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Optimization of pancreatic trypsin extraction in PEG/citrate aqueous two-phase systems

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ABSTRACT

Enzyme extraction using aqueous two-phase systems (ATPS) has been increasingly used as a primary recovery technique which integrates the clarification, concentration and partial purification of important biomolecules from their natural source in a single step. The goal of this work was to optimize the extraction of trypsin from pancreas homogenate with polyethylene glycol and sodium citrate (PEG/NaCit) ATPS by using the tools of experimental design.

The variables NaCl concentration – added inert salt –, the top/bottom phase volume ratio – V_r – and the biomass loaded into the system – in percentage – were selected as the main factors in the trypsin extraction. The yield (%) and the purification factor of trypsin were considered the responses to be optimized.

The central composite design and the response surface analysis proved to be suitable tools for a quick and efficient study. As a result, the optimal extraction conditions in PEG3350/NaCit system were 3.34% wt/wt for NaCl concentration, a biomass load which represented 9.30% wt/wt of the total ATPS mass and 6.37 top/bottom volume ratio giving a purification factor of 2.55 and a yield of 99.7% in top phase.

Introduction

Pancreatic trypsin (TRP)[1] is a serine protease widely used for different purposes in food, detergent, leather, pharmaceutical and medical industries [1]. This enzyme is synthesized and stored as an inactive precursor, the trypsinogen, in the mammalian pancreas. Traditional methods isolate TRP from pancreas homogenates which also contain alpha-chymotrypsin (ChTRP), another serine protease with similar structural and functional properties. Both enzymes are the major protein components TRP (16%) and ChTRP (19%) in mammalian pancreas [2]. They are purified and isolated with high degree of purity from the rest of the pancreatic proteins through protocols that involve affinity and ionic exchange chromatography. These processes are expensive, time-consuming and are not easily scalable. Consequently, it is desirable to find fast and efficient methods able to replace totally or partially any stage of the current purification procedures.

Enzyme extraction with aqueous two-phase systems (ATPS) has been increasingly used as a primary recovery technique which allows integrating different unit operations such as clarification, concentration and partial purification of important biomolecules from their natural source in a single step [3,4]. These systems present several advantages such as high biocompatibility (70–80% water content), high resolution, low cost and easy scalability [5].

Aqueous two-phase systems (ATPS) are formed when two aqueous solutions of different polymers, or a polymer and an inorganic salt, are mixed above a critical concentration [5,6]. One of the phases results enriched in one polymer and the other phase results enriched either in the salt or in the second polymer. The partitioning behaviour of proteins in ATPS depends on several parameters such as molecular mass and concentration of polymers, type of inorganic salt and concentration, pH, and protein properties (molecular mass, conformation, isoelectric point and surface hydrophobicity). When these systems are used to extract a target protein from a crude extract, other factors such as: the phase volume ratio of the system; the addition of an inert salt (NaCl)

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[1] *Abbreviations used:* ATPS, aqueous two-phase systems; PEG, polyethylene glycol; NaCit, sodium citrate; TRP, trypsin; ChTRP, alpha-chymotrypsin; CCRD, central composite design; RSM, response surface methodology; BAPNA, α-*N*-benzoyl-dl-arginine-*p*-nitroanilide; NaCl, sodium chloride; SOR, significance of the regression; LOF, lack of fit.

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and; the biomass load also affect the protein partitioning and therefore, the purification performance.

In previous works [7], we found that polyethyleneglycol/sodium citrate (PEG/NaCit) ATPS could be employed as a viable and potentially useful tool for separating proteins instead of the conventional PEG/phosphate systems. The main advantages of PEG/NaCit systems are the biodegradability and non-toxicity of the citrate anion when comparing with the high eutrophication potential of phosphate ions [8] and its consequent negative impact in the environment. According to preliminary studies, systems formed by PEG (molecular mass 3,350) and NaCit pH 5.20 showed the best capability of isolating TRP from bovine pancreas. An optimization of purification performance of this system by statistical techniques would be desirable.

