been applied to determine the optimum level of the variables. The design matrix and results of ATPS purification were summarized in Table 1 and 2. Analysis of the variance was employed for the determination of significant variables and their interactions with the response variables that affect partitioning of recombinant bromelain. After ANOVA analysis, the quadratic model for each response of enzyme activity, and partition coefficient may be predicted from the regression equation (3), and equation (4) where CPEG 6000 and CPP were the PEG 6000 and potassium phosphate concentrations % [w/w], respectively. The accuracy of the model was checked by the F-test and the analysis of variance (ANOVA) has been shown in Table 3.

The p-value that less than 0.05 indicated the model was significant, whereas the p-value greater than 0.1 suggested the model was insignificant. The determination of the coefficient (R^2) calculated from the regression equations for the enzyme assay, and partition coefficient has reached 0.9984, and 0.9555, respectively. The model had good fit interaction between the experimental and predicted as the R^2 values closed to one. The R^2 values were concordant with the adjusted R^2 of 0.9965, and 0.9110 for enzyme activity, purification factor and partition coefficient, respectively. The large difference between R^2 and adjusted R^2 suggests that the model contains many insignificant terms [39].

Accordingly, three dimensional (3D) plots were illustrated in Figure 1 to visualize the relationship between responses and variables tested. The 3D plots were generated as pair combination of three factors while keeping the third factor at center level. The effect of PEG 6000 and potassium phosphate concentration on enzyme activity and partition coefficient (KE) were depicted in the Figures 1 (b) and 1 (d). The plots exhibited a pronounced increase of enzyme activity and KE of recombinant bromelain as the potassium phosphate and PEG concentration reached at 18% [w/w] and 19% [w/w], respectively. The presence of PEG 6000 at higher concentration in aqueous two-phase medium will increase the hydrophobicity between polymer-rich phase with proteins.[40] The free-volume available due to the higher concentration of PEG in the top phase become one of the migration factors of the recombinant bromelain in that phase. [42] Ketnawa and colleagues [14] have observed the similar results using 18% [w/w] of PEG 6000 in the case of bromelain extraction from pineapple's peels. Apparently, due to the salting-out effect, the ability of salts to capture water molecules is improved and the ionic strength of salts in the ATPS medium also increased as the potassium phosphate concentration increased.[35]The plots of enzyme activity and KE against pH and potassium phosphate concentration were shown in Figures 1 (a) and 1 (c). It can be seen that the highest value of enzyme activity, and KE was obtained at pH 7.0. Furthermore, our results were in accordance with the reported ATPS results on bromelain extracted from pineapple's fruit and peel.[14, 24] In conclusion, the optimum values of enzyme activity, and KE appeared near the center of the plot, indicating the center point value chosen in this experiment.

$$\begin{aligned} \textit{Enzyme activity} = & -3.7601807012691 + 0.949493.\mathrm{pH} \\ & +0.038637.\mathrm{C_{PEG6000}} + 0.036679.\mathrm{C_{PP}} - 0.0786.\mathrm{pH^2} \\ & -0.00243.\mathrm{C^2_{PEG6000}} - 0.00101.\mathrm{C^2_{PP}} + 0.008749.\mathrm{pH.C_{PEG6000}} \\ & -5.3\mathrm{E}\text{-}05.\mathrm{pH.C_{PP}} - 0.00033.\mathrm{C_{PEG6000}}.C_{PP} \end{aligned} \quad (3)$$

$$\begin{aligned} \textit{Partition coefficient}\ (KE) = & -41.13 + 10.5844.\mathrm{pH} \\ & +0.129939.\mathrm{C_{PEG6000}} + 0.573874\mathrm{C_{PP}} \\ & -0.77532.\mathrm{pH^2} - 0.00413.\mathrm{C^2_{PP}} \\ & +0.029736.\mathrm{pH.C_{PEG6000}} - 0.01638\mathrm{pH.C_{PP}} \\ & -0.02137\mathrm{C_{PEG6000}}.C_{PP} \end{aligned} \quad (4)$$

3.2. Validation of model

Among the models based on purification responses (enzyme activity, and KE), the model for enzyme activity had high values of R^2 (0.9984) and close correlation between adjusted R^2 (0.9228) and predicted R^2 (0.9860), which become the desired function for the numerical optimization of ATPS purification. Three replicate experiments have been carried out to validate the model and the results were compared with the predicted results generated by the FCCCD of RSM as shown in Table 4. Under the optimized condition which comprised of 18.47% [w/w] of PEG 6000 and 13% [w/w] potassium phosphate at pH 7.0, the predicted enzyme activity value was 0.272 unit/mL. The validation of the predicted model was done by conducting the experiment using the suggested condition resulting enzyme activity of 0.277±0.0036 unit/mL, close to the predicted value. Minimal difference was observed between experimental and predicted values, suggesting the robustness of the model used and prove that RSM is a suitable tool for the optimization of ATPS condition for recombinant bromelain purification. Further, the KE value calculated using this model was found at 1.394±0.093. The partial purified samples from the optimized ATPS was subjected to SDS-PAGE analysis. The standard protein marker (lane M), the partial purified protein (lane 1) and crude sample (lane 2) is shown in Figure 2. The band profile demonstrated that number of bands have been reduced as compared to crude extract of recombinant bromelain. The molecular weight of recombinant bromelain was estimated to be 55 kDa which was similar reported by Amid et al. [3]. ATPS is a partial purification, therefore several protein contaminants was observed with target protein. Therefore, additional purification method can be utilized to achieve high purity of target protein.

