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# Protein Recovery From Shrimp Waste Using Aqueous Two Phase System: Effect Of Process Parameters On Partitioning Using Response Surface Methodology

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**Abstract:** Extraction by aqueous two phase system is a powerful technique for separation, concentration, and purification of biomolecules. In the present study, the partitioning of protein from shrimp waste in aqueous two phase system of PEG/ potassium citrate has been investigated. The influence of molecular weight, pH, and NaCl concentration had a pronounced effect on the partition behavior of protein and hence it is studied. The protein partitioning decreases on increasing the molecular weight of PEG, whereas the partitioning increases with an increase in pH and NaCl concentration. This has been attributed to strong electrostatic interactions between the proteins and the polymer and is also related to exclude volume effects. The optimal conditions of the process were investigated using central composite design based on $2^4$ full factorial designs. The best conditions of partitioning were achieved using ATPS composed of PEG 4000, sodium citrate salt, pH8, 1M Addition of sodium chloride, and 40 Tie Line Length. The maximum percentage yield of protein extracted from shrimp waste was found to be 83.64%. This work has presented the potential application of ATPS processes for the recovery of protein from shrimp waste, as an initial purification step in the development of a biotechnological process with commercial application.
**Key words:** Aqueous two phase system, shrimp waste, protein partitioning, Response surface methodology; Central composition design.

## Introduction

In recent years, industries demand for efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and gentle enough to preserve biological activity of proteins (ie.) purity. Thus separation and purification of proteins is a critical element of modern bioprocess engineering because it provides a vital link between laboratory technique and large scale operations. Aqueous two-phase system (ATPS) is a better alternative for many biomolecules in bioseparation process, especially in the early downstream stages for partitioning and purifying the mixtures of proteins. Mutually the ATPS removes the insoluble and major classes of contaminants and this technique has been developed as a primary purification step in the extraction protein from crude (1). Major Advantages of ATPS were scaling up feasibility, process integration capability and biocompatibility, low interfacial tension, high throughput, low process time, low energy requirements, scope for continuous operation, good resolution, high separation yield, and the possibility of polymer and salt recycling (2-4).

Aqueous two-phase systems are composed of aqueous solutions of either two water-soluble poly- mers, usually polyethylene glycol (PEG) and dextran (Dx), or a polymer and a salt, usually PEG

and phosphate or sulfate. A PEG - salt system was selected because this favors one sided partition of the compounds due to larger differences in the physicochemical properties of the two phases. For industrial purposes, polymer/potassium phosphate systems are the most commonly used but the high salt concentration required represents a waste disposal problem which leads to environmental concerns (5). Previous studies have demonstrated that substitution of phosphate by citrate, a biodegradable anion, could be considered a good alternative since it has lower environmental toxicity (6).The basis of separation in ATPS is achieved by the different distribution between the two phases of the target compound and the contaminants and is governed by number of parameters relating to the properties of the phase system and biomolecule, and the interaction between them (7).The general properties of the aqueous two-phase systems have been studied by several researchers. However, the mechanism governing the partition of biological materials is still not well understood. The observed partition coefficient (*K*p) is a result of van der Waals, hydrophobic, hydrogen bond, and ionic interactions of the biomolecules with the surrounding phase.ie (hydrophobicity, charge, size, molecular weight of biomolecules). Separation of compounds is usually attained by orderly variation of system parameters, namely the type and molecular mass of polymers, tie line (a function of the concentration of the system components), type and concentration of components or added salts, pH, etc. ATPS has been applied for the partitioning and recovery of various protein & enzymes such as β - galactosidase and β –glucosidase (8), α-amylase (9), Pectinase (10), β-xylosidase (11).

Processing techniques for sea food waste are needed to convert the underutilized wastes into more marketable, valuable and acceptable products. Processing of shrimp invariably generates solid waste in the form of head and body carapace. As the waste generation from processing of Indian shrimps ranges from 48% to 56% of the total weight depending on the species (12). The major components (on dry weight basis) of shrimp waste are protein (35–50%), chitin (15–25%), minerals (10–15%) and carotenoids (13). At present the shrimp waste generated in Indian shrimp processing industries is not commercially exploited for the recovery of valuable components, it forms one of the cheapest raw materials for recovery of globular proteins. These globular proteins have potential application in food and biopharmaceutical industries (14,15). Hence, there is scope for the recovery of valuable material from the shrimp waste.