An experimental design indicates the way in which the randomization and statistical aspects of an experiment are to be carried out. Analysis of results of an experimental design allows establishing a causal relationship between the independent and dependent variables, thus extracting the maximum possible information with the minimum expenditure of resources. In the last years, statistical optimization designs have been successfully employed to enzyme extraction with ATPS [9–11], but at present, there is no report focusing on TRP isolation.

The goal of this work was to optimize the extraction of trypsin from pancreas homogenate with PEG3350/NaCit pH 5.20 ATPS by using the tools of experimental design.

The purification factor and the yield of TRP were selected as responses to be optimized. The factors: NaCl concentration, top/bottom volume ratio of the system and biomass load (pancreas homogenate) were selected as independent variables. A central composite design (CCRD) and the response surface methodology (RSM) were applied to optimize the process and to analyse the factors affecting the extraction process.

Materials and methods

Chemicals

Trypsin (TRP) from bovine pancreas, sodium citrate (NaCit), polyethylene glycols of average molecular mass 3,350 (PEG3350) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were purchased from Sigma Chem. Co. and used without further purification. All the other reagents were of analytical quality.

Determination of TRP enzymatic activity

Trypsin activity was determined with the substrate α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) using a method modified from Gildberg and Overbo [12]. BAPNA was used in the assay at a final concentration of 0.85 mM in buffer 100 mM Tris–HCl pH 8.20. The reaction was followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm (molar absorptivity of 10.50 M^{-1} cm^{-1}) for 4 min. The assay was performed at a constant temperature of 22 °C. The activity was calculated from the initial linear portion of the absorbance vs. time curve.

Determination of total protein

The content of protein was estimated by the Warburg and Christian method [13] based in differential absorption of protein/nucleic acids at 280 and 260 nm [14]. Systems with phase medium were considered as blank in each case. Absorbance due to turbidity was estimated and subtracted. Alternative colorimetric methods, such as Bradford, were not used due to the well-known interference of PEG.

All the measurements were performed using a double beam UV/Vis spectrophotometer Jasco V-550.

Preparation of pancreatic homogenate

Pancreas was removed from freshly killed cow and stored at −80 °C. The pancreatic tissue was thawed, cut in small pieces of approximately 1 g and washed exhaustively with buffer 50 mM Tris–HCl pH 8.20. Then, they were mixed with three volumes of washing solution in a Phillips Minimize HR-1364 600 W mixer for 5 min. The resultant homogenate (suspension) was centrifuged for 5 min at low rate (2500 rpm) to precipitate impurities. Then, the supernatant was filtered, thus obtaining the working extract. The trypsinogen activation to active TRP was carried out under alkaline conditions by adjusting the pH at 8.20 with 1 M Tris–HCl [15]. This homogenate was left at a constant temperature (8 °C) and small aliquots were taken to check the changes in the proteolytic activity with time. The activation process was deemed complete when TRP activity reached a stable maximum. Finally, the pH was adjusted to 3.00 with 1 mM HCl solution to prevent autocatalytic hydrolysis. Finally, the extract was aliquoted and frozen (−30 °C) until used.

Preparation of the aqueous two-phase systems

To prepare aqueous two-phase systems, stock solutions of the phase components, PEG3350 40% (wt/wt) and NaCit 25% (wt/wt) pH 5.20 were mixed according to the binodal diagram previously obtained in our laboratory [16]. Table 1 shows the total compositions of ATPSs used in this work. The desired pH of NaCit solution was adjusted by the addition of sodium hydroxide. Low-speed centrifugation was used after a thorough gentle mixing of the system components to speed up phase separation. Then, 1 g of each phase was mixed to reconstitute several two-phase systems in which the partition of pancreas homogenate was assayed. Systems containing sodium chloride (NaCl) were prepared by dissolving the solid salt directly into the systems.

