Response Surface Methodology for the Evaluation of Lysozyme Partitioning in Poly (Vinyl Pyrrolidone) and Potassium Phosphate Aqueous Two-Phase System

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ABSTRACT: *The partitioning of lysozyme and extraction yield in an aqueous two-phase system containing Poly Vinyl Pyrrolidone (PVP) K25 and potassium phosphate were investigated as a function of weight percent of salt and PVP in the feed, temperature, and pH. To investigate partitioning behavior, the central composite design was considered using a quadratic model. According to the results of the model, the partitioning of lysozyme was mainly due to the impact of the weight percent of the salt in the feed. However, the partitioning was expanded a little following the increase in the weight percent of the polymer. Based on the results, there was an opposite relationship between the temperature and decrement in the viscosity of PVP as the increment in the former led to the decrement in the latter. Finally, the modification in the third factor was done by increasing the pH level. Before experimenting, some values were hypothesized such as 93.69% for the maximum extraction yield, 21.23% for PVP K25, 13.99% for the salt concentrations, 7.10 for the pH value and 35.57 for the temperature. The findings of the study suggest that the hypothesized values for different variables are in line with the experimental results.*

KEYWORDS: *Aqueous two-phase system; Lysozyme; Polyvinylpyrrolidone; potassium phosphate; RSM.*

INTRODUCTION

Aqueous Two-Phase System (ATPS) is a popular method employed for liquid-liquid extraction and this system is the results of mixtures containing two polymers or a polymer and a salt in water within a particular range of compositions [1]. ATPS is now recognized as a promising method due to several advantages namely, low interfacial tension [2], affordable material cost [3], low energy consumption [2], high production [4], relatively high load capacity[5], promoting the feasibility [6], selective extraction [7] and the number of beginning downstream steps are considerably decreased by using this method so that there is one integrated unit including all main properties of clarification, concentration, and partial purification [6]. One important function of the ATPS is separating various proteins and enzymes such as α-lactalbumin β-lactalbumin [8, 9], xylanase [10], bovine lactoferrin [11], β-glucosidase [12], and penicillin acylase [13]. Substantial amounts of Lysozyme (muramidase, EC 3.2.1.17)

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can be found in animal tissues and secretions [14] and hen egg-white forming about 3.5% of the total egg white proteins. Some applications of this enzyme are found in the food industry, pharmaceutical treatments and medicine due to its antimicrobial effects protecting the substance from toxins particularly in the food preservatives due to its antimicrobial effects protecting the foods from toxins [15-17]. Accordingly, such features make lysozyme a desirable tool to develop an efficient and economical separation and purification process for its large-scale production. Lysozyme was first prepared by direct crystallization from hen's egg white using an inorganic salt [18]. The alternative way to extract the enzyme out of the egg white is '' membrane processes'' in that the almost decontaminated lysozyme (80–90%) is produced using a process called ''hollow-fiber ultrafiltration membrane'' [19, 20]. A considerable number of protein decontamination procedures in light of adsorption and ion-exchange phenomena are accessible today, and the most practiced one is the chromatography. However, such techniques are not always successful and the procedures sometimes problematic and time-consuming, leading to low final yields [21, 22]. Considering these issues, ATPS is a promising substitute for a lysozyme partitioning and purification in the extended scales. *Silvério et al.* and *Sousa et al.* [23, 24] are some of the studies that investigated the partitioning of lysozyme in ATPSs. Determination of the partition coefficient of solute is an important step for designing the industrial-scale process in bioseparation research based on ATPS. Although there has been a great development in recent years, there is a gap for studies that predicts the value of the solute's partition coefficients in ATPS. Moreover, the partitioning mechanism is dependent on many factors such as the properties of the solute and environmental conditions including phase making polymers or salts, polymer molecular weight, pH, buffer, ion strength, and temperature. Accordingly, there is a need for appropriate mathematical models to evaluate the partitioning mechanisms and provide more information about the reliability of the mechanisms.

Response Surface Methodology (RSM) is a dominant means of the optimization process and contains different stages such as the experimental design, model fitting, approval of validity and condition enhancement [25, 26].

