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Aqueous two-phase systems extraction of α-toxin from *Clostridium perfringens* type A

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Abstract

Two sequential half-fraction designs were applied to studying the α-toxin partition produced by *Clostridium perfringens* type A in aqueous two phase systems (ATPS), as a function of four factors: PEG molar mass and concentration, phosphate concentration and pH. The highest purification factor, yield and partition coefficient results were obtained with PEG 8000 (15%, w/w), phosphate at 20% (w/w) and pH 8.0. This system allows, in a single step, an α-toxin purification of 4.6-fold with final activity yield of 230% and partition coefficient of 113.9 in the PEG rich phase.

Keywords: Aqueous two-phase system; PEG/phosphate; α-Toxin; Fractional design; Purification

1. Introduction

The *Clostridium perfringens* α-toxin is an important phospholipase C (EC 3.1.4.3) that plays a role in the pathogenesis of several diseases, such as Crohn's disease in humans and enteritis in domestic animals [1–3]. This α-toxin is also particularly associated with gas gangrene [2], against which, however, immunization with the α-toxin toxoid is effective [1].

Toxoid vaccines can be prepared from crude culture filtrates of *C. perfringens* type A. These vaccines have been shown to induce protection against experimental gas gangrene, but there is a recurring problem with the preparation of a toxoid with immunogenicity [2]. The α-toxin must be purified, or the vaccine will not be immunogenic. Several purification methods have been proposed, all using traditional separation techniques, such as chromatography and ammonium sulfate precipitation [4].

Aqueous two-phase systems (ATPS) have also been extensively used in pharmaceutical research for extraction, separation and purification of various biomolecules [5–10]. Several advantages are reported, including low cost, good reproducibility, easy scale-up and recycling of the phases [11].

Partitioning of proteins in ATPS depends mainly on the physico-chemical properties of the protein, such as its isoelectric point, surface hydrophobicity, molar mass, and on the medium variables like polymer molar mass, pH, added salt type and concentration. By controlling these factors, one can expect to selectively partition and recover a target protein from the culture medium [8].

Statistical design of experiments is a very widely used tool for process optimization and control. The multivariate design of experiments is a very efficient method for studying the influence of a certain number of variables on a given response of interest. The significant factors and their effects can be studied with fewer numbers of run using fractional factorial runs. With the proper design, the significant effects can be identified from a minimum number of experiments lowest operation cost [12,13].

In this work a fractional two-level factorial design was used to study the partition behavior of the *C. perfringens* α-toxin in a PEG-phosphate ATPS. Three responses (partition coefficient, purification factor and yield) were analyzed as a function of the levels chosen for pH, PEG molar mass, and PEG and phosphate concentrations.

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2. Materials and methods

2.1. Chemicals

Crude α-toxin from *C. perfringens* Type A (type I- P7633), poly(ethylene glycol) PEG 400, 3350, 6000, 8000, 10,000 g/mol, dibasic potassium phosphate (K_2HPO_4), monobasic sodium phosphate (NaH_2PO_4), Coomassie blue G-250, bovine serum albumin (BSA) and ρ-nitrophenyl-phosphorylcholine (ρNPPC) were obtained from Sigma Chemical Co (St. Louis, Mo, USA). All other chemicals were of analytical grade.

2.2. α-Toxin preparation

The α-toxin from *C. perfringens* Type A was diluted in 25 mM Tris–HCl pH 7.2 buffer to a final concentration of 9.9 U/ml activity and 150 μg/ml protein concentration.

2.3. Preparation of aqueous two-phase systems

A phosphate concentrated solution (40%, w/w) was prepared at different pH values (6.0, 7.0, 7.5, 8.0 and 8.5) by mixing appropriate amounts of solutions of sodium monobasic phosphate (NaH_2PO_4) and potassium dibasic phosphate (K_2HPO_4) at room temperature (25 ± 2 °C). The required amounts of each of these solutions were mixed with a 50% (w/w) PEG solution and an α-toxin solution representing 20% (w/w) of total mass, in 15 ml graduated tubes with conical tips. Water was added to a final amount of 10 g. After vortex shaking for 1 min, the two phases were separated by decantation (60 min). Then, the phase volumes were measured; top and bottom phases were withdrawn separately with pipettes and assayed for protein concentration and phospholipase activity.