Definition and estimation of parameters involved in the ATPS extraction

Partition coefficient of total protein (Kp_{TP}) is the ratio between the equilibrium concentrations of proteins at the top and bottom phases (C_T, C_B) respectively. It is calculated as follows:

$$Kp_{TP} = \frac{C_T}{C_B}$$

Note that the top phase is PEG-enriched while the bottom phase is NaCit-enriched.

Partition coefficient of trypsin (Kp_{TRP}) was calculated by the ratio between the enzyme activities in each phase (A_T, A_B):

$$Kp_{TRP} = \frac{A_T}{A_B} f$$

Table 1
Total system compositions for ATPS formed by PEG3350 and NaCit pH 5.20.

NaCl (% wt/wt)	PEG3350 (% wt/wt)	NaCit (% wt/wt)
Total concentrations		
Without	13.22	10.62
2.00	13.22	10.62
4.00	15.30	9.71
8.00	15.86	10.21

Table 2
Factors and value levels used in the central composite design.

Variables	Low value (−1)	Centre value (0)	High value (+1)
NaCl concentration (X_A, %)	2.00	4.00	6.00
V_r (X_B)	5.00	6.50	8.00
Biomass (X_C, %)	7.50	10.00	12.50

The correction factor (f), included in the above equation, considers the effect of phase composition on enzyme activity. It is calculated as the ratio between the activities (bottom/top) of reference solutions (of known concentration) of the enzyme in each phase.

Volume ratio (V_r) is defined as the ratio between top and bottom phase volumes (V_T, V_B):

$$V_r = \frac{V_T}{V_B}$$

Biomass load (%) was calculated as the ratio between the mass of pancreatic homogenate loaded and the total system mass (in percentage).

Percent yield ($Y_{TRP,Phase}$) of the trypsin in a given phase (either top or bottom), calculated as:

$$Y_{TRP,Phase}\ (\%) = \frac{A_{Phase}V_{Phase}}{A_{PH}V_{PH}} \times 100$$

where A_{Phase} and A_{PH} are the enzymatic activity in a given phase and in the starting pancreatic homogenate respectively; and V_{Phase} and V_{PH} are the corresponding volumes.

Purification factor ($PF_{TRP,Phase}$), was determined as the ratio between the specific activity of trypsin in a given phase (enzyme activity over total protein concentration, $A_{Phase}/C_{TP\ Phase}$) and the specific activity of trypsin in the starting pancreatic homogenate ($A_{PH}/C_{TP\ PH}$):

$$PF_{TRP,Phase} = \frac{(A_{Phase}/C_{TP\ Phase})}{(A_{PH}/C_{TP\ PH})}$$

The temperature was maintained constant at 22 °C and controlled within ±0.1 °C by immersing the glass tubes in a thermostatic bath. All the measurements were performed in triplicate. The mean values and their standard deviations were calculated.

Experimental design

A central composite design (CCD) was carried out to optimize the factors in TRP extraction and maximize purification factor and the yield simultaneously. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modelling and analysing systems where a response of interest is simultaneously influenced by several variables. In this work, the RSM was used to identify the region of the factor domain that include the optimum desired responses [17].

Three independent variables or factors, namely the *NaCl concentration* (X_A, % wt/wt) added as inert salt, the *volume ratio* V_r (X_B) and the *biomass load* (X_C, % wt/wt) were analysed according to previous studies [7]. The domain of each factor was selected in order to maintain the system integrity. Three different levels of each factor were considered with a central coded zero value (−1, 0, 1) (Table 2) and 17 experiments were run for this procedure. Yield and purification factor were analysed as response variables.

Data were analysed using analysis of variance (ANOVA) and the model significance was examined with Fisher's statistical test (F-test) by determining significant differences between sources of variation in experimental results, i.e., the significance of the regression (SOR), the lack of fit (LOF), and the coefficient of multiple determination (R^2). Parameters with less than 95% significance ($p > 0.05$) were excluded and added to the error term. The statistical design and the analysis were carried out using the Design Expert software, Version 6.1.10 for Windows.

