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# Optimization of aqueous two-phase partitioning for purification of recombinant *Eupenicillium terrenum* fructosyl peptide oxidase

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ABSTRACT

Fructosyl peptide oxidase (FPOX), a flavoenzyme belonging to the class of oxidoreductases, has been used as a diagnostic enzyme for HbA1c measurement test. Design of experiments methodology was applied to evaluate the partition behavior of recombinant *Eupenicillium terrenum* FPOX in aqueous two-phase systems (ATPS). Different ATPS parameters including polyethylene glycol (PEG) molecular weight, PEG concentration, salt type, salt concentration, pH, system buffer type, system buffer concentration, temperature, neutral salt type, neutral salt concentration, and crude enzyme loading were investigated using a Plackett–Burman design (PBD). Taking into consideration an increase in partition coefficient ($K_E$), PEG concentration, pH, system buffer concentration, and NaCl concentration, which played significant roles in enzyme partition, were chosen for further optimization by Box–Behnken design (BBD) in response surface methodology (RSM). The optimized condition led to maximum recovery (106.8%), yield (95.1%) and purification factor (42.4) values which were predicted to be achieved in ATPS formed by 14.0% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 5.0% (w/w) NaCl and 55.0 mM potassium phosphate buffer with pH 8.0. The partitioned recombinant FPOX was obtained as a single band into the upper PEG-rich phase and its specific activity was calculated to be 201.23 ± 2.1 U/mg. In summary, our data showed that the ATPS optimization using design of experiments can be an appropriate method for recovery of recombinant FPOX.

## 1. Introduction

Fructosyl peptide oxidase (FPOX) is a member of the family of oxidoreductases which catalyzes the oxidative deglycation of fructosyl peptide to unglycated peptide, glucosone and hydrogen peroxide. This enzyme has been identified from different microorganisms and used for the enzymatic measurement of glycated proteins including hemoglobin A1c (HbA1c) (Ichiyanagi et al., 2013; Ogawa et al., 2019). HbA1c is an important test for the diagnosis and management of patients with diabetes mellitus (Ahmed et al., 2005; Ferri et al., 2009). The FPOX from *Eupenicillium terrenum* which acts specifically on glycated peptides has great potential to be used as a diagnostic enzyme (Shahbazmohammadi et al., 2019; Shimasaki et al., 2017). The industrial applications of this biocatalyst require purified enzyme with appropriate kinetics properties and low cost. Several methods including chromatography and ammonium sulphate precipitation have been used to purify FPOX with varying degree of success. Generally, these conventional purification techniques are limited by their complicated and tedious operations, high cost and difficulty of scaling up. Hence, an efficient and inexpensive method for FPOX purification is highly sought (Hirokawa et al., 2003).

Aqueous two-phase systems (ATPS) have been applied for the separation and purification of various bio-molecules. When aqueous solutions of two incompatible substances, such as polyethylene glycol (PEG) and dextran or PEG and a salt are mixed above a critical concentration, two phase partitioning occurs (Shahbazmohammadi and Omidinia, 2013; Ehliers and Wilhelm, 2011). Since both phases are composed of water as well as the applied polymers have a stabilizing effect on protein structure, ATPS provide a non-denaturing environment for biological materials (Hatti-Kaul, 1999). Compared with the other purification methods, ATPS has advantages such as low cost, potential for easily scale-up, high yield, good resolution and preserving the biomolecules (Khayati et al., 2018; Lo et al., 2018). The optimum conditions are usually achieved by systematic variation of different parameters such as concentration and type of phases, temperature and pH. Empirical modeling can predict the partition behavior of proteins in ATPS (Shahbazmohammadi et al., 2015; Liu et al., 2013). The design of

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experiments methodology indicates a method in which the randomization and statistical aspects of an experiment are to be carried out (Fakhari et al., 2017). Analysis of results of an experimental design allows establishing a causal relationship between the variables, thus extracting the maximum possible information. Statistical optimization designs have been successfully employed to enzyme partition with ATPS (Garai and Kumar 2013; Kammoun et al., 2009). However, FPOX enzyme has not been subjected to two-phase partitioning yet. The goal of this communication was to investigate the performance of ATPS and the significant factors which may affect the FPOX partition feature. Plackett-Burman design (PBD) and Box–Behnken design (BBD) with response surface methodology (RSM) were used to identify the suitable conditions for recombinant *E. terrenum* FPOX partitioning.

## 2. Materials and methods

### 2.1. Materials

PEGs with different molecular weights and sodium chloride were purchased from Merck (Darmstadt, Germany). Fructosylvalyl-histidine (Fru-ValHis) was synthesized according to the method of Keil et al., (1985). 4-aminoantipyrine and horseradish peroxidase (HRP) was obtained from Sigma-Aldrich. Sodium *N*-ethyl--*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) was prepared from Santa Cruz Biotechnology. The other chemicals were of analytical grade.