RSM is a collection of mathematical and statistical techniques useful for designing experiments, building models and analyzing the effects of the several independent variables. The main advantage of RSM is the reduced number of experimental trials necessity of only to evaluate multiple factors and their interactions (16). This methodology can be used in developing suitable treatment technology considering the effects of operational conditions on the removal process or to determine a region that satisfies the operating specifications (17). Response surface methodology (RSM) is an effective statistical tool and widely used in process optimization, which includes experimental design, model fitting, validation and condition optimization.

The four-level factorial design was used to study the partition behavior of the protein in ATPS. To link to individual and interactive effects, a systematically statistical method response surface methodology (RSM) was applied to investigate how factors would affect the partitioning of protein. To expose the relationship between the partitioning of protein and the compositions of the system and also to simplify the further process optimization in examined Aqueous Two Phase system, a statistical analysis was performed by response surface methodology (RSM) which appeared itself to be very useful tool for modeling of processes of isolation and partitioning of biomolecules in ATPS.

The objective of this study is to analyze the feasibility of using an ATPS composed of PEG and potassium citrate, for the primary recovery of protein from shrimp waste. The effect of the polymer molecular weight, pH, Tie line length, and addition of neutral salts on the partitioning behavior of protein has been investigated. The present paper will also report the optimization of the ATPS and to determine the optimum conditions for the next step of protein purification directly from the crude waste.

## Materials and methods

### Materials

Polyethylene glycol with molecular weights of 4000, 6000 and 10000 was obtained from Merck-Schuchardt (Munich,Germany) and bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Tri-potassium citrate monohydrate [$K_3C_6H_5O_7$] was obtained from Loba, (with a minimum purity of 99 %). All chemicals were of analytical grade. The polymer and salts were used without further purification. Milli porewater was used throughout the experiments. Shrimp head waste was collected from local market in India.

### Preparation of shrimp waste

Fresh shrimp waste was collected from the local market. Known weight of shrimp waste was ground in cell lysis buffer, centrifuged and the supernatant was collected. The shrimp extract was stored at 4°C

and required quantities were taken as and when required for different experiments and directly subjected to Aqueous two phase system (ATPS).

**Aqueous two-phase diagrams**

The binodal curves were determined by the cloud point method (5). Potassium citrate of 30 % (w/w) and Poly Ethylene Glycol 4000, 6000 and 10000 of 40 % (w/w) was prepared. A known amount of the PEG solution was taken and titrated against salt, To ensure the end point, salt was added in drops till the appearance of turbidity, which indicates the two-phase formation. Water was then added until the disappearance of turbidity. The procedure was repeated to get the other binodal points. The determination of tie lines, samples were prepared (50g) by mixing polymer, salt and water in appropriate proportions in a centrifuge tube. The samples were thoroughly mixed and kept in a thermostat at constant temperature for 24 h. After separation of two-phases (PEG rich top phase and salt rich bottom phase), the concentration of PEG with desired molecular weight in top and bottom phases was determined using refractive index measurements. The concentration of Potassium citrate in the top and bottom phase was determined by using a flame photometry. The tie-line length (TLL) was determined by the square root of the sum of the squares of the difference in PEG and citrate concentrations between the top and bottom phases.

$$TLL = \sqrt{\left(C_P^T - C_P^B\right)^2 + \left(C_S^T - C_S^B\right)^2} \qquad \ldots\ldots (1)$$

where $C_P^T$ and $C_P^B$ are PEG concentrations (%, w/w) in the top and bottom phases respectively and $C_S^T$ and $C_S^B$ are salt concentrations (%, w/w) in top and bottom phases respectively. The tie line lengths are expressed in mass fractions.

**Preparation of Aqueous two-phase systems**

Partitioning of soluble proteins from shrimp waste was carried out in PEG+ potassium citrate +water system. BSA was used as model protein for partitioning. All partition experiments were carried out at different pH (6, 7 and 8) values at constant temperature (30°C). Phase systems were prepared in 50 ml graduated centrifuge tubes by weighing out appropriate quantities of the PEG of desired molecular weight and potassium citrate stock solutions and added to crude shrimp extract to make the total weight of the system 100 % (w/w).The pH of the system was maintained by using citric acid monohydrate. The amount of the shrimp extract added to the systems was 1 ml, which was the last added component. Complete phase separation was achieved by centrifugation at 3000 rpm for 20 min to speed up the phase separation, and then placed at room temperature (30°C) for 24 h to ensure complete equilibration. After equilibration, estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. In order to determine the concentration of proteins in each of the co-existing phases, samples from each solution phase was collected using a syringe and estimation of protein (18).