Table 2: Design of experiment for the optimization of ATPS of recombinant bromelain using face-centered central composite design (FCCCD).

Run	pH	Potassium Phosphate (% w/w)	PEG (% w/w)	Enzyme Activity (unit/mL)	K_E
1	6 (-1)	8 (-1)	15 (-1)	0.112±0.009	0.154±0.014
2	8 (+1)	8 (-1)	15 (-1)	0.071±0.001	0.170±0.005
3	6 (-1)	8 (-1)	19 (+1)	0.134±0.001	0.677±0.036
4	8 (+1)	8 (-1)	19 (+1)	0.167±0.002	0.950±0.067
5	6 (-1)	18 (+1)	15 (-1)	0.163±0.006	0.566±0.026
6	8 (+1)	18 (+1)	15 (-1)	0.125±0.004	0.274±0.012
7	6 (-1)	18 (+1)	19 (+1)	0.176±0.015	0.254±0.022
8	8 (+1)	18 (+1)	19 (+1)	0.204±0.007	0.180±0.007
9	6 (-1)	13 (0)	17 (0)	0.184±0.025	0.267±0.036
10	8 (+1)	13 (0)	17 (0)	0.175±0.008	0.569±0.028
11	7 (0)	13 (0)	15 (-1)	0.225±0.001	1.008±0.027
12	7 (0)	13 (0)	19 (+1)	0.272±0.004	1.316±0.070
13	7 (0)	8 (-1)	17 (0)	0.215±0.001	1.037±0.028
14	7 (0)	18 (+1)	17 (0)	0.250±0.006	1.143±0.055
15	7 (0)	13 (0)	17 (0)	0.257±0.001	1.424±0.035
16	7 (0)	13 (0)	17 (0)	0.255±0001	1.331±0.353
17	7 (0)	13 (0)	17 (0)	0.260±0.001	1.382±0.086

Table 3: Analysis of variance of quadratic model for enzyme activity, and K_E

Response variable	Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Enzyme activity	Model	0.055	9	6.142E-003	500.48	< 0.0001	significant
	Lack of Fit	*7.564E-005*	*5*	*1.513E-005*	*2.95*	*0.2727*	*Not significant*
	Pure Error	*1.027E-005*	*2*	*5.136E-006*			
	Cor Total	0.055	16				
R^2 = 0.9984, adjusted R^2 = 0.9965, predicted R^2 = 0.9860							
K_E	Model	3.49	8	3.49	21.47	0.0001	significant
	Lack of Fit	*0.16*	*6*	*0.026*	*12.17*	*0.0779*	*not significant*
	Pure Error	*4.33E-003*	*2*	*2.166E-003*			
	Cor Total	3.65	16				
R^2 = 0.9555, adjusted R^2 = 0.9110, predicted R^2 = 0.8015							

Table 4: Comparison of validated quadratic model and experiment result.

Model predicted		Experimental	
Enzyme activity (unit/mL)	**K_E**	**Enzyme activity (unit/mL)**	**K_E**
0.272	1.224	0.277±0.036	1.394±0.093