### 2.2. Expression and purification of recombinant FPOX

The gene encoding *E. terrenum* FPOX was cloned into the expression vector pET-28a(+) by *Nde*I and *Xho*I restriction enzymes. *E. coli* BL21 (DE3) harboring recombinant plasmid pET28FPOX was cultivated in 10 mL of Luria-Bertani (LB) medium containing 100 μg/mL of ampicillin at 37 °C and 200 rpm for a period of 16 h. 10 mL of pre-grown inoculum was added into 100 mL of LB medium in Erlenmeyerflask and incubated at 37 °C and 250 rpm. Expression of recombinant enzyme was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 24 h incubation at 20 °C. Induced cells were harvested, washed with 0.9% NaCl solution and stored at −20 °C. The cell pellet was dissolved in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 1% glycerol, pH 8.0) and disrupted by sonication using a pulse sequence of 20s on and 15s off and clarified by centrifugation at 9000 rpm at 4 °C for 30 min. The supernatant of cell lysate was employed as a crude enzyme in partition experiments (Shahbazmohammadi et al., 2019; Hirokawa et al., 2003).

### 2.3. Preparation of ATPS

ATPSs with final mass of 5 g were prepared by mixing the defined amounts of polymer (PEG-1000 or PEG-4000), salt ($Na_2CO_3$ or $K_2HPO_4$) and FPOX solution in 15 mL graduated tubes. Two-phase systems were mixed for 1 h and centrifuged at 3500 rpm at 25 °C for 50 min. Samples from both phases were removed and analyzed for FPOX activity and protein concentration. To avoid interference, samples were studied against blanks containing the same compositions, but without enzyme solution (Shahbazmohamadi and Omidinia, 2007).

### 2.4. Partition parameters

The efficiency of recombinant FPOX partition in ATPS was investigated using different parameters. Partition coefficient of enzyme ($K_E$) is specified as; $K_E = A_t/A_b$, where $A_t$ is the upper phase activity of enzyme, and $A_b$ is the correspondent value in the lower phase. Recovery ($R$%) is determined as; $R\% = A_t/A_{ori}$, where $A_t$ is the enzyme activity for top phase, and $A_{ori}$ is the initial enzyme activity for original sample. Also, Yield ($Y$%) is calculated as; $Y\ (\%) = 100V_tK_E/(V_tK_E + V_b)$, where $V_t$ and $V_b$ are the top and bottom phase volumes, respectively. Purification factor (PF) is determined by the ratio between the specific activity in the top phase and the specific activity in the initial sample (Hatti-Kaul, 1999).

**Table 1**
Variables and their levels employed in Plackett-Burman design for screening of parameters affecting on FPOX partition in ATPS.

| Variable code | Variable | Unit | Value | |
|---|---|---|---|---|
| | | | −1 | +1 |
| A | PEG concentration | % | 12.0 | 16.0 |
| B | PEG molecular weight | Da | 1000 | 4000 |
| C | Phase-forming salt type | – | $Na_2CO_3$ | $K_2HPO_4$ |
| D | Phase-forming salt concentration | % | 11.0 | 15.0 |
| E | pH | – | 7.0 | 8.0 |
| F | System buffer type | – | PBB | PBS |
| G | System buffer concentration | mM | 10.0 | 100.0 |
| H | Temperature | °C | 10.0 | 37.0 |
| J | Neutral salt type | – | NaCl | KCl |
| K | Neutral salt concentration | % | 2.0 | 5.0 |
| L | Crude enzyme Loading | % | 20.0 | 50.0 |

### 2.5. Enzyme activity and protein concentration

The activity of enzyme was assessed with the HRP-coupled reaction system. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 8.0), 3.0 mM substrate (Fru-ValHis), 0.45 mM 4-aminoantipyrine, 0.5 mM TOOS, 2 U/ml HRP and enzyme soultion in a total volume of 1 mL at 25 °C. The quinoneimine dye formation was measured spectrophotometrically at 555 nm. One unit of FPOX activity was defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute (Shahbazmohammadi et al., 2019). Protein concentration was determined according to the Bradford method (Bradford, 1976).

### 2.6. Enzyme purity analysis

The purity was analyzed by a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresis was run at 70 V and 15 mA for 4 h. The gel was stained with Coomassie brilliant Blue R-250 and then destained by diffusion in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid (Sambrook et al., 1994).

### 2.7. Design of experiments

The partition behavior of recombinant FPOX enzyme in ATPS was optimized using design of experiments methodology. Optimization strategy included the determination of the most significant components by PBD and evaluation of the optimal concentrations using BBD with RSM. Design Expert 11.1.0.1 software (State-Ease, Inc., USA) was used for the experimental design. 11 independent variables in ATPS system including PEG molecular weight, PEG concentration, phase-forming salt type ($Na_2CO_3$ and $K_2HPO_4$), phase-forming salt concentration, pH, system buffer type (potassium phosphate buffer (PPB) and phosphate buffer saline (PBS)), system buffer concentration, temperature, neutral salt type, neutral salt concentration, and crude enzyme loading were investigated using PBD. The response measured was the enzyme $K_E$. All variables were studied at two widely spaced levels. The low level (−1) and high level (+1) of each factor are listed in Table 1. The range of variables were defined based on our earlier experiences about the other oxidoreductases e.g. proline dehydrogenase (ProDH) (Shahbazmohammadi and Omidinia, 2013), phenylalanine dehydrogenase (PheDH) (Shahbazmohammadi and Omidinia, 2007), and glucose dehydrogenase and D-galactose dehydrogenase (Shahbazmohammadi et al., 2015). According to the Plackett-Burman experimental design, a total of 12 runs were performed and the effect of each variable was calculated by equation (1):