The partition coefficient is defined as the ratio of equilibrium concentration of protein extracted in top phase $(C_T)$ to equilibrium concentration of protein extracted in bottom phase $(C_B)$, and was determined using results from the Bradford protein assay.i.e.,

$$K = \frac{C_T}{C_B} \qquad \ldots\ldots.. (2)$$

The partition coefficient $(K)$ is used to quantify the degree of separation reached in an extraction process. The phase volume ratio $(R)$ is defined as ratio of volume of the top phase to volume of the bottom phase,

$$R = \frac{V_T}{V_B} \qquad \ldots\ldots.. (3)$$

where $V_T$ and $V_B$ are the upper and lower phase volumes, respectively. Yield percentage was calculated by using the following equation:

$$Y = \frac{100}{(KR)^{-1} + 1} \qquad \ldots\ldots. (4)$$

Protein quantification

Total protein was quantified by the Bradford method using a Coomassie assay reagent supplied by merck, Germany). To avoid interference from phase components, samples were analyzed against blanks containing the same phase composition but without proteins. Bovine serum albumin (BSA) was used as a protein standard and Absorbance was monitored at 595 nm.

**Experimental design**

To establish the optimum conditions for partitioning of protein in ATPS, response surface methodology (RSM) was used. The CCD system is an effective model and used for sequential experimentation which provides information for testing 'the goodness of fit' and does not require unusual large number of design points thereby reducing the overall cost associated with the experiment. A four-factor central composite design (CCD) obtained by using Design-Expert 8.0.5 software, (State-Ease Inc., Minneapolis MN, USA), was applied. The four factors considered to affect the protein partitioning in the ATPS systems were the PEG's molecular weight (A), pH (B), Addition of sodium chloride(C), and Tie Line Length (D). The level and ranges chosen for the factors are shown in Table 1. The complete design consisted of 30 experimental points which included six replications at the center point. The 30 samples

were prepared in random order. In each experiment the yield percentage was calculated and each trial was performed in duplicates.

**Statistical analysis**

The experimental data obtained from the design were analyzed by the response surface regression procedure using the following second-order polynomial equation:

$$Y_i = {}_o + \sum_i {}_i X_i + \sum_{ii} {}_{ii} X_i^2 + \sum_{ij} {}_{ij} X_i X_j \quad \ldots \quad (5)$$

where $Y_i$ is the predicted response, $\beta_o$, $\beta_i$ , $\beta_{ii}$ and $\beta_{ij}$ are regression coefficients for the intercept, linear, quadratic and interaction coefficients, respectively and $X_i$ and $X_j$ are the coded independent variables.

The statistical software package, Design-Expert 8.0.5 was used for regression analysis and graphical analysis of the data obtained during the experiment. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The second-order polynomial equation was employed to fit the experimental data. The significance of the model equation and model terms were evaluated by f-test. The quality of fit of the polynomial model equation was expressed by the coefficient of determination ($R^2$), adjusted $R^2$ and ‘‘adequate precision’’. The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the relationship between the responses and the experimental levels of each factor used in the design. To optimize the level of each factor for maximum response ‘‘numerical optimization’’ process was employed. The combination of different optimized parameters, which gave maximum response, i.e. maximum protein partitioning in PEG phase was tested experimentally to confirm the validity of the model.

## Results and discussion

**Phase Diagrams**

The binodal curve describes the border between the single-phase area and the two-phase area. The area above the binodal describes all compositions giving rise to two phase systems. Binodal curve is a border line between the homogeneous phase and heterogeneous two phase of ternary mixture. The two phase region formation is due to the incompatibility of system components. The tie-line describes the compositions of the two phases in equilibrium. Phase diagrams were determined for different PEG molecular weights (Fig. 1.). It can be noted from this figure that the binodal lines became more asymmetric and close to the origin with the increase in polymer molecular weight. This happens because as the polymer molecular weight increases, the components of the system become more different and so lower concentrations are required for phase separation. This may be caused by the increase in the hydrophobical character of PEGs of higher molecular weight (19).