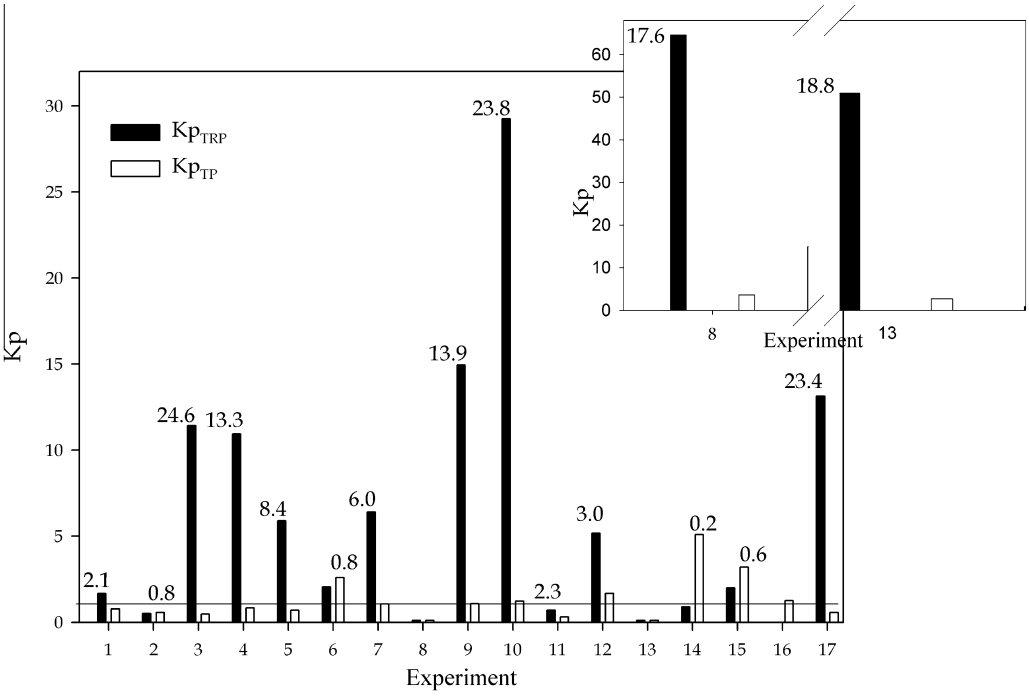

Fig. 1. Partition coefficient of TRP and TP in the experiments carried out. Temperature: 295 K. Total system compositions are those of Table 1. Numbers above the bars indicate the separating capability of ATPS in each experiment.

Table 3
Variables and responses of a central composite design carried out for trypsin purification from pancreas homogenate.

Run	Independent variables			Responses			
	X_A NaCl concentration (% wt/wt)	X_B V_r	X_C Biomass (% wt/wt)	$PF_{TRP,top}$	±	$Y_{TRP,top}$ (%)	±
1	4.00	6.50	10.0	8.85	0.19	68.8	2.0
2	4.00	5.00	7.5	0.99	0.01	64.0	0.3
3	4.00	6.50	10.0	2.42	0.01	93.2	2.3
4	2.00	5.00	10.0	1.61	0.01	94.8	1.4
5	2.00	8.00	10.0	11.88	0.58	96.9	1.5
6	6.00	8.00	10.0	4.61	0.16	75.1	1.4
7	4.00	8.00	7.5	5.06	0.14	97.4	1.4
8	4.00	8.00	12.5	1.25	0.02	84.1	2.6
9	4.00	5.00	12.5	1.52	0.01	64.5	0.4
10	4.00	6.50	10.0	0.95	0.01	70.9	3.9
11	6.00	5.00	10.0	1.37	0.01	73.2	1.5
12	4.00	6.50	10.0	0.94	0.01	86.5	1.7
13	6.00	6.50	12.5	0.68	0.01	6.2	0.1
14	6.00	6.50	7.5	4.44	0.03	97.8	0.6
15	2.00	6.50	12.5	2.06	0.01	97.4	2.8
16	2.00	6.50	7.5	0.86	0.01	72.7	0.5
17	4.00	6.50	10.0	1.84	0.02	72.2	3.0
(*)	3.34	6.37	9.30	2.55	0.02	99.7	0.1

(*) Optimal conditions.