2.4. Phospholipase C activity

The enzymatic activity was determined in both phases, by an assay based on the method described by Kurioka and Matsuda [14]. The substrate solution was prepared by mixing 60% (w/v) sorbitol, 1 mM $ZnCl_2$ and 50 mM ρ-nitrophenyl-phosphorylcholine (ρNPPC) in 25 mM Tris–HCl at pH 7.2. This solution was transferred to a glass tube (225 μL) and incubated for 15 min at 37 °C. Samples of the phases (75 μL) were then incubated at 37 °C under continuous agitation for 1 h. The rate of NPPC hydrolysis by phospholipase C was monitored through the ρ-nitrophenol absorbance at 415 nm. The molar extinction coefficient of ρ-nitrophenol in 60% sorbitol solution buffered by Tris–HCl (pH 7.2) was 1.51×10^4 mmol/min.

2.5. Protein determination

Total protein concentration was determined according to the Bradford method [15], using bovine serum albumin as the standard protein.

Table 1
Variable levels in the first 2^{4-1} design

Variables	Level		
	Lower (−1)	Central (0)	Higher (+1)
PEG molar mass (g/mol)	400	3350	8000
PEG concentration (%, w/w)	15	20	25
pH	6.0	7.0	8.0
Phosphate concentration (%, w/w)	15	20	25

Table 2
Variable levels in the second 2^{4-1} design

Variables	Level		
	Lower (−1)	Central (0)	Higher (+1)
PEG molar mass (g/mol)	6000	8000	10000
PEG concentration (%, w/w)	10	15	20
pH	7.5	8.0	8.5
Phosphate concentration (%, w/w)	15	20	25

2.6. Experimental design and statistical analysis

The influence of the variables molar mass of PEG (MMPEG), PEG concentration (CPEG), between 10 and 25%, phosphate concentration (CPHOSPH), between 15 and 25%, and pH on the three responses of interest was evaluated from the results obtained with two successive 2^{4-1} fractional factorial designs [12], augmented with a central point, which was run in replicate to allow estimation of experimental error (Tables 1 and 2). All statistical and graphical analyses were carried out with the Statistica 6.1 program [16].

2.7. Determination of partition coefficient, purification factor and yield

The α-toxin partition coefficient (K) was calculated as the ratio of the volumetric activity in the two phases,

$$K = \frac{A_t}{A_b}$$

where A_t and A_b are the enzyme activities in units/mL in the top and bottom phases, respectively.

The purification factor (P_f) was given by the ratio of the specific activity in the top phase to the specific activity in the crude extract:

$$P_f = \frac{A_t/C_t}{A_i/C_i}$$

where A_t and A_i are the enzyme activities in units/ml in the top phase and in the initial extract (before partition), respectively, and C_t and C_i are the total protein concentrations, in μg/mL, of the top phase and the initial extract, respectively.

The α-toxin yield (Y) was determined by the ratio between the total activity in the top phase and the total activity in the initial extract:

$$Y = \left(\frac{AVt}{A_i V_i}\right) \times 100,$$

Table 3
Results of the first 2^{4-1} design

Run	MMPEG	CPEG%	pH	CPHOSPH%	*K*	*Y*	P_f
1	400	15	6	15	–	–	–
2	8000	15	8	15	24.9	62.5	1.4
3	400	25	8	15	28.0	56.7	0.63
4	8000	25	6	15	3.85	29.9	2.07
5	400	15	8	25	114	80.3	1.34
6	8000	15	6	25	51.8	79.4	1.55
7	400	25	6	25	2.5	1.78	0.04
8	8000	25	8	25	22.2	68.9	1.6
9	3350	20	7	20	2.3	80.37	1.97
10	3350	20	7	20	3.56	72.9	1.79
11	3350	20	7	20	4.68	36.0	0.94

K, partition coefficient; *Y*, activity yield; P_f, purification factor as responses; MMPEG, PEG molar mass; CPEG%, PEG concentration; CPHOSPH%, phosphate concentration as variables studied. Formation of two phases was not observed in run no. 1.

Table 4
Contrast values calculated for the first 2^{4-1} design

Variables	Contrast estimate		
	K	P_f	*Y*
(1) MMPEG	**36.26 ± 1.49**	0.15 ± 0.68	−4.77 ± 29.65
(2) CPEG	**13.13 ± 1.58**	−0.98 ± 0.72	−46.51 ± 31.34
(3) CHOSPH	**80.13 ± 1.58**	−0.89 ± 0.72	−9.94 ± 31.34
(4) pH	**79.40 ± 1.58**	−0.67 ± 0.72	9.08 ± 31.34
12 + 34	**−38.49 ± 1.58**	1.34 ± 0.72	24.94 ± 31.34
13 + 24	**−57.49 ± 1.58**	0.73 ± 0.72	37.85 ± 31.34
14 + 23	**−83.72 ± 1.58**	0.36 ± 0.72	1.95 ± 31.34