To obtain the response surface optimization, an appropriate design is one of the priorities. Some of the RSM designs are reported in the literature including Plackett–Burman design, Box-Behnken design, Graeco–Latin square design and also central composite design as the most frequently selected design because of its characteristics such as ''orthogonally, uniform precision and rotatability'' [27]. Recently, a considerable number of studies applied the RSM in ATPS. For instance, *Dembczyński et al.* [28], studied the partitioning of lysozyme in ATPSs containing ethylene oxide-propylene oxide copolymer and potassium phosphates by RSM. Furthermore, *Alcântara et al.* [29] studied amyloglucosidase purification in polyethylene glycol and sodium polyacrylate with RSM and also some studies [30-32] investigated the partition and purification of biomolecules.

Poly Vinyl Pyrrolidone (PVP) is another polymer used in ATPSs that is favored for its quick solution in water and some salts that lack carbon (inorganic) [33,34]. PVP has been used in medical and pharmaceutical industries [35] due to the properties such as affordable expense, biocompatibility, and stability, therefore it has been the target of an increasing number of studies [36]. Some studies reported the effect of PVP on the phase partitioning in some drugs contacting both hydrophilic and hydrophobic parts (amphiphilic) [37-39] and measured the thermodynamic parameters at the phase separation point (i.e., cloud point) [40]. However, only a few reports on the partitioning of the proteins using ATPS of PVP–salt exist [41], while no study has reported the RSM use in the proteins partitioning process in ATPS based on PVP so far. Consequently, in the present study, the lysozyme solution was prepared in the laboratory from the egg white first. Then, the Central Composite Design (CCD) was utilized to concentrate on the impacts of various factors including salt and polymer weight percent in feed, temperature, and pH on the partitioning of lysozyme (commercial model) in ATPS of PVP K25–potassium phosphate. Based on the results of the experimental design, a regression analysis was completed and a model for protein separation was given. After identifying the impact of process parameters on partitioning, the partitioning process of lysozyme solution (lab mode) in the optimum condition of ATPS was investigated. The results of this research can provide a theoretical and practical basis for further design, optimization, and scale-up of such processes and also the development of models that explain the lysozyme partitioning behavior.

EXPERIMENTS SECTION

Materials

Polyvinylpyrrolidone K25 with molar mass of 24000 (CAS NO: 9003-39-8), dipotassium hydrogen phosphate(K2HPO4) (CAS NO: 7758-11-4) , monopotassium dihydrogen phosphate (KH2PO4) (CAS NO:7778-77-0) , ammonium sulphate ((NH4)2SO4)) (CAS NO: 7783-20-2) , tris (C27H18AlN3O3) (CAS NO:77-86-1), Coomassie brilliant blue (C45H44N3NaO7S2) (CAS NO: 6104-59-2) , acid orthophosphoric (H3PO4) (CAS NO: 7664-38-2), peptone(CAS NO: 73049-73-7), glucose(C6H12O6) (CAS NO50-99-7) , sodium chloride (NaCl) (CAS NO: 7647-14-5) , Yeast extract(CAS NO: 8013-01-2) and acetic acid(C2H4O2) (CAS NO: 64-19-7) were purchased from Merck company. Commercial lysozyme (C11H20NO6) (CAS NO: 9001-63-2) was purchased from Sinagen Company and *Escherichia coli* was prepared from Persian Type Culture Collection. Fresh chicken eggs were bought from the market, alcohol 99% was purchased from the Jahan Alcohol Teb Arak Corporation. Based on supplier information, the purities of Polyvinylpyrrolidone K25, dipotassium hydrogen phosphate, monopotassium dihydrogen phosphate, ammonium sulfate, tris, Coomassie brilliant blue, acid orthophosphoric, peptone, glucose, sodium chloride, Yeast extract, acetic acid, and commercial lysozyme are ≥ 99.8 %, ≥ 99.95 % ≥ 98 %, ≥ 99 % ≥ 99.8 %, ≥ 90% ≥ 99%, ≥ 95% ≥ 99% ≥ 99.5 %, ≥ 99% ≥ 99.7% and ≥ 99 %, respectively. The double-distilled deionized water was utilized as a part of the analyses.

