Optimization of Polymer/Salt Aqueous Biphasic System (ABS) for Direct Recovery of Recombinant *Bacillus Subtilis* Protease

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ABSTRACT

Introduction: The growing market demand for protease in various industries has sparked the interest among researchers to develop a simple, cost-effective and high yield method for the downstream recovery of proteases.

Objective(s): In this study, the partitioning behavior of recombinant *Bacillus subtilis* protease and different parameters are investigated using polymer/salt aqueous biphasic system (ABS).

Methodologies: Res1ponse surface methodology (RSM) and Box-Behken design (BBD), the multivariate statistical methodology, were employed to optimize the specific parameters including composition of polymer, composition of salt and pH of the biphasic system in order to achieve optimal partition coefficient and recovery yield of protease.

Results: The optimized ABS was exhibited at 17.50%(w/w) of polyethylene glycol (PEG) 1000 and 20.34%(w/w) of potassium phosphate, 20%(w/w) of crude feedstock, at pH 8.2, room temperature and without addition of sodium chloride (NaCl). Proteases preferentially partitioned to the PEG rich top phase with high partition coefficient of 5.387 and relatively high recovery yield of 84.82% were obtained although there are technical errors existed in the partition coefficient model analysis.

Conclusion: The results achieved with the optimized system held great promise on ABS, the single-step operation capable of surpassing conventional downstream processing methods and applying in the large-scale production of protease.

Keywords: Protease; Aqueous biphasic system; Response surface methodology; Box-Behken design; Polyethylene glycol

INTRODUCTION

Proteases are the proteins capable of breaking down peptide bonds with their catalytic function and found abundantly in nature as they are playing important roles in various physiological and biochemical functions of living organisms (Gimenes et al. 2019). Proteases are very important in diverse industrial sectors, major utilized in detergents, food and beverage processing, feather processing, silk gumming, pharmaceuticals, biosynthesis, biotransformation and bioremediation (Shine et al. 2016, Rawaliya et al. 2022). Conventional methods to be used to recover protease are specific, time consuming, expensive, difficult to scale-up in large-scale production and have low recovery yield which is insufficient to meet the increasing market demands on enzyme production (Golunski et al. 2011, Iqbal et al. 2016, Gimenes et al. 2019). Therefore, it is necessary to develop of a simple, low-cost, environmentally-friendly and efficient downstream processing method for large-scale recovery of protease.

Aqueous biphasic system (ABS) is a liquid-liquid fractionation technique that recovers a wide variety of products such as proteins, enzymes, cell organelles, antibodies and other biological products by forming two immiscible liquids (Iqbal et al. 2016, Teixeira et al. 2017). The ABS method has attracted the interest of many researchers because of its potential to extract, separate and isolate various products also to study the partitioning behaviour. The comparative advantages of ABS include single-step process, easy operation, cost-effective,

environmental friendly, reduced risk of destroying targeted biological products and their enzymatic activity (Ratanapongleka 2010, Iqbal et al. 2016). Polymer/salt ABS is applied in this study due to cost-effective, short phase separation time, low toxicity and low volatility (Rosa et al. 2010, Raja et al. 2011, Iqbal et al. 2016).

The bacterial strain used in this study is *Bacillus subtilis* PT5 also known as recombinant *B. subtilis* where the novel keratinase KerP within the bacteria were isolated from *B. polyfermenticus* B4 obtained from soil feather compost (Dong et al. 2017). *B. subtilis* strain is commonly used in enzyme production for several advantages such as generally recognized as safe (GRAS) status, able to produce numerous native or heterologous proteins efficiently, fast growth with short fermentation period, easy to cultivate on affordable substrates and changing environment which simplifies the downstream processing and reduces processing cost (Earl et al. 2008, Aslankoohi et al. 2015, Chen et al. 2016, Dong et al. 2017, Contesini et al. 2018, Su et al. 2020). With the GRAS status of *B.subtilis*, microbial proteases are applied in various food and beverages processing like food waste treatment, beer brewing process, meat tenderization, milk coagulation and casein hydrolysate preparation in cheese production (Patel et al. 2019, Su et al. 2020).

