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Original Article

Partitioning of microbial cellulase in aqueous two-phase system: Modeling and optimization using response surface methodology

Elham Asl Soleimani [1], Masoumeh Anvari [2], Gholam Khayati [3]

[1] Ph.D., Department of Biology Ra.C., Islamic Azad University, Rasht, Iran.
[2] Associate Professor, Department of Biology Ra.C., Islamic Azad University, Rasht, Iran.
[3] Associate Professor, Department of Chemical Engineering, Faculty of Eng., Guilan University, Rasht, Iran.

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Abstract

Background & objectives: Separating biological products is one of the key challenges in industrial fermentation processes. An aqueous two-phase system based on polymer-salt has been used for the fractionation of microbial cellulase obtained by fermentation.

Material & Methods: The optimal conditions for cellulase extraction were determined by response surface methodology an aqueous two-phase system. A second-order central composite design was applied to evaluate the effects of three independent variables (ammonium sulphate concentration, temperature and pH) on cellulase extraction yield.

Results: Maximum yield of extracted cellulase were determined as follows: 26.34 (%w/w) for ammonium sulphate concentration, 7.7 for pH, and a temperature of 31.61 (°C). The linear term of temperature and pH had significant effects on the yield of extraction (p-value < 0.05). Correlation analysis of the mathematical regression model indicated that the quadratic polynomial model could be employed to optimize the extraction.

Conclusion: Under both acidic and alkaline conditions within the system, there was a clear reduction in enzyme recovery, in contrast to the neutral pH.

Keywords: Cellulase, Aqueous two-phase system, Response surface methodology.

Corresponding author: **Masoumeh Anvari, PhD., Department Department of Biology Ra.C., Islamic Azad University, Rasht, Iran. Tel: +98-9112323482 E-mail: Anvari@iau.ac.ir**

Introduction

Cellulases are industrially important enzymes having application in diverse industries such as agriculture and food industry, textile, paper and pulp (1). Cellulases can be produced by microbial fermentation. They are relatively costly enzymes and a significant reduction in cost will be important for their commercial use. Studies indicate that the cost for producing a biological product is predominantly affected by purification to a degree ranging from 50% to 90%. (2). Partitioning using aqueous two-phase systems (ATPS) has proved to be a valuable tool for separating and purifying mixtures of biomolecules via extraction as it is a mild technique. ATPS integrates clarification, concentration and partial purification in a single step, thus it is recognized for offering reduction and shortening of the purification process of protein in comparison with other separation and purification procedures (3). These reasons led to the widespread utilization of ATPS in the downstream processing of compounds including enzymes (4-5), biopharmaceuticals (6-7), and other natural products (8). ATPS are produced through the combination of two flexible-chain polymers in water or by combining one polymer with a salt. These systems are typically biocompatible due to the high-water content in both phases and exhibit partition characteristics that are advantageous for their use in large-scale enzyme separation and purification (9-10). The distribution of proteins within a biphasic aqueous system is impacted by multiple factors. The biomolecule's dimensions, quantity, and surface characteristics are among those included. Additionally, the phases' composition, focusing on the type, concentration, and molecular weight of the polymers or salts, holds significant importance. Other important parameters include the system's pH and temperature (11). Response surface methodology (RSM) is a statistical technique that employs quantitative experimental data to uncover or simultaneously determine multi-variable relationships (12). This experimental methodology can generate a mathematical model and optimize the process levels (13). This technique is commonly used to optimize the extraction variables of biological products (such as polysaccharides, enzymes, phenolic compounds, and proteins) (14-15).

The aim of this work was to study the partitioning behavior of cellulase in aqueous two-phase systems composed by polyethylene glycol (PEG 4000) - $(NH_4)_2SO_4$ as a function of affecting parameters including salt concentration, temperature and pH using RSM was analyzed.

Materials and methods

A) Materials: The cellulase was obtained from Sigma-Aldrich (United States). The polyethylene glycol (PEG) with molecular weights of 4000, ammonium sulfate and other chemicals with analytical grade were obtained from Merck (Germany). All chemicals were used without further purification. The salts were dried in an oven at about 100 °C for 24 h before use.

B) Phase diagram: The experimental apparatus employed is essentially similar to the one used previously (16). The titration method was used to determine binodal curves. A salt solution of a specific concentration was titrated with a polymer solution of known concentration until the solution became turbid, and vice versa. The composition of this mixture was noted and distilled water was added drop wise until the mixture become clear again. Salt solution was added again till turbidity was obtained. This procedure was repeated for 8-10 times to obtain the complete range of composition. The composition of the mixture was determined by

mass using an analytical balance with a precision of ± 0.0001 g. The data points were plotted with "%PEG 4000 vs. %salt" to obtain binodal curves (17).