Apparatus and Procedure

To concentrate on the impact of some exploratory factors on the extraction performance of lysozyme in ATPS the partition behavior of commercial lysozyme in PVP K25 and potassium phosphate salt ATPS were considered by RSM. According to the findings of the experimental design, a regression analysis was performed and a model for lysozyme partitioning was provided. The optimum conditions were determined based on the model and this condition was applied for the purification of lysozyme solution extracted from the egg white. Fig. 1 shows the flow diagram of the procedure applied in this study.

Preparation of aqueous two-phase system PVP, K_2HPO_4, and solution of the enzyme lysozyme

The ATPS of PVP, K_2HPO_4 and lysozyme enzyme solution were prepared in 30 mL containers. The concentration of PVP and K_2HPO_4 in the stock solution was prepared up to 20 and 40 percent by weight, respectively. Next, a stock solution of commercial lysozyme with a constant concentration (1 g/L) was added to the prepared ATPS. According to the reports of prior studies, there is a direct relationship between the increment in size of the two-phase region in diagrams and increment in polymer molecular weight [42, 43]. One feature of the higher molecular weight of the polymer is that it generates lower mixing entropy per volume unit than the lower weight. The diminishment of the mixing entropy in the systems assists the phase partition. Fig. 2 show the binodal curve of *Mokhtarini et al.* [41] and *Rahmani et al.* [44] at three various pH. According to the figure and the literature, the polymer molecular weight of this work PVP K25 (Mw=24000) is bigger than both of them

To save time and gain a higher accuracy the commercial Lysozyme was added to the previously prepared stock solution (4 mg/ml), at a final concentration of 0.05 mg/ml in the ATPS. The reference solution in double-distilled water was mixed with certain weight and a vortex mixing was used for the phase dispersion. The final weight of each aqueous the two-phase system was considered as approximately 10 g, pH of salt K_2HPO_4 in ATPSs was adjusted by KH_2PO_4 in the range of 5.6 to 5.8. Moreover, the ATPS were prepared based on the data in Table 1. Containers of ATPS were placed at equilibrium for 24 hours in the water bath temperature range (0-25 ºC). The temperature was controlled with an accuracy of 0.1 ± ºC. Then, the sample tubes were used to be centrifuged by Hermle Z206A (made in Germany) at 6000 rpm for 5 minutes to easily separate the top and bottom samples from the resultant non-turbid phases. Then the top phase was carefully separated from the bottom phase using a syringe made from plastic with a long needle and it was measured to obtain the activity and the concentration of the enzyme.

Experimental design and regression analysis

The lysozyme partitioning in ATPS is dependent on several factors namely polymer and salt concentrations, temperature, and pH. The experimental design suits best