Various parameters that affect the partitioning behaviour of protease in polymer/salt ABS significantly: polymer phase composition, salt phase composition and pH were studied to determine the optimal conditions of polymer/salt ABS on the recovery of recombinant *B. subtilis* protease. In order to optimize the direct recovery of protease in ABS effectively, the response surface methodology (RSM) is applied to obtain the optimal conditions for experimental design and validate them with actual experiments. Box-Behken design (BBD) with three-factor design, consisting of 15 experimental runs according to the three most significant factors mentioned is used to determine the ideal Box-Behnken model for the optimum partition coefficient and recovery yield of recombinant *B. subtilis* protease in polymer/salt ABS in this study.

METHODOLOGIES

Fermentation of *Bacillus subtilis* for production of protease

25 g/L of Luria-bertani (LB) broth, pH 7 was prepared by adding appropriate amount of LB broth powder into distilled water for fermentation. A total of 90 mL of the LB broth were added into clean 250 mL conical flasks respectively. All conical flasks together with their contents were sterilized by using an autoclave machine at 121°C for 20 minutes. The recombinant *B. subtilis* was revived in the LB broth with inoculum size of 10%(v/v) and incubated at 37°C in an orbital shaker with speed of 150 rpm for 24 hours. The 10%(v/v) of inoculum from the culture medium was transferred into 90mL of LB medium and incubated at 37°C in an orbital shaker with speed of 200 rpm for another 24 hours to produce protease. The fermented broth was centrifuged at 4000 rpm for 30 minutes to isolate extracellular protease from the fermentation medium. The supernatant collected was sealed and stored at 4°C in the chiller for future use (Dong et al. 2017).

Stock solutions preparation

The 50%(w/w) PEG1000 stock solution was prepared by adding appropriate amount of PEG and distilled water in a 100-mL beaker and mixed well. The 10mM Tris-HCl buffer solution of pH 9 was prepared by weighing appropriate amount of Tris-HCl and added into distilled water to mix well. 1M sodium hydroxide solution was prepared to adjust the desired pH of Tris-HCl buffer solution. The 0.4M TCA solution was prepared by weighing appropriate amount of TCA and added into distilled water to mix well. The stock solutions were stored in Schott bottles or centrifuge tubes of appropriate sizes.

Box-Behnken design (BBD) configuration

The 3 selected independent parameters (concentration of PEG1000, concentration of potassium phosphate and pH of ABS) were undergoing the screening step, applying BBD matrix at the two factor levels so that significant variables can be studied using statistical software, MiniTab®. The parameters selected along with 15 experimental runs were performed so that predicted equations for both partition coefficient and recovery yield of protease could be formed.

ABS partitioning experiments using Box-Behnken design (BBD)

ABS of 5g was prepared by adding a known amount of PEG1000 stock solution and potassium phosphate into a 15mL centrifuge tube. The desired pH of the ABS system and concentration of potassium phosphate were prepared by different combinations of dipotassium hydrogen phosphate (K2HPO4) and potassium dihydrogen phosphate (KH2PO4). Then, 20% (w/w) crude protease was added. Distilled water was added to make up the ABS system to a total weight of 5g. The system was mixed thoroughly using a vortex mixer and centrifuged at 4000 rpm for 10 minutes for phase separation to take place completely. The volumes of top and bottom phases of the system were recorded. Samples from both top and bottom phases were collected respectively for sample analysis to determine protease enzyme activity.

Sample analysis using protease activity assay

The method of this assay was adopted and modified from Jin et al. (2019) using casein as the substrate. 0.25%(w/v) casein was added into an appropriate amount of 10mM Tris-HCl buffer solution, pH 9. 250 µL of the samples from top and bottom phases were pipetted into 2 mL microcentrifuge tubes, followed by the addition of 250

µL of preheated mixture of 0.25%(w/v) casein and 10mM Tris-HCl buffer (pH 9.0). The mixture was gently mixed with vortex mixer and incubated at 40°C for 10 minutes. 500µL of 0.4M TCA solution was added in order to stop the enzymatic reaction. The mixtures were centrifuged at 10,000 rpm for 10 minutes. The absorbance of the supernatant was measured and recorded at the wavelength of 280 nm using a spectrophotometer for tyrosine concentration determination. Protease enzyme activity was expressed as µg/mL based on the tyrosine standard curve. One unit of protease enzymatic activity was defined as the increase of 0.01 absorbance unit at 280nm per minute under the described assay conditions.