**Effect of molecular weight on protein partitioning**

The selection of the best molecular weight of polymer is very important and is the first step in the ATPS experiments because partition behavior of protein depends on the PEG molecular weight in ATPS. The effect of increasing molecular weight of PEG on protein partitioning behaviour has been explained on the excluded volume effect of PEG (20) and protein hydrophobicity (21). In Fig.2 it has been observed that the partitioning of soluble proteins from shrimp waste can be altered by varying the molecular weight of PEG. The increase in the molecular weight of PEG from 4000, 6000 and 10 000, the partition coefficient of protein was decreased. Due to the presence of excluded volume effect, the partition coefficient decreases with increase in the Molecular Weight of PEG (22).The PEG molecular weight is increased with increasing degree of polymerization. And hence PEG chain length increases and due to which there will be less hydroxyl groups for the same concentration of the polymer and so the polymer-richer upper phase increase in hydrophobicity. At the same time, the chain length increase and it can cause less space available for the protein accommodation in the top phase, so the protein is automatically pushed to the bottom phase. Another tendency that affects the partitioning is the high PEG molecular weight which will might affect the viscosity of system and thus incompatible for processing. The globular protein has a comparatively high surface hydrophobicity, so the size-exclusion effect is suggested to be the main reason for the decrease in the partition coefficient. It was observed for that better protein partitioning was achieved with lower molecular weight PEG. This will agree with the report of enzymes/proteins from different sources (23-25).

**Table1. Factors and value levels used in the central composite design**

| Variables (factor) | Low value (-1) | Centre value (0) | High value (+1) |
|---|---|---|---|
| Molecular weight (A) | 4000 | 6000 | 10000 |
| pH(B) | 6 | 7 | 8 |
| Addition of Nacl(C) | 0 | 0.5 | 1 |
| Tie Line Length (D) | 28 | 32 | 36 |