Results and Discussion

Trypsin partition coefficient

Fig. 1 The experiments using pancreatic homogenate as biomass in ATPS revealed that TRP exhibited partition coefficients (Kp_{TRP}) greater than unity, while partition coefficients of total protein (Kp_{TP}) were smaller than one (Fig. 1). This trend indicates that TRP concentrates at the top phase (PEG-enriched phase), whereas nucleic acids – from cellular disruption – and proteins of high molecular weight are partitioned to the salt-enriched phase (bottom phase). As a consequence of this uneven behaviour, a high separating capability (Kp_{TRP}/Kp_{TP}) is observed in all the assayed systems, thus reaching values near to 20. The numbers above bars in Fig. 1 indicate the separating capability for each experiment.

Yield and purification factor were calculated for TRP in both the top and the bottom phases. However, the most significant purification parameters ($Y_{TRP,top}$ and $PF_{TRP,top}$) were obtained in the top phase in agreement with the uneven distribution of TRP and the high selectivity mentioned.

In a few ATPSs (see runs 2 and 9 of Table 3), a thin and translucent precipitate – corresponding to insoluble material such as debris of cellular membranes, proteins, organelles – appeared at the interface when biomass was loaded. This is the visual manifestation of a clarification process which takes place together with the protein partitioning. In this way, two unit operations – clarification and primary recovery – are integrated in a single step, thus decreasing costs and time consumption [18]. The balance mass calculated for these systems showed that approximately the 63% of total TRP was partitioned into the two-phases, suggesting that the remainder protein co-precipitated at the interface with the insoluble material.

Optimization of trypsin extraction. Validation of model

The factors with their levels and the results of the CCD experiments are listed in Table 3. The ANOVA (Table 4) confirms the suitability of the PF_{TRP} and Y_{TRP} models since they do not exhibit lack of adjustment ($p = 0.29$). The determination coefficients $R^2 = 0.93$ (for $Y_{TRP,top}$) and $R^2 = 0.79$ (for $PF_{TRP,top}$) show that most of the variations in the responses can be explained by the model.

Table 4
ANOVA for the central composite design carried out for trypsin purification from pancreas homogenate.

	Source	SS[a]	DF[b]	MS[c]	*F*-value	*P*-value
$PF_{TRP,top}$	Model	39.83	6	6.64	30.03	<0.0001
	A	8.77	1	8.77	39.66	<0.0001
	B	5.39	1	5.39	24.39	<0.0001
	C	21.50	1	21.50	97.24	<0.0001
	AB	0.30	1	0.30	1.34	0.25
	AC	7.55	1	7.55	34.16	<0.0001
	BC	6.36	1	6.36	28.76	<0.0001
	Residual	10.39	47	0.22		
	Lack of fit	7.22	30	0.24	1.29	0.29
	Pure error	3.17	17	0.19		
	Cor total	50.22	53			
	$R^2 = 0.79$; r^2 pred = 0.70; r^2 ajus = 0.77; Adeq precision = 22.47					
$Y_{TRP,top}$	Model	$3.035\ 10^4$	9	$3.372\ 10^4$	66.11	<0.0001
	A	$2.666\ 10^3$	1	$2.666\ 10^3$	52.27	<0.0001
	B	$5.864\ 10^3$	1	$5.864\ 10^3$	114.96	<0.0001
	C	$2.479\ 10^3$	1	$2.479\ 10^3$	48.60	<0.0001
	A^2	7.14	1	7.14	0.14	0.71
	B^2	$6.871\ 10^2$	1	$6.871\ 10^2$	13.47	0.01
	C^2	1.415 10	1	1.415 10	0.28	0.60
	AB	$4.276\ 10^2$	1	$4.276\ 10^2$	8.38	0.01
	AC	6.980 10	1	6.980 10	1.37	0.25
	BC	$4.005\ 10^2$	1	$4.005\ 10^2$	7.85	0.01
	Residual	$2.245\ 10^3$	44	51.02		
	Lack of fit	$1.607\ 10^3$	27	59.53	1.59	0.16
	Pure error	$6.373\ 10^2$	17	37.49		
	Cor total	$3.026\ 10^5$	53			
	$R^2 = 0.93$; r^2 pred = 0.90; r^2 ajus = 0.92; Adeq precision = 29.40					