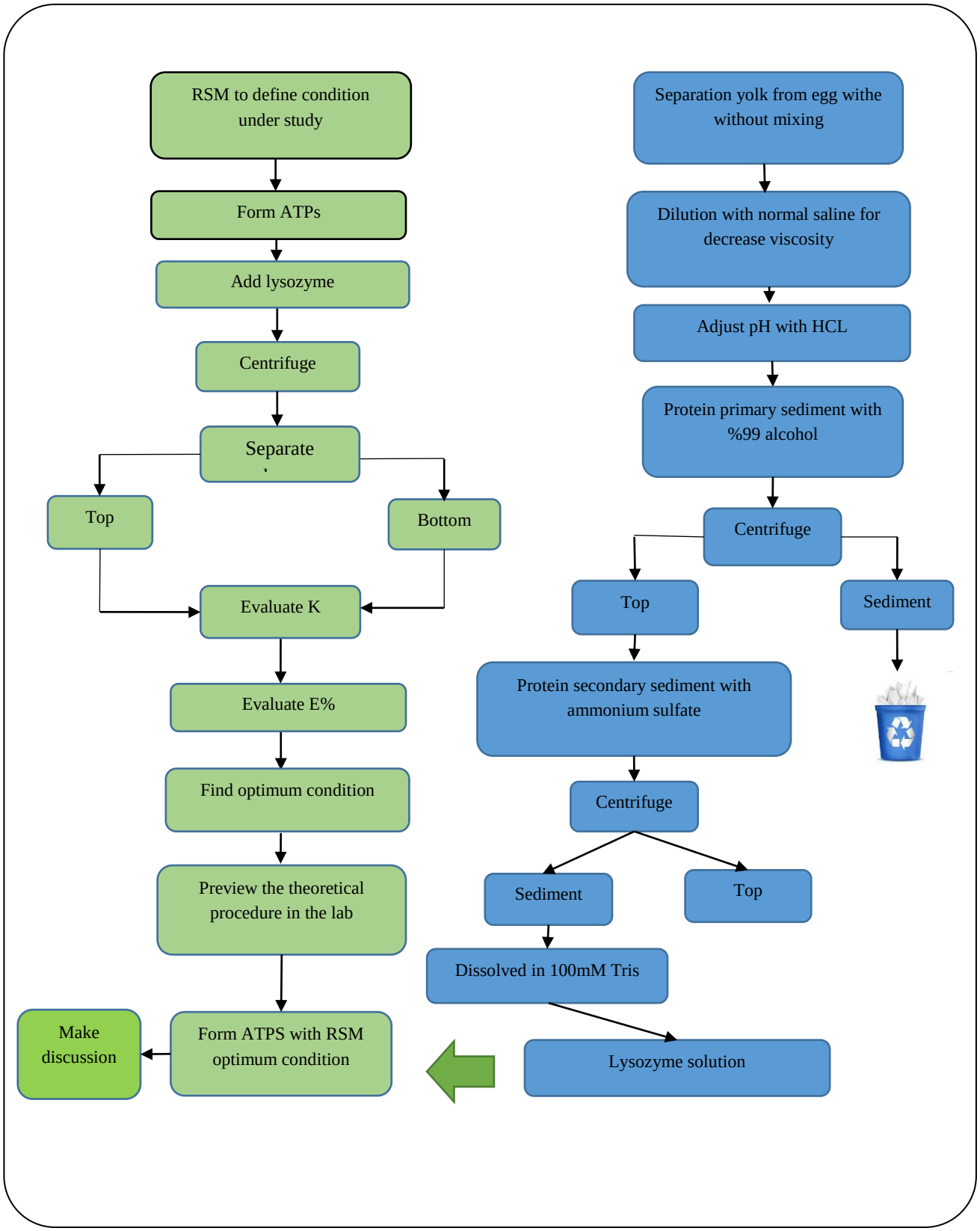

Fig. 1: Flow diagram of the procedure.

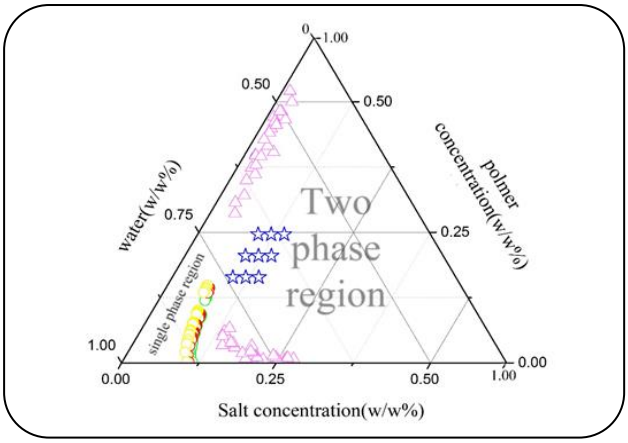

Fig. 2: Binodal curve of PVP and potassium phosphate: ● Rahmani et al.(2017) binodal; ▲Mokhtariani et al.(2011) binodal

to investigate the effect of these factors and their interactions on the lysozyme partitioning. In this study, the CCD is considered to measure the partitioning of the protein in ATPS containing PVP–potassium phosphate. Table 1 indicates the independent variables, experimental ranges and statistical levels for this system are presented. The independent variables are, the salt weight percent in the feed (X1), the PVP weight percent in the feed (X2), temperature (X3) and pH (X4). On the other side, the weight percent of PVP, salt, and protein in the upper and lower phases are the dependent variables. To select the range of the independent variables, the study took advantage of the preliminary experiments.