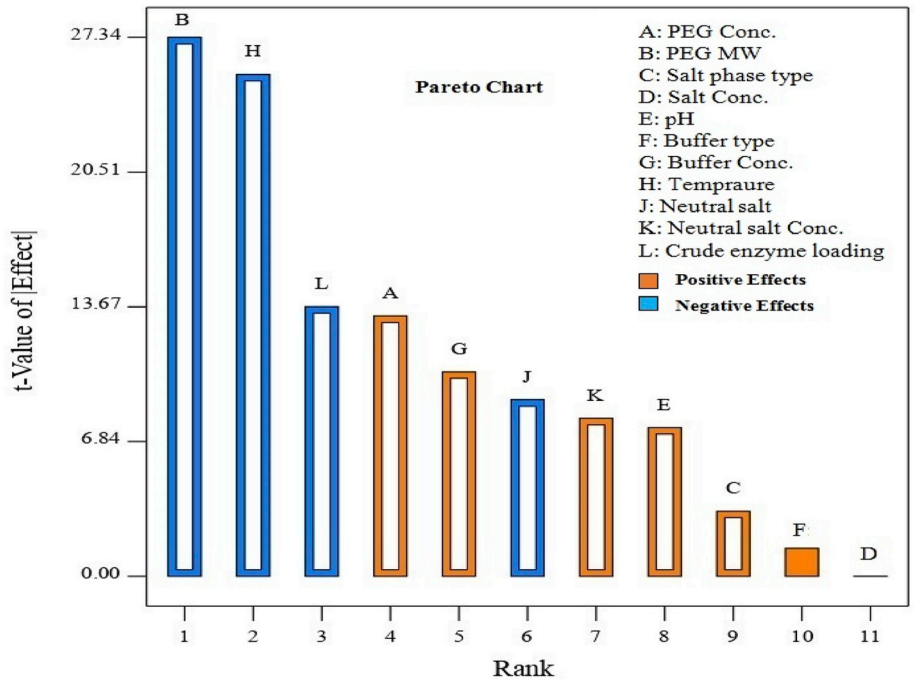


**Fig. 1.** Effect of different parameters on recombinant FPOX partition coefficient in ATPS based on Plackett-Burman experimental design (Y: main effects X: variables).

$$E(X_i) = 2\left(\sum M_i^+ - M_i^-\right)/N \quad \text{(Eq. 1)}$$

Where, E is the effect of the tested variable ($X_i$), $M_i^+$ and $M_i^-$ are responses of experiments ($K_E$) at which the factor is at its high or low levels, respectively and N is the number of trails. All the experiments were carried out in duplicate and the average value was reported as the final response. Since, PBD cannot describe interaction effects among the variables and their optimal levels; it was followed by BBD in RSM. The four variables (PEG concentration, pH, system buffer concentration, and neutral salt concentration), which showed positive and significant effects were studied at three levels (-1, 0, 1) and a set of 29 experiments was conducted (Table 3). The PEG molecular weight, phase-forming salt concentration, system buffer type, temperature and crude enzyme loading were set to be PEG-4000, 15.0% (w/w) $Na_2CO_3$, PPB, 37 °C and 20.0% in these experiments. The *R*, *Y* and PF were taken as the responses of the designed experiments. The significance of variables was studied by analysis of variance (ANOVA). Also, response surface plots were used to assess the visible relationships between the significant independent factors (Rahimpour et al., 2016; Muruchi and Jimenez, 2017; Pérez et al., 2015).

## 3. Results and discussion

### 3.1. Selection of significant factors by PBD

There are many important parameters affecting partition of proteins in ATPS including physicochemical properties and environmental conditions (Wanga et al., 2016; Lo et al., 2018). To elucidate the main factors included in modeling of recombinant FPOX partitioning, Plackett–Burman factorial design was performed. Fig. 1 contains a Pareto chart representing the estimated effects relative to PEG molecular weight, PEG concentration, phase-forming salt type, phase-forming salt concentration, pH, system buffer type, system buffer concentration, temperature, neutral salt type, neutral salt concentration, and crude enzyme loading. Pareto's chart presents each of the estimated effects and interactions, and the measure of each bar is proportional to the estimated effect. As shown in Fig. 1, PEG concentration (A), pH (E), system buffer concentration (G) and neutral salt concentration (K) had positive effects on $K_E$. In contrast, PEG molecular weight (B), temperature (H), neutral salt type (J), and crude enzyme loading (L) exhibited negative effects on $K_E$. Statistical analysis of Plackett-Burman experimental design for 10 variables is described in Table 2. Analysis of *p*-values showed that PEG concentration, neutral salt concentration, system buffer concentration and pH played significant roles in enzyme partition ($p < 0.05$). The PEG concentration (t-value = 13.02) is the main determinant of $K_E$, followed by system buffer concentration (t-value = 10.44), neutral salt concentration (t-value = 8.09), and pH (t-value = 7.1). PEG molecular weight demonstrated the most negative influence on $K_E$. Taking into account the best $K_E$, PEG-4000 was more favorable for the partitioning of desired enzyme. This behavior can be attributed to hydrophobicity influence. As PEG molecular weight increases, the hydrophobicity of the upper phase also increases (Xu et al., 2013). Since FPOX is a hydrophobic protein, this increment in hydrophobicity also drives its partition to the bottom phase. Similar behaviors have been in partitioning of other proteins such as human serum albumin (Garza-Madrid et al., 2010). The precision of a model can be checked by the adjusted coefficient of determination ($R^2$). The value of