For the determination of the tie-lines, feed samples were prepared by mixing right amounts of polymer, salt and water in the flask. The thermostat was set at a desired temperature, and the sample was stirred for 2-3 h. Then the mixture was allowed to settle for 24 h. Portions of solutions in both phases were taken out carefully for analysis.

The concentration of PEG was determined by refractive index measurements at 31.5° C using a Kruss Abbe refractometer model AR3D. Since the refractive index of phase samples depends on PEG and salt concentration, calibration plots of refractive index versus polymer concentration were prepared for different concentration of salt. The relation between the refractive index, n_D, and the mass fractions of polymer, W_1 and salt, W_2 is given by equation 1 (18).

$$n_D = n_D^0 + a_1 w_1 + a_2 w_2 \quad (1)$$

where n_D^0 is the refractive index of pure water for which the value 1.3322 was measured at 31.5° C. a_1 and a_2 are constants. The values of coefficients a_1 and a_2 for the applied system are given in Table 1.

C) Cellulase partitioning in aqueous two-phase system: The aqueous two-phase systems in different salt and PEG 4000 concentrations were prepared in 21.59, 25, 30, 35 and 38.41 (%w/w) for $(NH_4)_2SO_4$ and 30 (%w/w) for PEG 4000 solutions respectively. All aqueous two-phase systems were done in 10 ml graduated centrifuge tubes at equal volume of salt and PEG solution in different conditions (Table 2). A known amount of cellulase was added in ATPSs and mixed thoroughly and centrifuged for 10 min at 3000 rpm to assist phase separation. The volume of each phase was measured to estimate the volume ratio when the system turned clear. In order to determine the cellulase activity in each of the phases, samples of the top and bottom phases were carefully withdrawn by using a plastic syringe with short and long needle, respectively (25).

D) Experimental design and statistical analysis: RSM were used to determine the optimum condition for extraction of cellulase. The effect of three independent variables: Ammonium sulfate concentration (X_1), temperature (X_2), and pH (X_3) on the response variable (%Y, the yield of extracted cellulase) was evaluated using central composite design (CCD) (Table 2). The five coded levels of each variable were incorporated into the design and were analyzed in 20 experimental trials (Table 3).

The central point of the design was repeated six times to calculate the reproducibility of the method (19). For each experimental trail of the independent variables in the experimental design, the dependent parameter (the yield of extracted cellulase) was determined. The effect of these independent variables X_1, X_2 and X_3 on the response Y was investigated using the second-order polynomial regression equation. The equation 2 (14), derived using RSM for the evaluation of the response variable, is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i x_j + \sum_{i<j} \sum \beta_{ij} x_i x_j \quad (2)$$

Table 1. Values of parameters of Eq. (1) for PEG 4000 (1) + salt (2) + H2O (3) systems at 31.5° C.

System	α_1 [a]	α_2
PEG 4000 + (NH4)2SO4+ H2O	0.1722	0.1394

[a] coefficients of equation (1)

Table 2. Central composite experimental design and responses.

Run	Independent variables [a]			R [b]	response (Y %) [c]
	x_1	x_2	x_3		
1	-1	-1	-1	0.61	38.69
2	1	-1	-1	0.61	26.41
3	-1	1	-1	0.62	34.23
4	1	1	-1	0.63	28.82
5	-1	-1	1	0.97	44.05
6	1	-1	1	1.02	34.19
7	-1	1	1	1.14	43.14
8	1	1	1	1.11	42.20
9	-1.68	0	0	0.59	45.53
10	1.68	0	0	0.59	32.22
11	0	-1.68	0	0.46	34.29
12	0	1.68	0	0.61	38.05
13	0	0	-1.68	0.69	27.01
14	0	0	1.68	0.64	45.35
15	0	0	0	0.90	44.91
16	0	0	0	1.00	44.41
17	0	0	0	0.62	45.17
18	0	0	0	0.64	44.56
19	0	0	0	0.68	45.23
20	0	0	0	0.66	43.65

a: x_1 : ammonium sulphate concentration (% w/w), x_2 : Temperature (°C), x_3 : pH
B: the volume ratio (the top phase to the bottom phase)
C: yield of extracted cellulase

Table 3. Independent variables and their coded and actual levels.