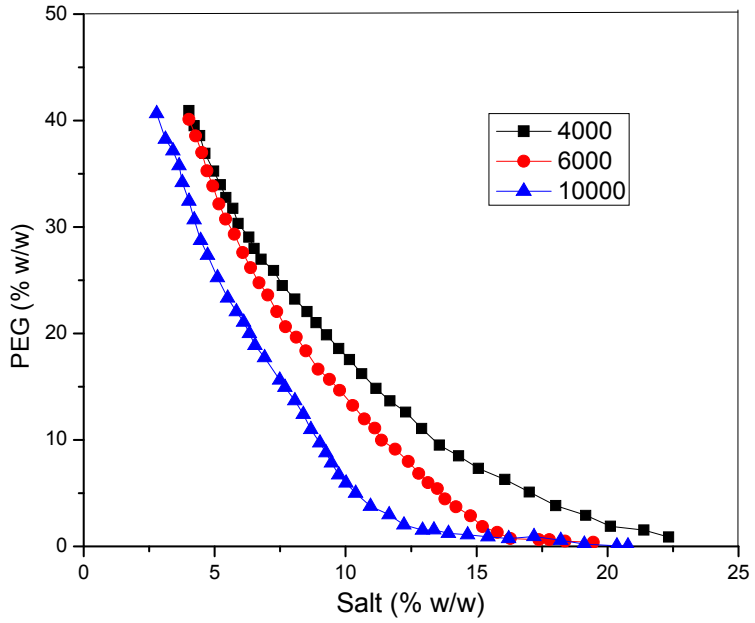


**Figure. 1 Effect of molecular weight on Binodal Curve of PEG/potassium citrate aqueous two phase system.**

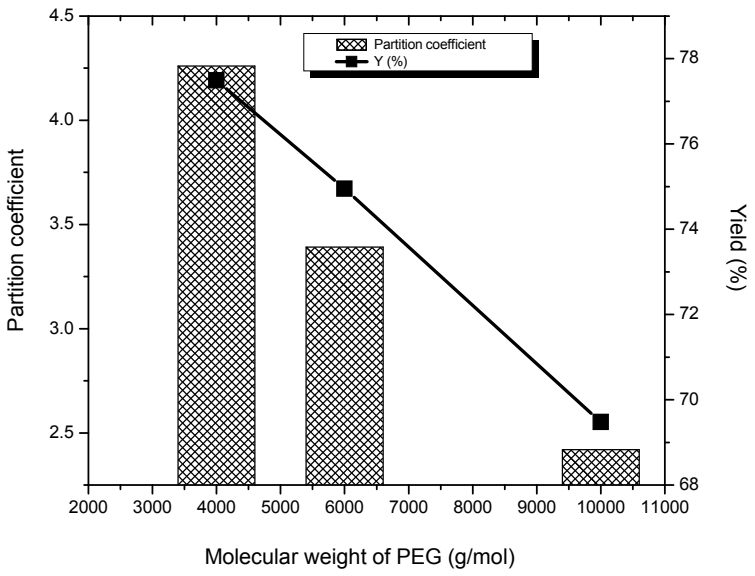


**Figure. 2 Effect of PEG molecular weight on protein partitioning**

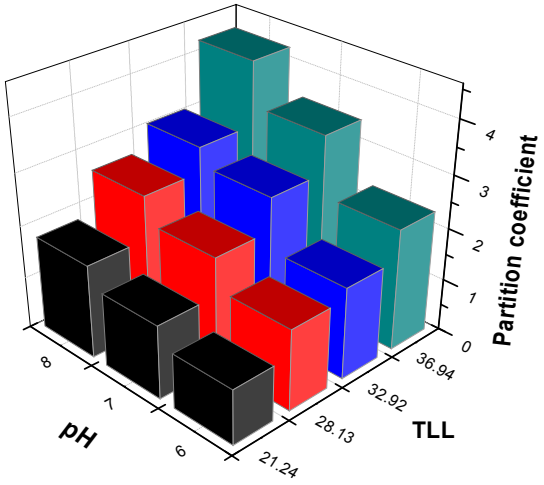


**Figure. 3 Effect of pH on the partition coefficients of protein partitioning**

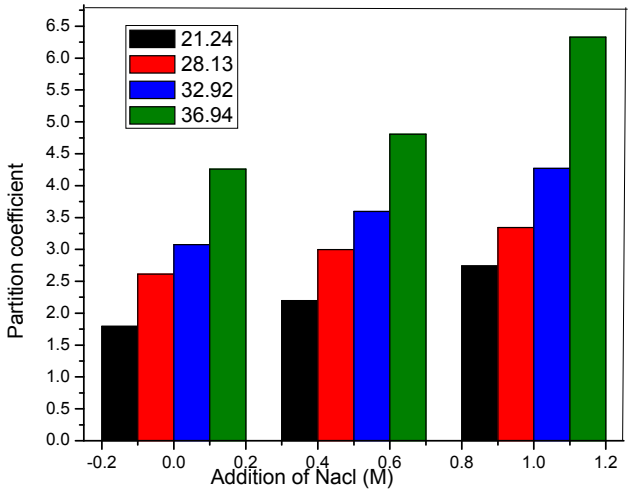


**Figure. 4 Effect of NaCl concentration on the partition coefficients of protein partitioning**

**Effect of pH on protein partitioning**

The system pH is another important factor which influences on the partitioning of biomolecules. The pH affected the charge of target protein and ion composition, as well as surface character of contaminating materials. Effect of pH on protein partitioning from shrimp waste using PEG-potassium citrate system without sodium chloride is shown in Fig. 3. It was observed that the partition coefficient increased with increase in pH of the system from 6 to 8.It is observed that the partition coefficient increases with increase in pH of the system from 6 to 8. The pH could affect the partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. At low pHs, the protein has a net positive charge because the amine gains an extra proton and at high pHs, it has a net negative charge because the carboxyl loses its proton. The intermediate pH at which protein has a net charge of zero is called the isoelectric point (26). The aqueous two phase system composed of PEG and potassium Citrate salt, top phase (PEG phase) carries a positive charge while the bottom phase (salt phase) have a negative charge (27). Whereas the negatively charged proteins partition which refer to the top phase (PEG rich) and the positively charged protein to the bottom phase (salt rich) in the PEG–salt system (28). At higher pH, the protein is more negatively charged than at low pH, and therefore, the partition coefficient and percentage yield of the protein increases with increasing the pH, which may be caused by the electrostatic interactions between the protein and PEG units. The shrimp waste contains large amount of globular proteins, which aggregates and its reach the negative charge on increasing the pH of the system. The increase in partition coefficient value could be due to hydrophobic interaction and net

charge effect, which is the function of polymer concentration and solution pH. Protein partitioning in ATPS is mainly depend on the hydrophobicity and charge of protein (29). This similar behavior was observed with other acidic proteins (30-32).