Equation (1) is the empirical second-order polynomial model used that accounts for the system function:

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii} x_i^2 + \sum_{i=1}^{k-1}\sum_{i=2}^{k}\beta_{ij} x_i x_j + \varepsilon \qquad (1)$$

Where *Y* indicates the anticipated response, *xi*, *xj*, ..., *x* are the input variables, which affect the response, *Y*, x_i^2, x_j^2, ..., x_k^2 stand for the square effects, $x_i x_j$, $x_i x_k$ and $x_j x_k$ are the interaction effects, β_0 is the intercept term, *βi* (i =1, 2, ..., k) is the linear effect, βii (i =1, 2, ..., k) is the squared effect, β(i =1, 2, ..., k;j =1, 2, ..., k) shows the interaction effect and *ε* denote a random error [45-46].

Partitioning Coefficient and efficiency of Aqueous phase system PVP, K_2HPO_4 and enzyme lysozyme

Lysozyme partitioning coefficient (K) is the ratio of the weight of lysozyme in top phase to bottom phase as following

$$K = \frac{[\text{Solute concentration}]_{top}}{[\text{Solute concentration}]_{bottom}} \qquad (2)$$

And (Y) is defined as the percentage of yield based on the following formula:

$$\%Y = \frac{100}{[RK+1]} \qquad (3)$$

Where *R* stands for the phase volume ratio of both top and bottom phases, and *K* is the partition coefficient of lysozyme. Accordingly, the results are reported in Table 4.

Analytical Method

The concentration of the enzyme lysozyme in top phase and bottom phase was determined using the Bradford method [47] with the spectrophotometer UNICO model UV 2100. Accordingly, the concentration of certain Bovine Serum Albumin (BSA) was prepared and then the mixture of salt and polymer with a solution of Bradford (absorption wavelength of 595 nm) were obtained. The concentration of proteins (lysozyme) was determined in both phases using a standard curve of BSA. Experimental data were fitted to the linear expansion:

$$Z = a_0 + a_1 w_p \qquad (4)$$

Where z is optical density (O.D) in g/cm and w_p is BSA concentration. a_0, and a_1 denote the adjustment coefficients of the model.

Preparation of egg white solution

The study by *Boeck et al.* [48] reported that almost 37% of the proteins in the membrane of the hen's egg vitelline are filled by the Lysozyme. The lysozyme solution was prepared in the laboratory. For this purpose, the fresh eggs were used to extract the egg white using hands. Chicken egg white solution was prepared using the method of *Guérin-Dubiard et al.* [21] with a few modifications. The egg whites were separated from the yolks and to reduce the viscosity without mixing and it was diluted with 10 ml of sodium chloride solution 0.05, and the mixture was modified to pH 4 with 1 mol/L HCl. The solution was blended carefully at 2°C during the night, then it was centrifuged at 4°C and 4000 rpm for 10 minutes to remove the precipitate. The supernatant containing lysozyme was isolated and then with

Table 1: The experiment range and level of independent variables in coded units

Independent variable	Range and level		
	-1	0	+1
X_1 (salt weight percent in feed)	9.80	11.90	14.00
X_2 (polymer weight percent in feed)	16.40	20.50	24.60
X_3 (temperature ºC)	14.40	25.00	35.60
X_4 (pH)	6.80	7.50	8.20

Table 2: The value of the coefficients obtained from Equation (1).

	a_0	a_1	R^2
Z	0.104	0.494	0.9934

the proportion of 1: 2 to ammonium sulfate was added to precipitate lysozyme. Adapted from the study by *Chicks et al* [49], the precipitate was separated and it was dissolved in Tris 100 mM and kept in a freezer at -20 °C.