Independent variables (factors)	Symbol	Actual values of coded levels				
		-a	-1	0	+!	+a *
ammonium sulfate concentration (% w/w)	X_1	21.59	25	30	35	38.41
Temperature	X_2	20.56	25	31.50	38	42.43
pH	X_3	5.32	6	7	8	8.68

* a = 1.68 (star point for orthogonal CCD for the case of 3 independent variables)

where x is independent variable, β_0 is defined as the constant, β_i the linear coefficient, β_{ii} the quadratic coefficient and β_{ij} the interaction coefficient, while k equals to the number of the tested factors (k =3). The analysis of variance (ANOVA) table was generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the p-value at a probability of 0.05. The regression coefficients were then used to make statistical calculations to generate response surface and contour maps from the regression models. The analysis of data and the optimizing process were generated using Minitab statistical software version 15.

E) Analytical methods: The filter paper cellulase (FPase) activity was determined in accordance with the International Union of Pure and Applied Chemistry procedures as reported by Ghose (20). The activity was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No. 1 filter paper as substrate in 50 mM sodium citrate buffer (pH 4.8) at 50° C, after 60 min. Reducing sugars were assayed by dinitrosalicylic acid method of Miller (21). “One unit of

enzyme activity was defined as 1 µmol of reducing sugar released per min under the assay conditions."

The partition coefficient (K) for cellulose activities in the aqueous two-phase systems were defined as

$$K = activity_{\text{top phase}} / activity_{\text{bottom phas}} \quad (3)$$

The partition coefficient (K) is used to quantify the degree of separation reached in an extraction process. Yield (%) of cellulase in top phase was also calculated based on the partition coefficient to study the efficiency of the system by using the following equation (10):

$$Y\ (\%) = \frac{100}{((RK)^{-1} + 1)} \quad (4)$$

The (R) was defined as the volume ratio of the top phase to the bottom phase (V_t/V_b) (Table 3).

Results

A) Phase diagram for PEG /salt aqueous two-phase system: ATPS using PEG/salts, the top phase is rich in PEG while the bottom phase is rich in salt. ATPSs composed of PEG 4000 with ammonium sulfate were used for partitioning of cellulase. Experimental binodal data of the aqueous two-phase system PEG 4000 - (NH4)2SO4 determined at 31.5° C is given in Fig 1 and the tie-line data for this system is given in Table 4.

B) Effects of independent variables on cellulase separation: Response surface were used to illustrate the effect of ammonium sulfate concentration, extraction temperature and pH on the extraction of cellulase from dilute water. The response surfaces and contour plots of cellulase extraction conditions are shown in Figs 2-4. Fig 2 represents the three-dimensional response surface and contour plots of extraction efficiency at pH=7 to investigate the interactive effect of ammonium sulfate concentration and temperature.

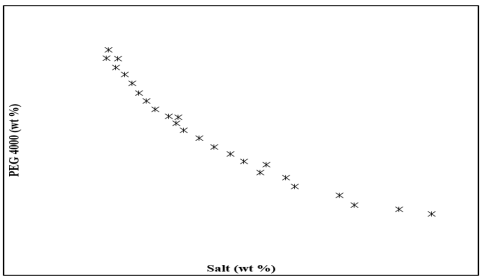

Fig 1. Experimental binodal data for the PEG 4000 (1) + (NH4)2SO4 (2) + H2O (3) system at 31.5° C.

Comparing run 5 and run 8 showed that low initial concentration of salt would require low temperature to obtain the optimum extraction yield of cellulase and vice versa.

The interaction between pH and concentration of ammonium sulfate in the three-dimensional response surface and contour plots at temperature 31.5° C showed that pH has a higher effect on the yield of cellulase activity in top phase at high initial salt concentration than that at low initial concentration (Fig 3). It could be observed by comparing the run 1, 5 (increased 14%) versus run 2, 6 (increased 29%). which can be explained by salting out effect of the ammonium sulfate on the extraction of enzyme.

Table 4. Experimental phase equilibrium compositions for the the PEG 4000 (1) + salt (2) + H2O (3) system at 31.5° C.

PEG4000 + (NH4)2SO4+ H2O			
Top phase		Bottom phase	
100 $w1$ [a]	100 $w2$	100 $w1$	100 $w2$
35.41	3.99	7.71	22.52
37.82	3.78	5.73	25.61
39.84	3.49	5.49	26.85
54.18	2.42	2.99	36.72

[a]; mass fraction

Fig 2. Response surface plot (upper) and its contour plot (lower) for the effects of salt concentration (%w/w) and temperature (°C) on yield of extracted cellulase at its center level.