**Table 2**
Statistical analysis of PBD for each variable affecting on FPOX partitioning.

| Variables | Coefficients | df | Standard error | *F*-value | *P*-value |
|---|---|---|---|---|---|
| Model | 778.67 | 9 | 0.1768 | 230.72 | 0.0043 |
| A-PEG concentration | 65.33 | 1 | 0.1768 | 174.22 | 0.0057 |
| B-PEG MW | 280.33 | 1 | 0.1768 | 747.56 | 0.0013 |
| C-Phase-forming salt | 4.08 | 1 | 0.1768 | 10.89 | 0.0809 |
| E-pH | 21.33 | 1 | 0.1768 | 56.89 | 0.0171 |
| G-System buffer concentration | 40.33 | 1 | 0.1768 | 107.56 | 0.0092 |
| H-Temperature | 243.00 | 1 | 0.1768 | 648.00 | 0.0015 |
| J-Neutral salt type | 30.08 | 1 | 0.1768 | 80.22 | 0.0122 |
| K- Neutral salt concentration | 24.08 | 1 | 0.1768 | 64.22 | 0.0152 |
| L-Crude enzyme loading | 70.08 | 1 | 0.1768 | 186.89 | 0.0053 |

**Table 3**
Experimental BBD matrix along with experimental and predicted values results for FPOX partitioning in ATPS.

| Run | $X_1$ (PEG, %.) | $X_2$ (pH) | $X_3$ (Buffer, %) | $X_4$ (NaCl, %) | *R* (%) | | *Y* (%) | | PF | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted |
| 1 | 12 | 7.5 | 55.0 | 5.0 | 88.3 | 92.20 | 77.4 | 81.40 | 21.30 | 27.38 |
| 2 | 14 | 7.5 | 100.0 | 2.0 | 90.1 | 90.61 | 92.3 | 87.20 | 31.30 | 28.30 |
| 3 | 14 | 7.5 | 100.0 | 5.0 | 101.5 | 99.84 | 94.5 | 91.60 | 38.90 | 34.64 |
| **4** | **14** | **8.0** | **55.0** | **5.0** | **106.8** | 105.48 | **95.1** | 94.08 | **42.40** | 40.15 |
| 5 | 14 | 7.5 | 10.0 | 2.0 | 89.5 | 89.38 | 86.1 | 84.59 | 30.20 | 31.25 |
| 6 | 12 | 8.0 | 55.0 | 3.5 | 91.4 | 90.26 | 87.6 | 84.82 | 32.10 | 30.39 |
| 7 | 14 | 7.0 | 10.0 | 3.5 | 90.8 | 95.41 | 85.4 | 89.91 | 34.50 | 35.25 |
| 8 | 16 | 7.5 | 100.0 | 3.5 | 84.2 | 84.18 | 74.6 | 78.77 | 27.30 | 27.86 |
| 9 | 16 | 7.5 | 55.0 | 2.0 | 79.5 | 79.65 | 71.4 | 71.85 | 24.10 | 21.78 |
| 10 | 14 | 7.0 | 55.0 | 2.0 | 91.9 | 90.96 | 80.4 | 81.38 | 28.30 | 30.00 |
| 11 | 14 | 8.0 | 100.0 | 3.5 | 97.3 | 96.73 | 94.4 | 94.33 | 33.40 | 36.42 |
| 12 | 16 | 8.0 | 55.0 | 3.5 | 82.6 | 84.09 | 73.5 | 73.67 | 27.10 | 26.72 |
| 13 | 14 | 7.5 | 55.0 | 3.5 | 99.5 | 96.20 | 91.2 | 86.28 | 32.50 | 32.02 |
| 14 | 14 | 7.5 | 10.0 | 5.0 | 106.1 | 103.81 | 94.8 | 95.49 | 37.50 | 37.29 |
| 15 | 12 | 7.5 | 10.0 | 3.5 | 88.5 | 86.26 | 85.5 | 81.30 | 31.20 | 30.08 |
| 16 | 16 | 7.0 | 55.0 | 3.5 | 87.5 | 86.86 | 79.5 | 77.87 | 28.50 | 27.00 |
| 17 | 14 | 7.5 | 55.0 | 3.5 | 94.8 | 96.20 | 86.5 | 86.28 | 32.50 | 32.02 |
| 18 | 12 | 7.5 | 55.0 | 2.0 | 77.5 | 79.46 | 68.4 | 73.20 | 22.20 | 22.65 |
| 19 | 12 | 7.5 | 100.0 | 3.5 | 84.4 | 85.20 | 75.1 | 77.87 | 26.30 | 25.43 |
| 20 | 14 | 8.0 | 55.0 | 2.0 | 91.5 | 89.95 | 86.2 | 86.58 | 24.50 | 26.61 |
| 21 | 14 | 7.5 | 55.0 | 3.5 | 95.5 | 96.20 | 85.3 | 86.28 | 27.80 | 32.02 |
| 22 | 16 | 7.5 | 55.0 | 5.0 | 88.5 | 90.58 | 79.3 | 78.95 | 26.10 | 29.42 |
| 23 | 14 | 7.0 | 100.0 | 3.5 | 93.5 | 94.45 | 84.1 | 85.23 | 24.50 | 29.05 |
| 24 | 14 | 7.0 | 55.0 | 5.0 | 99.8 | 99.10 | 89.6 | 89.18 | 31.50 | 28.83 |
| 25 | 14 | 7.5 | 55.0 | 3.5 | 95.5 | 96.20 | 81.4 | 86.28 | 32.20 | 32.02 |
| 26 | 16 | 7.5 | 10.0 | 3.5 | 88.9 | 85.85 | 79.4 | 76.60 | 28.50 | 28.81 |
| 27 | 12 | 7.0 | 55.0 | 3.5 | 85.4 | 82.13 | 75.1 | 70.52 | 25.00 | 22.17 |
| 28 | 14 | 7.5 | 55.0 | 3.5 | 95.7 | 96.20 | 87.0 | 86.28 | 35.10 | 32.02 |
| 29 | 14 | 8.0 | 10.0 | 3.5 | 95.4 | 98.50 | 87.6 | 90.91 | 36.60 | 35.82 |