Optimization of protease extraction and validation of predicted extraction equations

Optimization was done with Analysis of Response Surface Design tool and Response Optimizer tool in MiniTab® to plot out predicted optimum extraction equations, response surface design analysis, surface and contour plots for protease extraction in this study. Validation experiments were performed to verify the optimal partition coefficient and recovery yield of proteases using the predicted equations, and both results were recorded. The random equations and validation experiments were conducted with the similar procedures.

Determination of partition coefficient, recovery yield and percentage similarity

Partition coefficient (K_E) of protease was calculated using Equation 1 (Amid et al. 2012).

$$\text{Partition coefficient } (K_E) = \frac{\text{protease enzyme activity at top phase } (A_T)}{\text{protease enzyme activity at the bottom phase } (A_B)}$$

Recovery yield (Y) of protease was represented in percentage (%) and calculated using Equation 2 (Amid et al. 2012).

$$\text{Recovery yield } (\%Y) = \frac{100}{1 + \frac{1}{[\text{volume ratio of top and bottom phases } (V_R) * K_E]}}$$

The percentage similarity between experimental values and theoretical values by MiniTab® (% Similarity) were measured with Equation 3 (Aziz et al. 2017).

$$\%\ \text{Similarity} = 100\% - \left(\frac{\text{Theoretical value - Experimental value}}{\text{Theoritical value}} \times 100\% \right)$$

RESULTS AND DISCUSSION

Optimization of response surface model

Table 1 summarised the Analysis of variance (ANOVA) for both partition coefficient and recovery yield regression models. The significance of the model terms was identified by its p-value similar to significance of the model, with p-value < 0.05 was significant and p-value > 0.05 was statistically insignificant (Garai and Kumar 2013). From Table 1, the p-value (0.037) for recovery yield model indicated that this model was significant with good reliability and high F-value (5.54), but not for the partition coefficient model with p-value (0.537) and low F-value (0.99) (Polat and Sayan 2019). The lack of fit p-values for partition coefficient model (0.063) and recovery yield model (0.508) implied that these models were insignificant relative to pure error (P > 0.05) and appropriate (Garai and Kumar 2013, Aziz et al. 2017, Zhu and Zhang 2019).

Model determination coefficient (R^2) value is closer to 100% indicates that the higher accuracy of the model. Correction determination coefficient (adjusted R^2) provides variability in test data that can be explained by equation models based on the obtained values (Zhu and Zhang 2019). Adjusted R^2 value should theoretically be close to R^2 value because the model may contain too many extraneous terms if there is too large difference (Garai and Kumar 2013). The results above proved that the recovery yield model was fitting well, statistically significant model and its regression equation was able to precisely forecast the actual situations with R^2 value of 90.9% and adjusted R^2 of 74.5%. In contrast, partition coefficient model was not suitable for further analysis due to statistically insignificant results with R^2 value of 64.0% and adjusted R^2 of 0%, leading to poor fitted model and weak correlation between measured and predicted responses (Polat and Sayan 2019). Both quadratic and interaction terms were significant with p-values of 0.048 and 0.032 respectively in the recovery yield model as shown in Table 1.

Table 2 presented the regression coefficients for partition coefficient and recovery yield regression models. The terms in Table 2 were expressed as concentration of PEG1000 (A), concentration of potassium phosphate (B) and pH (C). The independent parameters involved in this study of concentration of potassium phosphate (B), second-order effect of concentration of potassium phosphate (B^2) and interaction effect of concentration of potassium phosphate and pH (BC) were significant parameters for the recovery yield model with p-values < 0.05 based on Table 2. The interaction effect found in the recovery yield model was further analysed by plotting response surface plot and contour plot using Minitab®. These plots provide useful information on the fittings of the model and estimated responses but may not represent the true behaviour of the system (Baş and Boyacı, 2007).