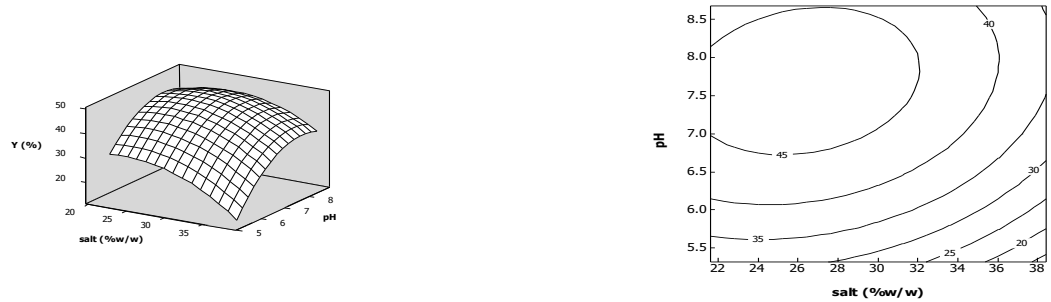

Fig 3. Response surface plot (upper) and its contour plot (lower) for the effects of salt concentration (%w/w) and pH on yield of extracted cellulase at its center level.

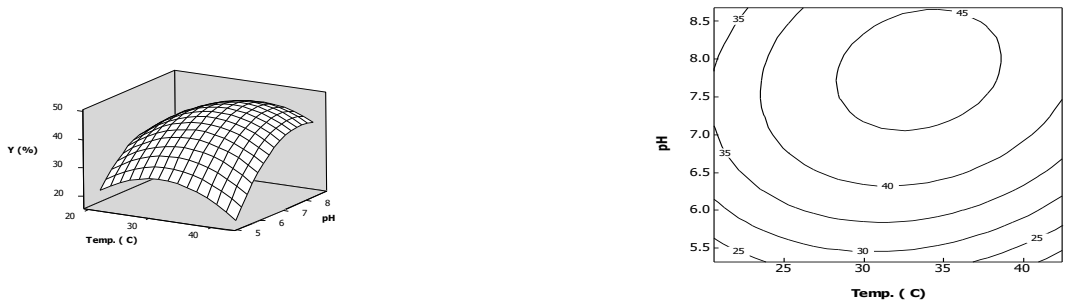

Fig 4. Response surface plot (upper) and its contour plot (lower) for the effects of temperature (°C) and pH on yield of extracted cellulase at its center level.

The response surface and contour plots of extraction yield of cellulase at 30 (%w/w) ammonium sulfate with different temperatures and pH is shown in Fig 4. It showed that pH had a significant effect on yield of extracted cellulase; however, the extraction efficiency was less affected by the temperature. The effect of temperature was more significant when applying at high pH. It can be seen that the extraction efficiency increased slightly with increasing temperature from 30-35 °C and then decreased slightly at higher temperature.

The effects of extraction conditions on the yield of extracted cellulase by the regression coefficients of fitted second-order polynomial are presented in Table 5.

C) Fitting the model (The equation 2) and Response surface analysis: RSM was used to optimize cellulase extraction conditions and the experimental results were presented in Table 2. The yield of cellulase activity in top phase was found to range from 26.41 to 45.53% in response to the variation in the experimental conditions. The yield of extracted cellulase was analyzed to get a regression model. The approximating function of top phase yield of cellulase activity (in terms of uncoded units) obtained by the software is given in equation 5.

$$Y\ (\%) = -132.95 + 1.59\ X_2 + 36.54X_3 - 0.08X_1^2 - 0.07X_2^2 - 3.02X_3^2 + 0.06X_1X_2 \quad (5)$$

where Y is the yield of extracted cellulase, whereas X_1, X_2 and X_3 are the independent variables for salt concentration, temperature, and pH concentration respectively.

Table 6 summarizes the ANOVA results.

Table 5. Significance of regression coefficients of the fitted second-order polynomial model for response (Y [a]).

Term [b]	Regression Coef.	SE [c] Coef.	*P*- value
β_0	-132.951	24.038	< 0.001
	Linear		
β_1	1.118	0.373	0.161
β_2	1.595	0.538	0.014
β_3	36.542	3.874	< 0.001
	Quadratic		
β_{11}	-0.038	0.009	< 0.001
β_{22}	-0.072	0.005	< 0.001
β_{33}	-3.029	0.222	< 0.001
	Interaction		
β_{12}	0.061	0.009	< 0.001
β_{13}	0.172	0.059	0.016
β_{23}	0.176	0.046	0.003

[a] yield of extracted cellulase
[b] coefficients of equation (2)
[c] *standard error*

Table 6. Analysis of variance (ANOVA) of the regression parameters for the response surface model.