**Effect of sodium chloride on protein partitioning**

Effect of sodium chloride on protein partitioning from shrimp waste using PEG-potassium citrate system at pH 8 is shown in Fig. 4. From the figure it has been observed that with increase in NaCl concentration, the partition coefficient of the system was increased. The increasing in NaCl has been reported to drive most proteins to the top (PEG) phase due to a decrease in the difference of electrostatic potential between two phases. The addition of neutral salts is known to generate an electrical potential difference between the two phases that were able to drive proteins from one phase to another phase depending on the charge. Generally, the addition of sodium chloride affects the partitioning in aqueous two phase system by speeding up phase separation, by influencing the phase potential or by protein hydrophobicity (5).The addition of sodium chloride salts in ATPS can affect water Structure and hydrophobic interactions differently (6) in which the interaction between hydrophobic chain (ethylene group) of PEG and hydrophobic surface area of the protein will be facilitated. The addition of Nacl affects the partitioning of proteins in the ATPS because of the differential distribution of the salt ions in the two phases. The added salts contain ions with different hydrophobicities, which can direct the partitioning behavior of the proteins. The most hydrophobic anions or cations will drive the partitioning of their counterions to the more hydrophobic phase, and the less hydrophobic co-ions will be forced to partition to the more hydrophilic phase (33,34). Several authors (21-22, 35-36) found that addition of NaCl to PEG–salt systems increases the difference in hydrophobicity of the phases; more hydrophobic proteins are prompted to partition into the upper phase. On the other hand, when the concentration of neutral salts is further increased, proteins tend to partition into the upper phase.

**Optimization of protein partitioning conditions using response surface methodology**

The partitioning of protein in a PEG + potassium citrate ATPS was optimized using a statistical experimental design involving four variables, namely PEG molecular weight (A), pH(B), addition of Nacl(C), and tie line length (D) at two levels**.** Table 2 presents the design matrix of the variables and data where the actual and the predicted data for protein partitioning were compared. Actual values were the measured response data for a particular run and the predicted values were determined by approximating functions employed for the models. The analysis of variance (ANOVA) was performed in order to verify the validity of the models and the results are presented in Table 3. According to the analysis of variance, f-value for the overall regression model (135.55) is significant at 5% level and the lack of fit is insignificant indicating that the first-order model with interaction is very adequate in approximating the response surface of the experimental design. The regression analysis of the experimental design showed that the linear model terms (A, B, C, and D), quadratic model terms ($A^2$) and interactive model term (AB, AD, BC, BD, and CD) are significant ($P < 0.05$). However, quadratic model terms ($B^2$, $C^2$and $D^2$) and interactive model terms (AC) were found to be insignificant ($P > 0.05$). The coefficient of determination ($R^2$) and adjusted $R^2$ values were 0.9922 and 0.9848, respectively. This indicates that the model could explain 99.22% of the variability in response. Adequate Precision measures the signal to noise ratio and a ratio greater than 4 is desirable. In this case, a ratio of 53.19 was achieved indicating an adequate signal and so this model can be used to navigate the design space.

The analysis of variance is employed for the determination of significant variables. The regression equations were submitted to the *F*-test in order to determine the coefficient $R^2$. The *F*-values and 'probability > *F*' values of all the regression equations show that these models are significant and the model determination coefficient $R^2$ indicates a good response between model prediction and experimental data. For further convenience, the relative model equations of uncoded variables fitted by regression analysis are given by:

$$\text{Yield} = 69.48 - 6.00A + 5.19B + 3.72C + 6.62D + 1.60AB + 0.51AC + 1.14AD - 0.98BC - 0.80BD - 0.85CD - 4.63A^2 - 0.78B^2 + 0.59C^2 - 0.84D^2 \quad \ldots\ldots\ldots\ldots(5)$$

The response surface curves were plotted to assess the interaction of the variables and to find out the optimum level of each variable for maximum response. The regression models obtained were used to calculate the response surface for each response variable. The response surface plots for the protein extraction yield (Fig. 5a, 5b and 5c) showed a pronounced increase the molecular weight of PEG, Tie Line Length and pH and a slight increase when the concentration of NaCl increases. The model predicted maximum recovery yields (83.64%) with 4000 PEG, pH 8, 36% TLL and 1M NaCl.

**Table 2. Central composite design matrix measured and predicted responces of protein partition in PEG + pottasium citrate + water system.**