RESULT AND DISCUSSION

Fitting parameters of the calibration equation

The values of the coefficients a_0 and a_1 for the studied system are shown in Table 2.

The partition coefficient and extraction yield of commercial lysozyme in the PVP (K25) + potassium phosphate salt + water system are given in Table 3.

Fitting the model and statistical analysis

Four main factors affect the partitioning of commercial lysozyme namely, the salt weight percent in the feed, the PVP weight percent in the feed, the temperature, and the pH. The study provided a model, derived from the findings of the experimental design to investigate the possible effect of these factors on the protein partitioning. The function of this system was presupposed to be according to a quadratic equation presented below:

$$K = -10.3532 + 0.6891X_1 - 0.1691X_2 + 0.6989X_3 + 0.06195X_4 - 0.002700X_1X_2 - 0.05078X_1X_3 - 0.002744X_2X_4 - 0.006151X_1^2 + 0.05024X_2^2 \quad (5)$$

The experimental data were interpreted using a software called Design Expert 7 (Stat-Ease Inc., Minneapolis, MN, USA) to obtain the effects of these factors. Then the coefficients of the selected quadratic equation were calculated. Moreover, the validity of this model was checked by ANOVA (Table 4) Since the p-values for the independent variables were greater than the selected level ($p < 0.05$), the difference was not significant, so their interactions were removed from the equation.

Effect of salt weight percent in feed on the lysozyme partition

Fig. 3A illustrates the effect of the salt weight percent in the feed on the studied protein. The increment of the weight percent had a direct impact on the lysozyme partitioning coefficient and increased the partitioning value. The highest impact on the partition coefficient among the studied factors is dedicated to the weight percent, which can be justified by hydrophobicity of the proteins. The same effect was confirmed in the study by *Bora* and *Chen* [50, 51]

Effect of polymer weight percent in feed on the lysozyme partition

The effect of PVP weight percent in the feed on the lysozyme partitioning coefficients is provided in Fig. 3B. Based on this figure, there was a minor change (increment) in the partitioning coefficients of lysozyme after increasing the PVP weight percent. Such observation was also made in another related study as *Alves et al.* [52] and *Rodrigues et al.* [53] for the ATPS of PEG–salt. Since the pH level of the ATPS is located above the isoelectric point of the proteins, after the increment of the PVP weight percent, some great tendencies were observed for the lysozyme toward the phase that was enriched with PVP.

Table 3: Experimental design based on central composite design for five independent variables along with observed response.

Run	pH	polymer concentration(w/w)	Salt concentration(w/w)	Temperature(ºC)	K	Y(%)
1	7.50	20.50	14.00	25.00	5.85	90.89
2	8.20	24.60	9.80	35.60	2.21	74.44
3	7.50	20.50	11.90	25.00	3.87	84.62
4	6.80	24.60	9.80	35.60	2.71	78.55
5	7.50	20.50	11.90	25.00	3.80	84.33
6	8.20	16.40	14.00	35.60	5.66	90.41
7	6.80	24.60	9.80	14.40	2.29	75.21
8	7.50	20.50	9.80	25.00	2.18	75.01
9	6.80	16.40	14.00	14.40	5.43	89.8،
10	6.80	24.60	14.00	14.40	6.22	91.79
11	6.80	20.50	11.90	25.00	4.24	86.06
12	6.80	16.40	14.00	35.60	6.16	91.65
13	6.80	24.60	14.00	35.60	6.41	93.27
14	6.80	16.40	9.80	14.40	1.20	52.94
15	7.50	20.50	11.90	25.00	3.91	82.79
16	8.20	24.60	14.00	35.60	6.09	90.05
17	8.20	16.40	9.80	14.40	1.57	64.53
18	7.50	20.50	11.90	35.60	4.04	85.30
19	8.20	16.40	9.80	35.60	1.98	71.86
20	7.50	20.50	11.90	25.00	3.17	81.34
21	7.50	20.50	11.90	14.40	3.81	84.37
22	8.20	20.50	11.90	25.00	3.62	83.55
23	7.50	24.60	11.90	25.00	3.60	81.12
24	8.20	24.60	14.00	14.40	4.92	88.32
25	8.20	24.60	9.80	14.40	1.40	60.22
26	6.80	16.40	9.80	35.60	2.70	78.48
27	8.20	16.40	14.00	14.40	5.60	90.25
28	7.50	16.40	11.90	25.00	3.78	84.25
29	7.50	20.50	11.90	25.00	3.85	84.54
30	7.50	20.50	11.90	25.00	3.60	83.46