Figure 1 showed the effect of interaction between pH and concentration of potassium phosphate (BC) on recovery yield of protease in the form of a response surface plot. It is observed that a higher recovery yield presents when the pH is increased and the potassium phosphate concentration increased. This phenomenon may occur due to the electrochemical interactions and salting-out effect of protease. Proteases partitioned to

Table 1: ANOVA for the regression models for partition coefficient (K_E) and recovery yield (Y) of protease

Source	Sum of square	Degree of freedom	Mean square	F-value	p-value Prob > F
Partition Coefficient					
Regression	237.930	9	26.437	0.99	0.537
Linear	91.771	3	30.590	1.14	0.417
Square	12.248	3	4.083	0.15	0.924
Interaction	133.911	3	44.637	1.66	0.288
Residual error	134.069	5	26.814		
Lack of fit	128.338	3	42.779	14.93	0.063
Pure error	5.731	2	2.866		
Total	371.999	14			
Recovery Yield					
Regression	862.58	9	95.84	5.54	0.037
Linear	222.70	3	74.23	4.29	0.075
Square	285.86	3	95.29	5.51	0.048
Interaction	354.01	3	118.00	6.83	0.032
Residual error	86.43	5	17.29		
Lack of fit	53.85	3	17.95	1.10	0.508
Pure error	32.58	2	16.29		
Total	949.00	14			

Table 2: Estimated regression coefficients for partition coefficient (K_E) and recovery yield (Y) of protease

Term	Coefficient	Standard error coefficient	p-value Prob > F
Partition Coefficient			
Constant	7.62000	2.990	0.051
A	-2.81875	1.831	0.184
B	-1.71375	1.831	0.392
C	-0.76750	1.831	0.692
A^2	-0.89875	2.695	0.752
B^2	-1.63875	2.695	0.570
C^2	-0.00625	2.695	0.998
AB	-1.97250	2.589	0.481
AC	-2.89500	2.589	0.314
BC	4.60500	2.589	0.135
Recovery Yield			
Constant	89.777	2.400	0.000
A	-2.126	1.470	0.208
B	-4.107	1.470	0.038
C	-2.539	1.470	0.145
A^2	3.744	2.164	0.144
B^2	-6.603	2.164	0.028
C^2	-4.136	2.164	0.114
AB	-4.897	2.079	0.065
AC	-4.215	2.079	0.098
BC	6.838	2.079	0.022

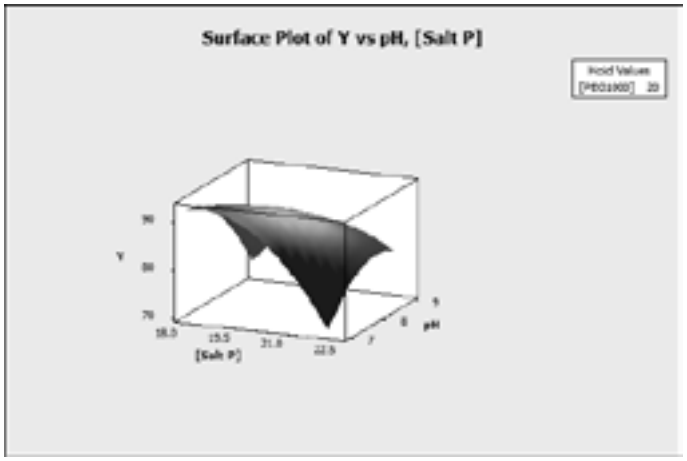

Figure 1: Response surface plot displaying interaction effect between pH and concentration of potassium phosphate variables on the recovery yield of protease. The curve shown is a concave curve; it clearly shows that when potassium phosphate concentration was at the range of 19.5 – 21.0 and pH was at the range of 7 – 8, high recovery yield can be achieved.