**Table 4**
Results of ANOVA performed for the *R*, *Y* and PF of reduced models for FPOX.

| Response | Source | DF | SS | MS | *F*-value | *p*-value | |
|---|---|---|---|---|---|---|---|
| *R* of FPOX | Model | 14 | 1295.85 | 92.56 | 11.47 | <0.0001 | Significant |
| | Lack of fit | 10 | 98.90 | 9.89 | 2.81 | 0.1657 | Not Significant |
| | Pure error | 4 | 14.08 | 3.52 | | | |
| | Total | 28 | 1408.83 | | | | |
| *Y* of FPOX | Model | 14 | 1308.01 | 93.43 | 5.39 | 0.0016 | Significant |
| | Lack of fit | 10 | 193.21 | 19.32 | 1.56 | 0.3547 | Not Significant |
| | Pure error | 4 | 787.67 | 12.39 | | | |
| | Total | 28 | 1550.77 | | | | |
| PF of FPOX | Model | 14 | 555.09 | 39.64 | 3.15 | 0.0197 | Significant |
| | Lack of fit | 10 | 148.15 | 14.81 | 2.13 | 0.2422 | Not Significant |
| | Pure error | 4 | 27.788 | 6.94 | | | |
| | Total | 28 | 731.04 | | | | |

DF: degree of freedom, SS: sum of squares, MS: mean squares.

adjusted- $R^2$ was 0.9947, indicating a close agreement between the experimental results and the theoretical values predicted by the model. Based on the obtained results in PBD, phase-forming salt and its concentration, system buffer type, temperature, neural salt type and crude enzyme loading were kept at 15.0% (w/w) $Na_2CO_3$, PPB, 37 °C, NaCl salt and 20.0% (v/v), respectively in the following experiments. As a result, PEG concentration, pH, PPB concentration and NaCl concentration were chosen for further optimization by BBD in the remaining steps.

### 3.2. Analysis of BBD from RSM

For the development of a successful two-phase partitioning, RSM using BBD was used to optimize four variables that affected FPOX $K_E$. Experiments according to the design matrix of variables were carried out and the experimental and predicted results are shown in Table 3. A considerable similarity was observed between the predicted and experimental values reflecting the correctness of the models to predict the desired responses The best result was obtained with the ATPS composed of 14.0% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 5.0% (w/w) NaCl and 55.0 mM potassium phosphate buffer with pH 8.0. ANOVA was employed for the determination of significant variables and the all possible linear and quadratic interactions. The model determination coefficient ($R^2$) was calculated to be 0.91, 0.84 and 0.86 for *R*, *Y* and PF of recombinant FPOX, respectively. The $R^2$ is always between 0 and 1.0, and a value > 0.75 indicates suitability of the model (Kammoun et al., 2009). Table 4 lists the significant parameters and statistical test results of the models. As shown in Table 4, the quadratic effects of PEG concentration, PPB concentration, NaCl concentration and pH significantly ($p < 0.05$) affected the *R*, *Y* and PF of FPOX. The *F*-value parameter for *R*, *Y* and PF were obtained 11.47, 5.39 and 3.15, respectively, which imply the accuracy of the models. The significance of the *p*-value is also shown in Table 4. Larger values of *F*-value and smaller values of *p*-value exhibited that the variables would be more significant ($p < 0.05$). The Lack of fit test measures the failure of the model to represent experimental data in the experimental domain at point which are not included in regression analysis. Lack of fit was found to be non-significant (*p* value > 0.05) and it suggested that the model equation was adequate. Hence, ANOVA confirmed the suitability of *R*, *Y* and PF models and