Standard uncertainties: u (polymer/salt concentration)=0.02; u (pH)=0.01; u (T)=0.10ºC; u (K)=0.01

Effect of pH on the lysozyme partition

Fig. 3C illustrates the effect of the system pH on the lysozyme partitioning. According to the figures, the net charge of the protein may be responsible for the reduction in the value of the partitioning coefficients after increasing the pH value. It was observed that the pH level above the isoelectric point of lysozyme carries negative electrical charges while keeping the distance from the statistical mean and it provides positive values due to the enhancement of the underlying phase by the phosphate anion. Consequently, the electrostatic term of Albertsson equation would be negative. Also, the range of increment in either lysozyme net charge or the charge positioned in the same boundary of the two phases (interfacial potential) was increased at the medium pH. Hence, this behavior adds to the negativity of the electrostatic resulting in the reduction of K value.

Effect of temperature on the lysozyme partition

As can be seen in Fig. 3D, the ATPS temperature has an inconsiderable effect on the partitioning coefficient of lysozyme due to the viscosity or resistance against

Table4: Analysis of variance (ANOVA) for response surface quadratic model.

Source of variation	Sum of Squares	Degree of freedom	Mean square	F-value	p-value
Model	68.090	9	7.570	101.18	< 0.0001
pH	0.170	1	0.170	2.32	0.1432
salt concentration	64.590	1	64.590	863.76	< 0.0001
Polymer concentration	1.030	1	1.030	13.81	0.0014
Temperature	1.690	1	1.690	22.65	0.0001
Polymer* salt	0.008	1	0.008	0.12	0.4373
Polymer*pH	0.340	1	0.340	4.55	0.0456
Salt*temperature	0.061	1	0.061	0.82	0.3772
$Polymer^2$	0.037	1	0.037	0.49	0.4909
$Salt^2$	0.170	1	0.170	2.26	0.1482
R^2	0.978				
R^2_{adj}	0.9688				

%CV 0.9715

the deformation of the PVP. However, temperature increment indirectly affects the movement of the proteins to other phases by decreasing the viscosity of the PVP solution.

Optimum conditions and model verification

Table 3 indicates the results of the partitioning. Accordingly, the extraction result of the lysozyme varied from 52.94% to 93.27% considering the variation of the experimental conditions provided in Table 1. Moreover, the RSM was used to estimate the best suitable conditions to extract the lysozyme (Table 5). Taking these conditions into account, the study predicted the extraction yield process to be 87.26%

According to the results, the extraction yield of lysozyme (87.26%) was not significantly different from the predicted value (93.69%).

CONCLUSIONS

Lysozyme has a high nutritional value as one of the priorities for usage in food production companies. Even though ATPS is a dominant method for extraction enzymes such as the lysozyme, the number of studies providing experimental data in this regard is inadequate. Therefore, the current study provided new experimental data for the partitioning of lysozyme in ATPS of PVP K25. According to the findings, three main factors determine the partitioning of the lysozyme in the target system namely, weight percent of salt and PVP in the feed, temperature, and pH. The impact of these factors on lysozyme partitioning was revealed using the CCD and fitting this design into the polynomial model for the ATPS. This model was in line with the results derived from the experimental data and it showed a direct relationship between the increment of the weight percent of salt in feed and the value of the lysozyme partitioning coefficient. Furthermore, one of the factors that had an impact on the partitioning coefficient was the weight percent of PVP in the feed, which enhanced the partitioning coefficient's effect on the lysozyme partitioning coefficient. The study found that the differences in the viscosity of PVP solution resulted in the impact of temperature on the partitioning coefficients of lysozyme. Accordingly, after increasing the temperature, there was a reduction in the viscosity of PVP providing the opportunity for the lysozyme to move to other phases. However, the experimental results indicated that the partitioning coefficients of lysozyme were decreased by increasing pH and protein net charge can be a reason for the reduction. In conclusion, the described ATPS process of PVP–K_2HPO_4 in this study was found to be a practical and effective process featuring low expenses for the lysozyme partition. The findings of this study can be used in larger-scale plans and projects.