| Run | Factors | | | | Response | |
|---|---|---|---|---|---|---|
| | A | B | C | D | Actual | Predicted |
| 1 | 1 | 0 | 0 | 0 | 56.81 | 58.85 |
| 2 | -0.333 | 0 | 1 | 0 | 72.62 | 73.64 |
| 3 | -0.333 | 0 | 0 | 1 | 73.87 | 74.92 |
| 4 | -0.333 | 0 | 0 | 0 | 70.03 | 69.48 |
| 5 | 1 | -1 | -1 | 1 | 55.36 | 55.26 |
| 6 | -1 | 1 | -1 | 1 | 75.63 | 76.87 |
| 7 | 1 | 1 | -1 | -1 | 53.27 | 53.42 |
| 8 | -1 | -1 | -1 | -1 | 53.74 | 54.74 |
| 9 | -0.333 | 1 | 0 | 0 | 74.53 | 73.44 |
| 10 | -1 | 1 | 1 | -1 | 71.34 | 71.90 |
| 11 | -0.333 | 0 | 0 | 0 | 70.03 | 69.48 |
| 12 | 1 | -1 | 1 | -1 | 49.3 | 48.51 |
| 13 | -0.333 | -1 | 0 | 0 | 61.77 | 63.96 |
| 14 | -0.333 | 0 | 0 | 0 | 70.03 | 69.48 |
| 15 | -1 | -1 | -1 | 1 | 70.19 | 69.06 |
| 16 | 1 | 1 | 1 | 1 | 74.78 | 74.04 |
| 17 | -0.333 | 0 | -1 | 0 | 66.43 | 66.51 |
| 18 | 1 | 1 | 1 | -1 | 61.29 | 61.69 |
| 19 | -1 | 1 | -1 | -1 | 66.05 | 65.73 |
| 20 | -1 | 1 | 1 | 1 | 79.53 | 79.62 |
| 21 | -1 | -1 | 1 | 1 | 75.8 | 75.74 |
| 22 | -1 | 0 | 0 | 0 | 71.79 | 70.85 |
| 23 | -0.333 | 0 | 0 | 0 | 70.03 | 69.48 |
| 24 | -0.333 | 0 | 0 | 0 | 70.03 | 69.48 |
| 25 | -0.333 | 0 | 0 | -1 | 62.31 | 62.36 |
| 26 | 1 | -1 | -1 | -1 | 36.93 | 36.31 |
| 27 | 1 | 1 | -1 | 1 | 69.48 | 69.19 |
| 28 | 1 | -1 | 1 | 1 | 64.1 | 64.05 |
| 29 | -1 | -1 | 1 | -1 | 65.27 | 64.84 |
| 30 | -0.333 | 0 | 0 | 0 | 70.03 | 69.48 |

**Table 3. Analysis of variance for the experimental results of the Central composite design**

| Source | SS | DF | MS | F-V | Prob > F |
|---|---|---|---|---|---|
| Model | 2488.75 | 14 | 177.77 | 135.55 | < 0.0001 |
| A* | 648.24 | 1 | 648.24 | 494.29 | < 0.0001 |
| B* | 485.06 | 1 | 485.06 | 369.86 | < 0.0001 |
| C* | 249.02 | 1 | 249.02 | 189.88 | < 0.0001 |
| D* | 789.90 | 1 | 789.90 | 602.31 | < 0.0001 |
| AB* | 40.90 | 1 | 40.90 | 31.18 | < 0.0001 |
| AC | 4.10 | 1 | 4.10 | 3.13 | 0.0973 |
| AD* | 20.66 | 1 | 20.66 | 15.75 | 0.0012 |
| BC* | 15.48 | 1 | 15.48 | 11.81 | 0.0037 |
| BD* | 10.14 | 1 | 10.14 | 7.74 | 0.0140 |
| CD* | 11.66 | 1 | 11.66 | 8.89 | 0.0093 |
| $A^{2*}$ | 55.64 | 1 | 55.64 | 42.43 | < 0.0001 |
| $B^2$ | 1.59 | 1 | 1.59 | 1.21 | 0.2877 |
| $C^2$ | 0.90 | 1 | 0.90 | 0.69 | 0.4193 |
| $D^2$ | 1.85 | 1 | 1.85 | 1.41 | 0.2538 |
| Residual | 19.67 | 15 | 1.31 | | |
| Lack of Fit | 19.67 | 10 | 1.97 | | |
| Pure Error | 0 | 5 | 0 | | |
| Core Total | 2508.42 | 29 | | | |
| $R^2$ | 0.9922 | | | | |
| Adj $R^2$ | 0.9848 | | | | |