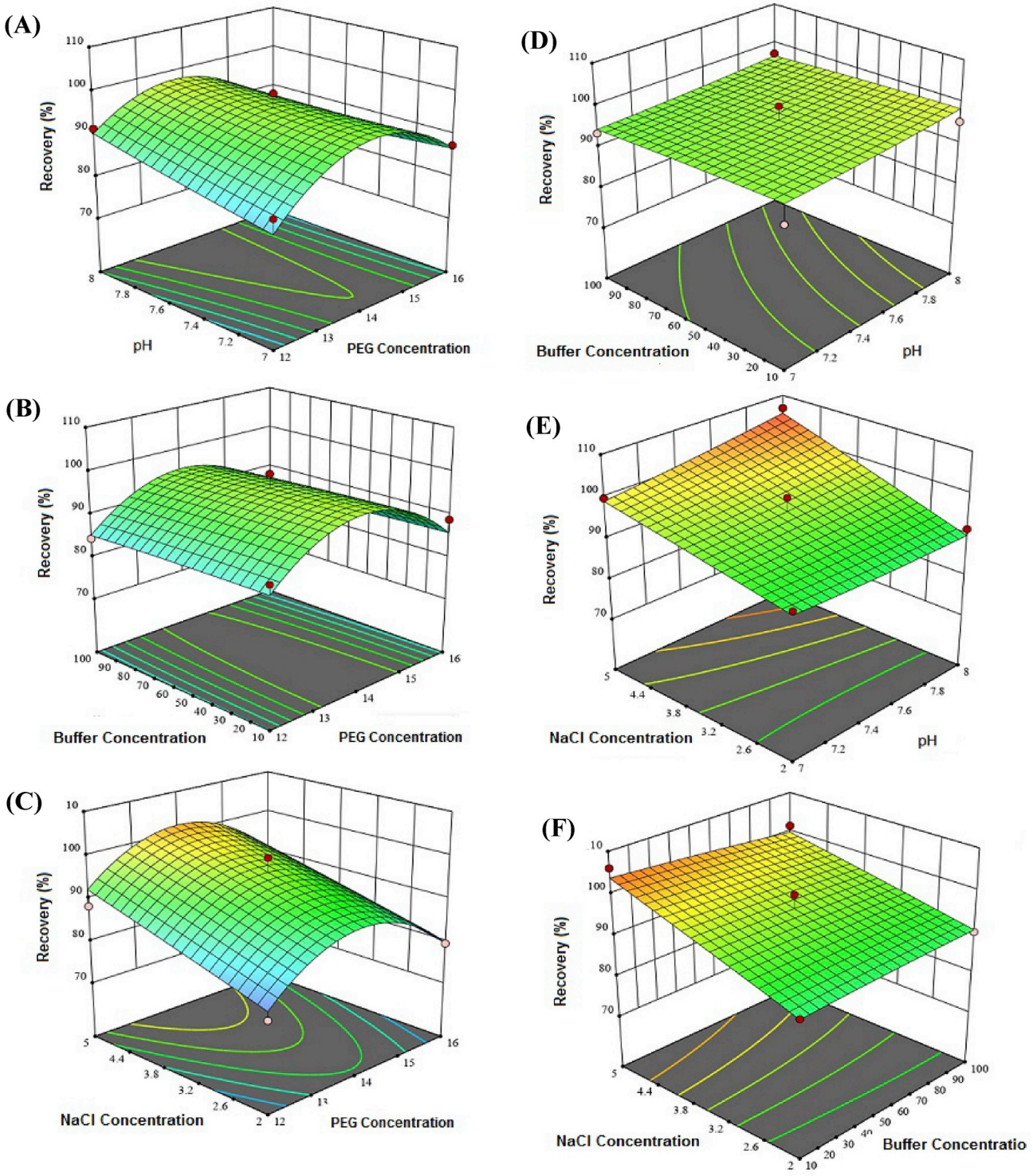


**Fig. 2.** Response surface plots for the interactive effect (A) PEG-4000 concentration and pH, (B) PEG-4000 concentration and buffer concentration, (C) PEG-4000 concentration and NaCl concentration, (D) pH and buffer concentration, (E) pH and NaCl concentration, (F) buffer concentration and NaCl concentration on *R* of FPOX.

exhibited that the four factors were very critical for partitioning of FPOX enzyme. The three-dimensional graphs were generated, in which the effect of four defined factors are shown. The response surface plots provide a method to visualize the relation between responses and experimental levels of each variable and the type of interactions between two test variables. Through the response surface plots, the interactions of the variables and the optimum level of each variable for maximum response can be well understood. The plots of *R* and *Y* for FPOX against different values of PEG concentration, pH, PPB concentration, and NaCl concentration are depicted in Figs. 2 and 3, respectively. It can be observed that the intermediate values of PEG-4000 concentration favored the *R* and *Y* for enzyme. The plots (Fig. 2 (A, B, C) and Fig. 3 (A, B, C)) exhibited a pronounced increase in *R* and *Y* of FPOX when the concentration of PEG was increased to intermediate value, and a slight decline as the concentration of PEG was decreased. Also, increasing of pH value from 7.0 to 8.0 resulted to increase in enzyme