Table 5: Optimum condition for lysozyme extraction.

Optimum condition				The extraction yield	
Polymer concentration	Salt concentration	pH	Temperature	Cal. value	EXPT. value
21.23	13.99	7.10	35.57	87.26%	93.69%

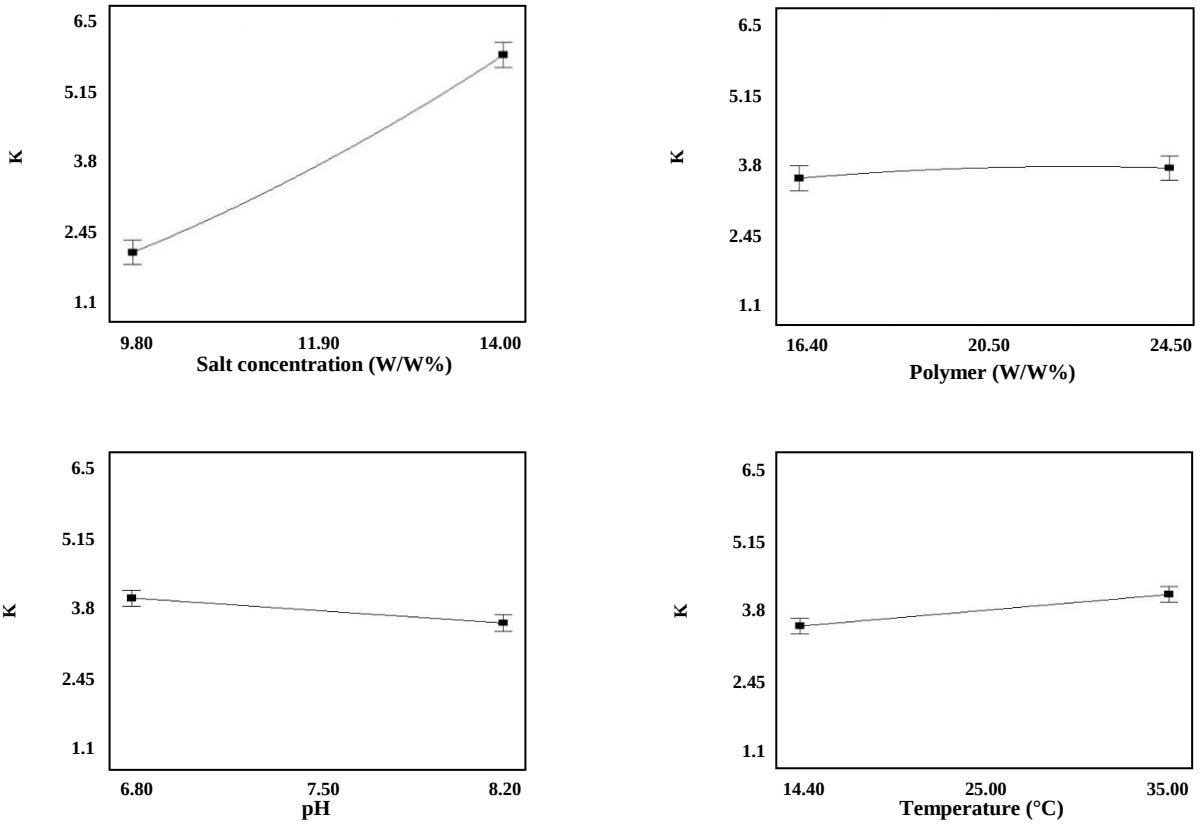

Fig. 3: Effective parameter on lysozyme partition coefficient.

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