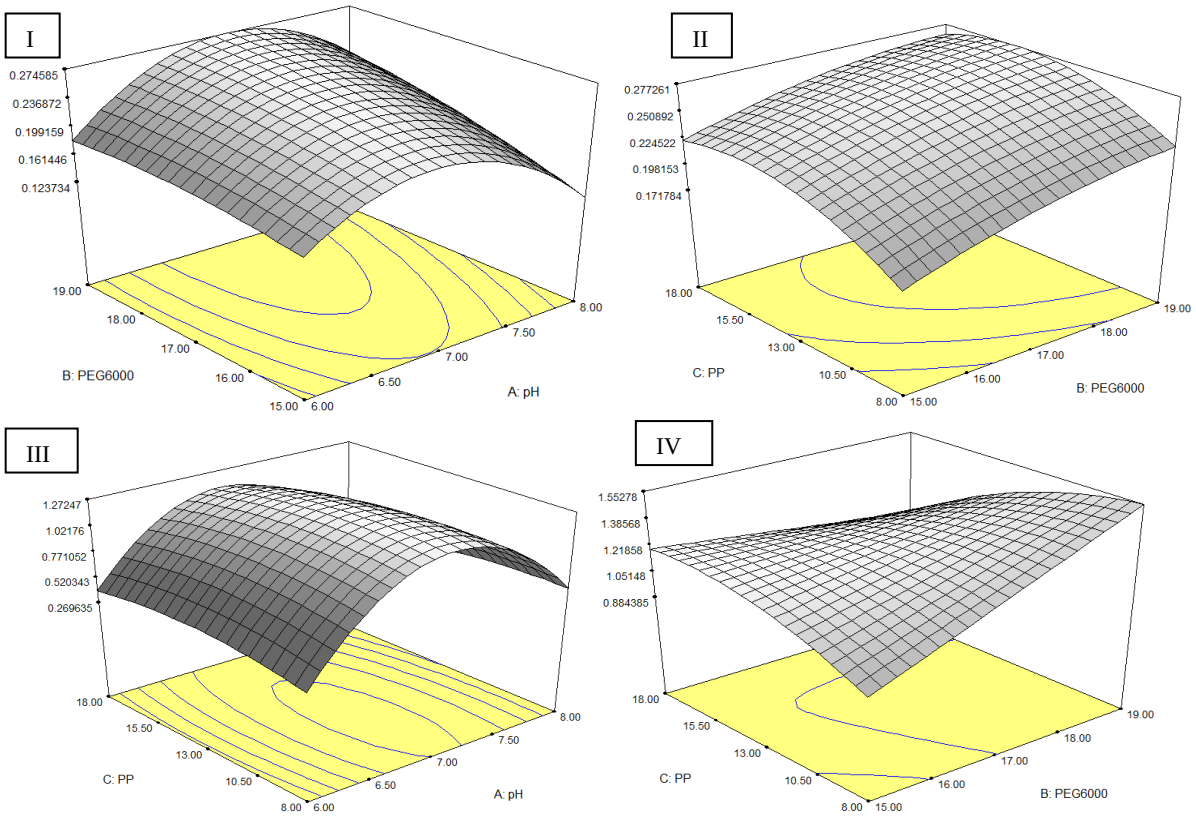

Figure 1: 3-D surface plot of RSM showing effects of pH, potassium phosphate and PEG 6000 concentration on enzyme activity (I, II) and KE (III,IV) of recombinant bromelain.

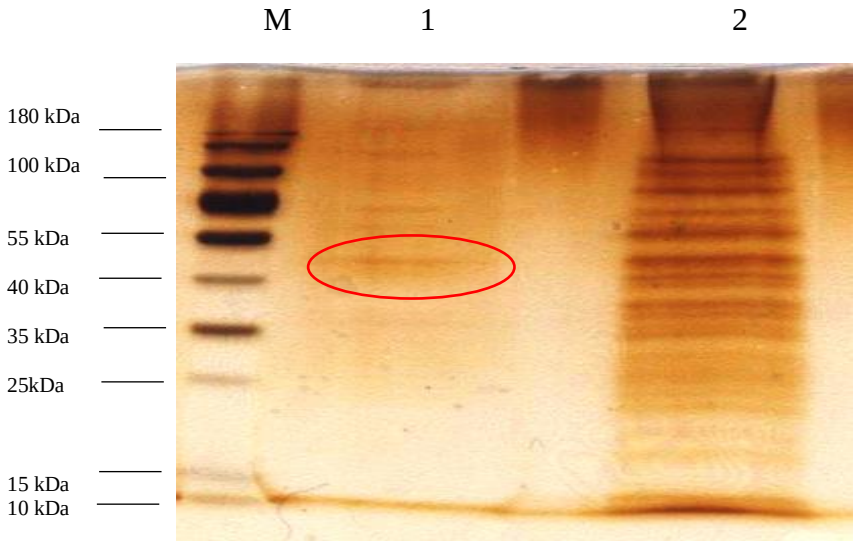

Figure 2: SDS-PAGE result for partial purified of recombinant bromelain. Lane M corresponds to standard protein marker. Lane 1: partitioned recombinant bromelain from the top phase; lane 2: crude extract recombinant bromelain. Note: For SDS-PAGE analysis, 20 µl of the sample (5 times dilution factor) with sample buffer was loaded on a 12% resolving and 4% stacking gel. The gel was stained with silver staining after electrophoresis was performed at 200 V, 700 mA for 37 minutes.

4. Conclusion

This study showed that aqueous two-phase system consists of a combination of PEG 6000 and potassium phosphate is efficient for the purification of recombinant bromelain. Response surface methodology experimental design with three factors was successfully optimize the process variables such as pH, PEG 6000 and potassium phosphate concentration. After optimization, the ATPS consisted of 18.47 % [w/w] of PEG6000 and 13 % [w/w] potassium phosphate at pH 7.0 was the most suitable system to recover the recombinant bromelain. The enzyme was successfully partitioned into the top phase, with enzyme activity and KE of 0.277±0.0036 unit/mL and 1.394±0.093, respectively. Besides, a good agreement between experimental and predicted values were obtained after the validation experiment. Therefore, ATPS technique using RSM approach is suitable for the purification of recombinant bromelain and can be attractive for recovery of other enzymes.

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