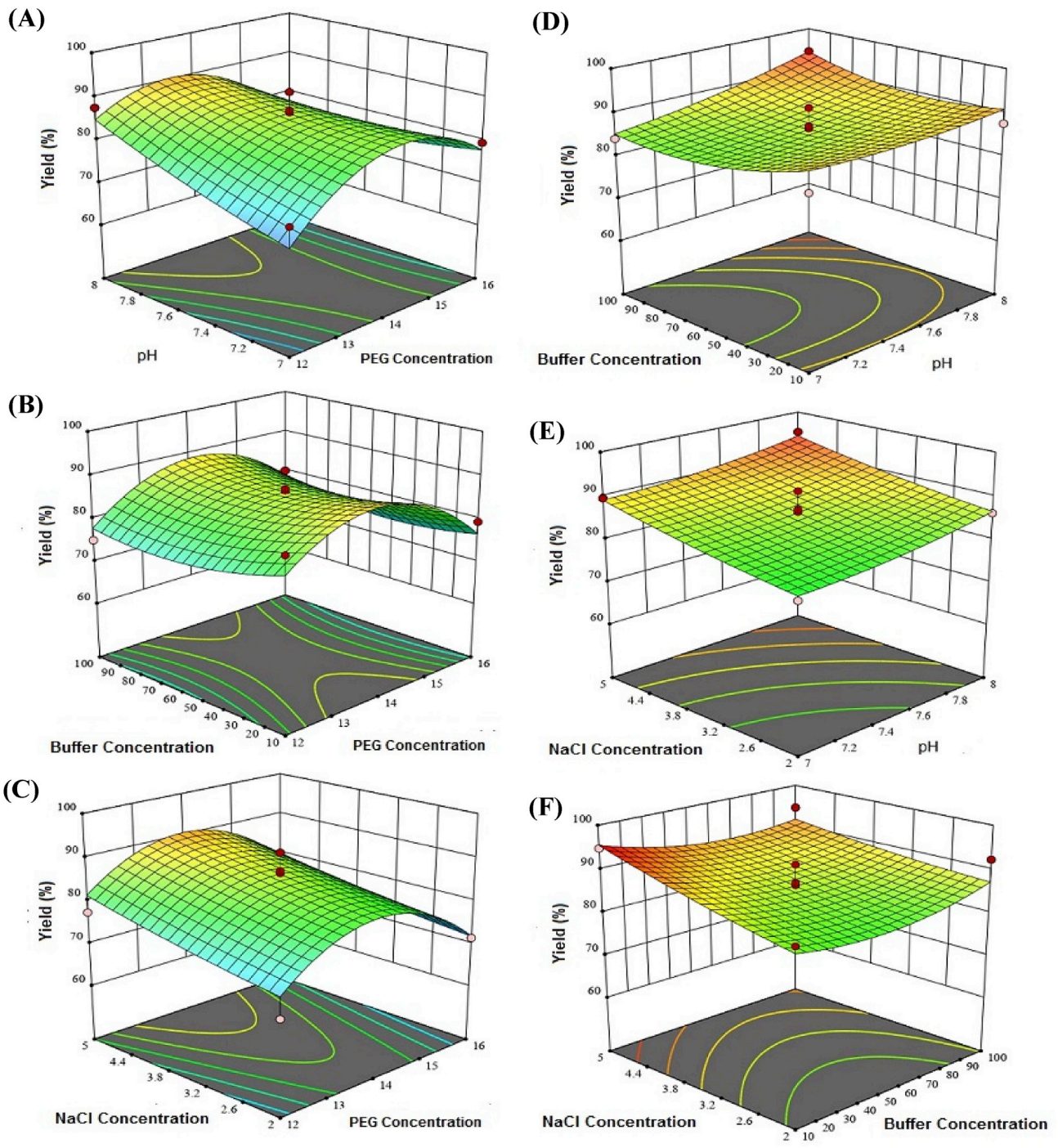


**Fig. 3.** Response surface plots for the interactive effect (A) PEG-4000 concentration and pH, (B) PEG-4000 concentration and buffer concentration, (C) PEG-4000 concentration and NaCl concentration, (D) pH and buffer concentration, (E) pH and NaCl concentration, (F) buffer concentration and NaCl concentration on *Y* of FPOX.

partitioning (Fig. 2 (A, D, E) and Fig. 3 (A, D, E)). The highest *R* and *Y* were obtained at pH 8.0. It could be attributed to the charge effect. FPOX was negatively charged protein (p*I* of *E. terrenum* FPOX is 6.68) at pH 7.0–8.0. With pH increasing, FPOX has more negative charge and PEG behaved as a positively charged molecule and thereby polyanions of the target enzyme were attracted by the PEG–rich phase. As a result, FPOX partitioned preferentially to the top phase. Our results were in accordance with the reported results for other enzymes such as lipase (Li et al., 2010) and lysozyme (Dembczynski et al., 2013). The pH of two-phase system was controlled using PPB. As observed in Fig. 2 (B, D, F) and Fig. 3 (B, D, F), system buffer concentration tends to fall along a straight line in surface plots. It means that the change of buffer concentration was not significant for both models. Based on the presented results in Table 3, 55.0 mM potassium phosphate buffer was selected as the maximum point. As illustrated in Fig. 2 (C, E, F) and Fig. 3 (C, E, F), inclusion of NaCl concentration increased significantly *R* and *Y* of FPOX. The best partitioning was obtained in ATPS containing 5.0% (w/w) NaCl. Neutral salts such as NaCl changes the phase diagram, alters the properties of the partitioning solute and produce electrical potential difference between two phases (Hatti-Kaul, 1999). Although adding salts have been reported to be beneficial to target molecules (Pérez et al., 2015), negative results have been obtained as well