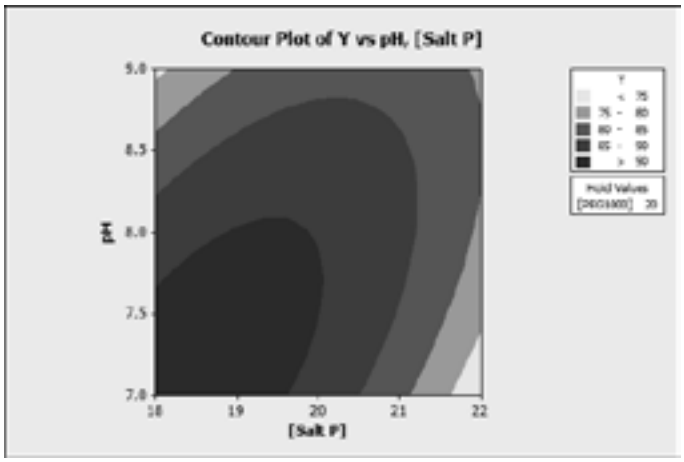

Figure 2: Contour plot displaying interaction effect between pH and concentration of potassium phosphate variables on the recovery yield of protease. Contour plot demonstrated a significant interaction in the system where the area that can be having a high recovery yield values are within the potassium phosphate concentration range of 18%(w/w) -21%(w/w) and the pH range of 7 – 8.5.

PEG rich phase due to salting-out effect in the salt-rich phase where the higher salt concentrations (high ionic strength) in polymer/salt ABS causes a decrease in the solubility of proteins in the salt-rich phase, forcing them to migrate towards the PEG rich phase where they are more soluble, hence improve the enzyme partition to top phase (Hasmann et al. 2008). It is also observed high concentration of potassium phosphate minimize the recovery yield of protease.

Besides, the pH range more than 7 is applied in PEG/ phosphate system due to the compatibility of biomolecule distribution (Goja et al. 2013). Negatively charged proteins are preferable for extraction from the polymer-rich phase because they repelled from the salt-rich bottom phase and increase the partition coefficient (Hatti-Kaul 2000, Ng et al. 2021, Ketnawa et al. 2017). Using the pH above the pI of the protein may result in an increase of electrostatic interactions between the protein and polymer molecules, lead to additional affinity towards the polymer-rich phase. However, extreme pH environments can affect protease recovery yield, as high alkaline pH can lead to protein denaturation and possible loss of enzyme stability as shown in Figure 1 (Benavides et al. 2000, Goja et al. 2013).

Figure 2 illustrated the contour plot of the effect on recovery yield with the interaction between concentration of potassium phosphate and pH (BC). The higher intensity of the colour in the plot shows the increases of recovery yield percentage. The middle of the contour plot showed the significant interaction between these two independent variables since there is a cascading effect in the curve. The peak and intensity of colour in the plot also showed a good interaction between pH and concentration of potassium phosphate on the effects of recovery yield of protease.

Experimental validation of model predicted values

The optimum condition on the selected parameters of the polynomial equation model for recovery yield was predicted using the response optimizer function in Minitab® software. Table 3 presented the results of optimal validation experiment performed on the recovery yield model. Using the conditions given by the software, the maximum predicted protease recovery yield of 96.00% could be achieved at pH 8.2, 17.50%(w/w) of PEG1000 concentration and 20.34%(w/w) potassium phosphate concentration in the ABS. Experimental values obtained were recorded and further calculated towards the differences between the predicted and experimental values in percentages for recovery yield of protease using the Equation 3. High percentage in similarity and small difference values show that the optimized recovery yield equation model is valid and appropriate for protease extraction.

Table 3: Optimal validation of experimental and predicted values of the partition recovery yield (Y) of protease

Recovery yield (Y)		Experimental Mean	Difference	% Similarity
Predicted	Experimental			
96.00 %	1) 87.24 %	84.82 %	11.18	88.35 %
	2) 85.55 %			
	3) 81.66 %			

CONCLUSION

ABS was successfully demonstrated in the optimization of proteases recovery from crude feedstock in polymer/salt ABS. Experimental data showed that pH of ABS and concentration of potassium phosphate had possessed a significant impact on the recovery yield of protease in polymer/salt ABS. The highest predicted recovery according to the optimized polynomial predicted response model using ABS was 96.00%, with the optimal condition at pH 8.2, 17.50%(w/w) of PEG1000, 20.34%(w/w) of potassium phosphate, room temperature, constant crude load of 20%(w/w) and without the addition of NaCl. The validation data of recovery yield obtained from the actual experiment was 84.82% with a similarity percentage of 88.35%. Hence, this result proved that the optimization of the recovery yield model for protease extraction was valid for use and approved. In connection with this study, polymer/salt ABS are continue to be proposed as one of the potential approaches for downstream processes of protease from the fermentation of various microorganisms at industrial scale.

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