[a] SS: sum of squares.
[b] DF: degree of freedom.
[c] MS: mean squares.

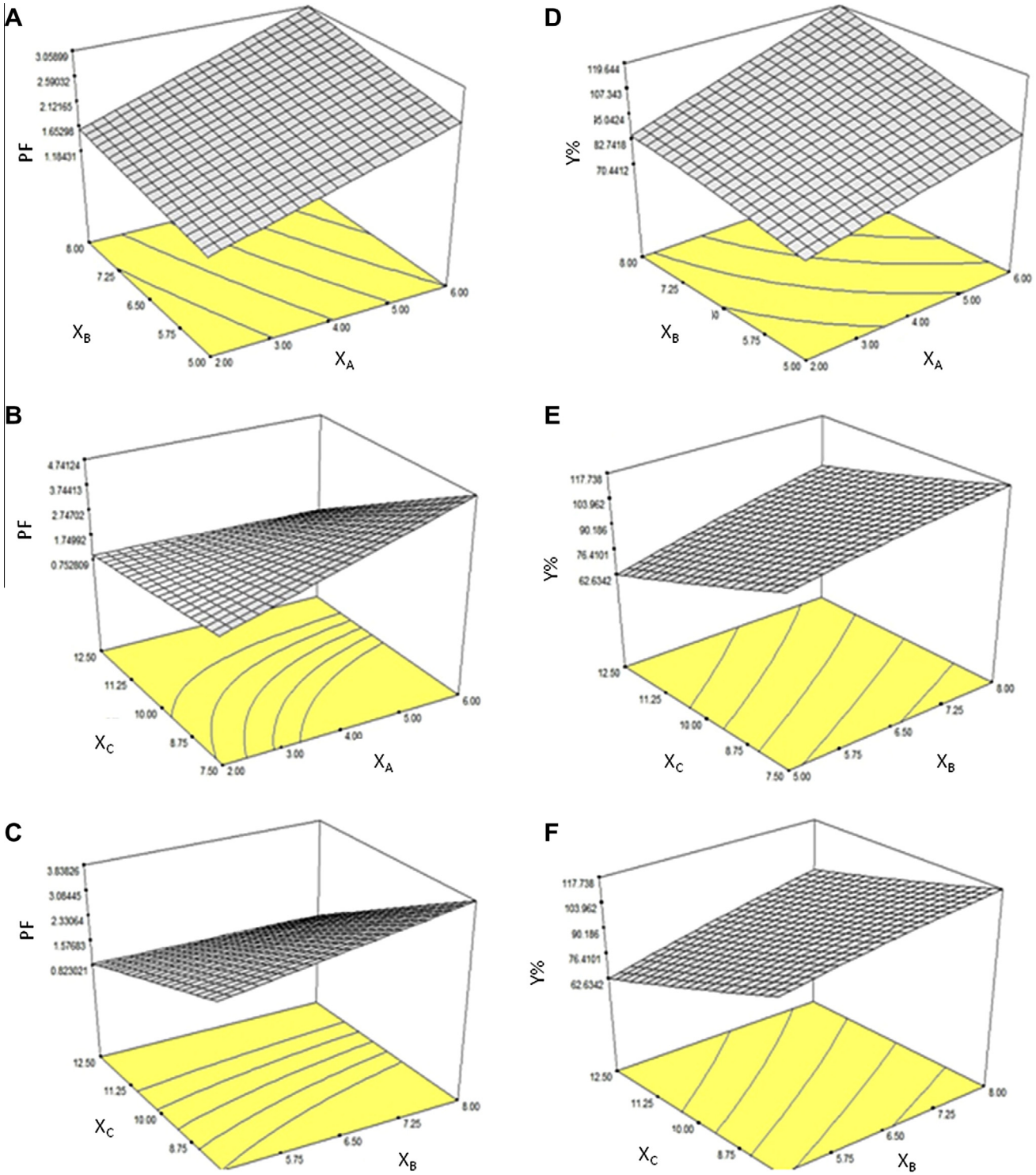

Fig. 2. Respone surface plots for the interactive effect of (A) V_r (X_B) and NaCl concentration (X_A), (B) biomass (X_C) and NaCl concentration (X_A), (C) biomass (X_C) and V_r (X_B), on PF_{TRP} and (D) V_r (X_B) and NaCl concentration (X_A), (E) biomass (X_C) and NaCl concentration (X_A), (F) biomass (X_C) and V_r (X_B), on Y_{TRP}.

Regression analysis

The *F*-test showed that in top phase, $PF_{TRP,top}$ and $Y_{TRP,top}$ were influenced by linear contributions of the studied factors and their interactions. $Y_{TRP,top}$ also presented a quadratic contribution of V_r factor (X_B). After eliminating the statistically insignificant terms (p-value > 0.05), the coefficients were adjusted and the models resulted to be:

$$PF_{TRP,top} = -6.13 + 1.82 * X_A + 0.87 * X_B + 0.59 * X_C - 0.015 * X_{AC} - 0.072 * X_{BC}$$

$$Y_{TRP,top}\ (\%) = 150.34 - 0.56 * X_A - 0.26 * X_B - 12.33 * X_C - 0.50 * X_B^2 + 1.19 * X_{AB} + 0.93 * X_{BC}$$

where *Xi* are the independent variables.

Response surface plot

Factors influencing trypsin extraction at ATPS are shown in Fig. 2 as response surface plots for a pair of factors by keeping the third factor constant at its middle level. Maximum $PF_{TRP,top}$ (3.05) was obtained at high level of NaCl concentration (X_A) and V_r (X_B) factors at a constant middle level of biomass (X_C) factor. Nevertheless, further increase of this purification parameter (4.74) is observed at high level of factor X_A, low value of factor X_C, X_B being at its centre value. Similar trends were observed for $Y_{TRP,top}$.