**Table 5**
Experimefntal validation of model predicted values.

| | ATPS composition | Model | Experimental |
|---|---|---|---|
| *R* (%) | 14.22% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 4.91% NaCl and 56.43 mM PPB, pH 7.83 | 95.1 | 98.0 ± 0.57 |
| *Y* (%) | 14.22% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 4.91% NaCl and 56.43 mM PPB, pH 7.83 | 106.8 | 107.3 ± 1.02 |
| PF | 14.22% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 4.91% NaCl and 56.43 mM PPB, pH 7.83 | 44.6 | 42.4 ± 0.88 |

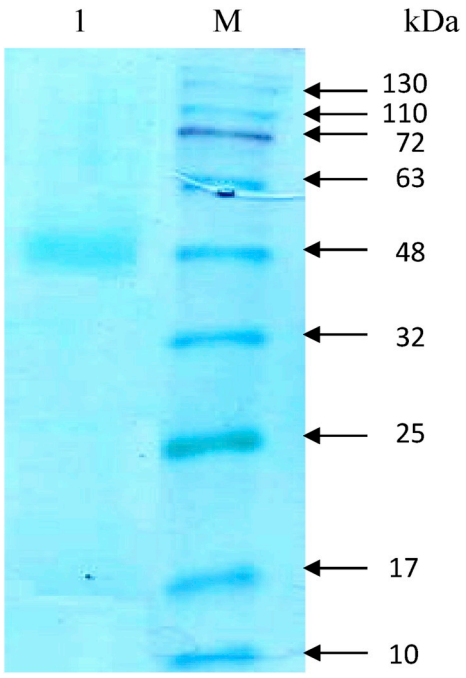


**Fig. 4.** SDS-PAGE analysis of the recombinant FPOX partitioned in ATPS. Lane M: protein molecular marker; Lane 1: top phase obtained from ATPS [14.0% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 5.0% NaCl, and 55.0 mM potassium phosphate buffer with pH 8.0].

(Shahbazmohammadi and Omidinia, 2013). In conclusion, these results revealed that PEG concentration, pH, and NaCl concentration were the most significant factors affecting FPOX partitioning compared to the other effects.

### 3.3. Experimental validation of models

Generally, it is important to consider the optimization of multiple response variables together by the desirability function (Grahovac et al., 2012). This function allows the superposition of response surfaces and permits the determination of the optimal point. To optimize the process with two or more output response it is helpful to utilize the desirability function. The optimum conditions for the selected variables were predicted using the desirability function. The maximum predicted *R*, *Y* and PF were achieved in 14.22% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 4.91% (w/w) NaCl and 56.43 mM potassium phosphate buffer with pH 7.83. Under these conditions, predicted values of *R*, *Y* and PF were found to be 107.3%, 98.9% and 44.60. To validate the predicted results, experiments were carried out under optimized conditions. It was found that the responses predicted by the models were close to the experimental data (Table 5).

### 3.4. Analysis of activity and purify of FPOX partitioned by ATPS

To support the partition results, an electrophoresis analysis was performed using samples extracted from the ATPS system (14.0% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 5.0% (w/w) NaCl and pH 8.0). As shown in Fig. 4, partitioned FPOX was observed as a single band in the upper PEG-rich phase. Recombinant *E. terrnum* FPOX had a molecular weight of approximately 48.0 kDa (Gan et al., 2015) and its specific activity was measured to be 201.23 ± 2.1 U/mg. These results suggest the experimental design can be a suitable tool for the optimization of two-phase partition of FPOX enzyme.

## 4. Conclusion

This study showed for the first time the potential application of ATPS as an attractive alternative for partitioning of recombinant FPOX. Design of experiments method was proved to be an effective tool in modeling and optimizing the best partition features. The influence of various partition factors on two-phase partitioning of FPOX was investigated and it was observed that PEG concentration, pH, and NaCl concentration play significant roles in enzyme partition behavior. An ATPS composed by 14.0% (w/w) PEG-4000, 15.0% (w/w) $Na_2CO_3$, 5.0% (w/w) NaCl and 55.0 mM potassium phosphate buffer with pH 8.0 was developed for recombinant FPOX recovery. Under the optimized condition, FPOX was purified with *R* of 106.8%, *Y* of 95.1%, PF of 42.4 and specific activity of 201.23 ± 2.1 U/mg. Our experimental results for enzyme activity and purity analysis also confirmed the validity of the proposed model.

## Conflicts of interest

The authors declare that no conflict exists and the study has not involved human participants or animals.

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