*significant at 5% level, A Molecular weight, B pH , CAddition of Nacl, D TLL

**Fig.5(a)**

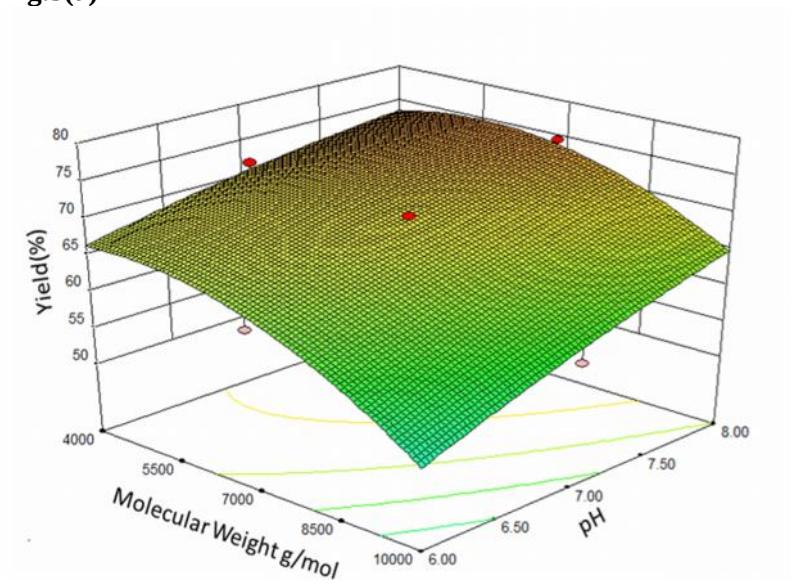


**Fig.5(b)**

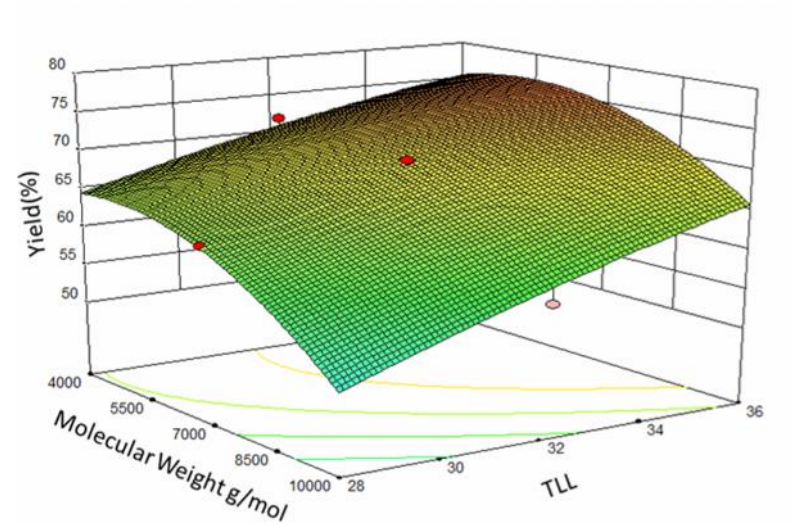


**Fig.5(c)**

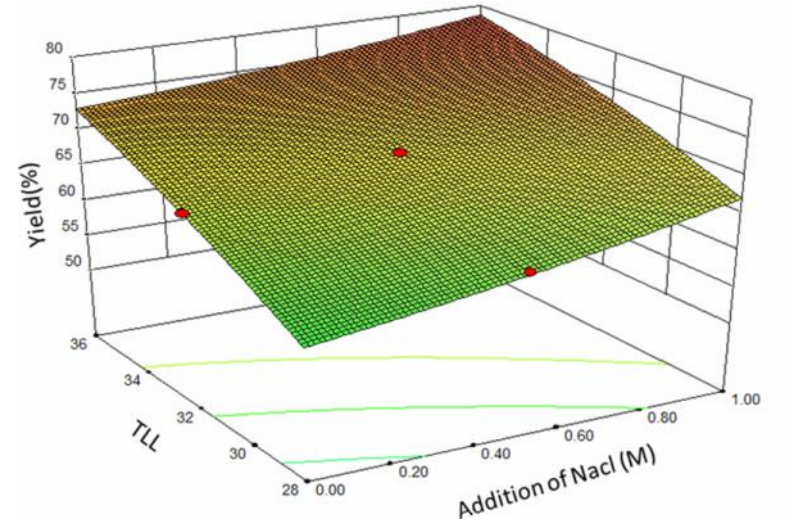


**Figure.5** 3D response surface plot for percentage yield of protein partioning in shrimp waste
(a) PEG Molecular weight and pH;
(b) PEG Molecular weight and TLL;
(c) Addition of Nacl and TLL;while other variables are set at optimized value.

## Conclusions

This work has demonstrated the potential application of ATPS processes for the recovery of protein from shrimp waste extracts, as a first step in the development of a biotechnological process with commercial application. A central composite design allowed a thorough analysis of the factors that influence the partition of protein in the aqueous two phase systems, leading to the definition of the conditions that maximize the recovery yield. The partition coefficient of 6.327 and yield of about 83.64% are undoubtedly a good result for a first step purification. It predicted that the optimized condition could be then applied to crude extract for the purpose of instantaneous extraction and purification. The overall results indicated that ATPS is an effective method for separating and concentrating protein from shrimp extract.

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