Extraction of trypsin with PEG3350/NaCit aqueous two-phase system

According to the previous results, the optimised conditions, predicted by the software, for the extraction of trypsin with ATPS, occur when NaCl concentration, the biomass% and V_r are 3.34% wt/wt, 9.30% and 6.37 respectively giving theoretical $PF_{TRP,top}$ and $Y_{TRP,top}$ values of 2.3 and 97% respectively. Under the mentioned conditions, a purification factor of 2.55 and a yield of 99.7% were obtained experimentally. This confirms the robustness of the model used and suggests that the experimental design using RSM is a suitable tool for a quick and efficient study on the optimization of experimental conditions for trypsin extraction.

Conclusions

Aqueous two-phase extraction of trypsin from bovine pancreas in PEG/NaCit system was studied by statistical analysis. The integration of two methodologies such as the central composite design and the response surface analysis proved to be adequate to design and optimize the bioprocess. It was possible to determine the optimum extraction conditions of trypsin with a reduced number of experiments. A good compromise between the purification factor and the yield was achieved within the experimental domain investigated. A purification factor of 2.55 and a yield as high as 99.7% was obtained for trypsin in a single step which integrates clarification and primary recovery. Further chromatography techniques, could be easily coupled to the ATPS extraction if a high quality product were desirable.

Acknowledgments

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