Source	D. F.[a]	Sum of Square	Mean Square	*F*-value	*p*-value
Model	9	843.044	93.672	131.70	< 0.001
Residual	10	7.112	0.711		
Lack of fit	5	5.362	1.072	3.06	0.122
Pure error	5	1.750	0.350		
Total	19	850.157			

[a] degree of freedom

The model *F*-value of 131.70 implied that the model was significant for the yield of extracted cellulase and there was only a 0.1% chance that a model *F*-value of this large could occur due to noise.

Fig 5 shows the predicted output values versus actual experimental values for the yield of cellulase activity in top phase.

The figure showed that was good agreement between model (Eq.5) and experimental data. Therefore, the model developed is suitable for predicting the yield of extracted cellulase in the conditions investigated.

From the model, optimum conditions for cellulase extraction were obtained 26.34 (%w/w), 31.61 (°C) and 7.69 for ammonium sulfate

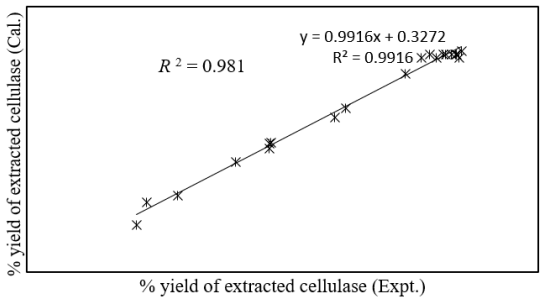

Fig 5. The relationship between the calculated yield of extracted cellulase and experimental data.

concentration, temperature and pH respectively. Furthermore, Chaiwut et al. show that the highest protease recovery and partition coefficient were obtained in the system pH of 7.0 (22). At alkaline and acidic condition, decrease of the recovery was found when compared to that of neutral pH.

Discussion

Aqueous two-phase systems have been used in biochemistry for the separation and purification of biomolecules, cells and cell particles. When these systems used for purification, there are many possible phase systems to choose. Access to reliable equilibrium data in multi-component systems is very useful for designing extraction processes. Therefore, the solubility curve of the two-phase system under study was determined (Fig 1). Partitioning of cellulase in an aqueous two-phase system, composed of polyethylene glycol 4000 and ammonium sulfate was studied. The effects of salt concentration, temperature and pH were analyzed by RSM. The extraction yield of cellulase decreases with increase in temperature of the system (Fig 2). It has been reported that as the temperature increases, the PEG structure becomes more extended and as a result its preferential interaction with the protein decreases thereby decreasing the yield of extraction (10).

It was evident that the linear terms except for ammonium sulfate concentration were significant (p-value < 0.05), whereas all the quadratic and interaction terms were significant (p-value > 0.05). The results indicated that the pH was the major contributing factors to cellulase extraction. Chaiwut et al. show that the pH values in the system have a pronounced effect on the partitioning of proteins between the two phases (22). In general, the pH had influence on protein partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. Within the experimental range, however, salt concentration had no significant effects (p-value > 0.05) on the recovery of cellulase (Table 5).

In general, exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit, which makes checking of the model adequacy essential (23). For Eq. (5), lack of fit F-value of 3.06 implied that the model of cellulase extraction yield developed was insignificance. The non-significant lack of fit indicates good predictability of the model (14). There was a 12.2% chance that a lack of fit F-value could occur due to noise. This value confirmed that the model fitness was acceptable. The quality of the model developed was evaluated based on the value of coefficient of determination (R^2). Coefficient of determination (R^2) is defined to be the ratio of the explained variation to the total variation and is a measurement of the degree of fitness (24).The R^2 value for Eq. (5) was 0.98 which was relatively high (close to unity). The high R^2 value indicated that the developed model was able to give a convincingly good estimate of response in the studied range. The closer of R^2 value to unity, the better the empirical models fit the actual data (14). This implied that 98% of the variations for the yield of cellulase activity in top phase are explained by the independent variables and only 2% of the total variability in the response was not explained by the model.

Conclusions

The second-order polynomial model gave a satisfactory description of the experimental data. Cellulase showed high affinity for the polyethylene glycol-rich phase. In this system acidic and alkaline conditions, the reduction of enzyme recovery was clearly observed compared to that of the neutral pH.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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