K, partition coefficient; *Y*, activity yield; P_f, purification factor as responses; MMPEG, PEG molar mass; CPEG%, PEG concentration; CPHOSPH%, phosphate concentration as variables studied. Three-factor interactions are not shown. The errors are estimated from the replicate runs at the central point. Statistically significant values (95% confidence level) are shown in boldface. The error is descriptive around of the contrast estimate, after the symbol ±.

where *A* and A_i are the enzyme activities, in units/mL, in the top phase after extraction and in the initial medium (before extraction), respectively; V_t and V_i are the top phase and initial volumes.

3. Results and discussion

3.1. First 2^{4-1} design

The results obtained in the first half-fraction design are shown in Table 3. Since this is a resolution IV screening design, its analysis results in contrasts where the main effects are confounded with the three-factor interactions, while the two-factor interactions are confounded with each other. As is well-known, a factoring model can be interpreted as modeling a given response by a special kind of power series where higher-order terms are assumed to be less important than those of a lesser order, otherwise the series would not converge, and the model would be useless [12]. It is natural, therefore, to assume that third-order terms be much less important than first-order ones, and, in a first approximation at least, may be neglected.

The contrast values calculated for this design are given in Table 4. Interpreting these values, however, requires much care, because the calculations do not include the results from run no. 1, which did not form a two-phase system. Only the contrasts for the partition coefficient (*K*) are statistically significant, at the 95% confidence level. For the other two responses the contrasts resulting from changing the levels of the variables are indistinguishable from experimental error. Assuming that third-order interactions are negligible, as discussed above, the results for the partition coefficient would be interpreted as follows. All four variables have very significant positive main effects on the *K* values, indicating that raising any one of them from their lower to their higher levels will on average increase the value of the partition coefficient. This influence appears to be most significant for phosphate concentration and pH, and less so for the PEG concentration. Moreover, the two-factor interaction contrasts are all highly significant and have negative algebraic signs. This indicates, at least, that the (positive) effect of a certain variable on the partition coefficient tends to be lessened if the other variables have their levels raised at the same time. In short, this is a quite complex situation, best visualized in the cubic plot of Fig. 1, where the observed responses are displayed against the level combinations of the four variables. The two highest *K* values (114.0 and 51.9), are located on the top face of the cube, which corresponds to 25% phosphate, but are obtained with opposite level combinations for MMPEG and pH: (400 g/mol, 8) and (8000 g/mol, 6), respectively. Both, however, were obtained with 15% CPEG.

Salts can change the electrostatic charge of ATPS and influence the distribution of charged amino acids [17] or proteins. Generally, phosphate ions shift to the bottom phase (i.e. dextran or salt rich phase), which has a higher density, and the bottom phase thus becomes more negative than the top phase (i.e. PEG rich phase), which is less dense [18]. Most proteins with isoelectric points in the acidic region, and consequently negative surface

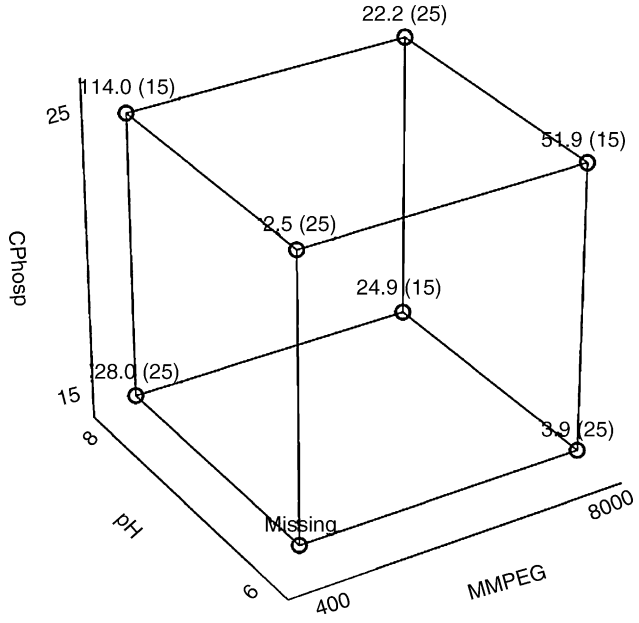

Fig. 1. Cubic plot of the partition coefficient values obtained in the design of Table 1. The values in parentheses are the PEG concentration values.

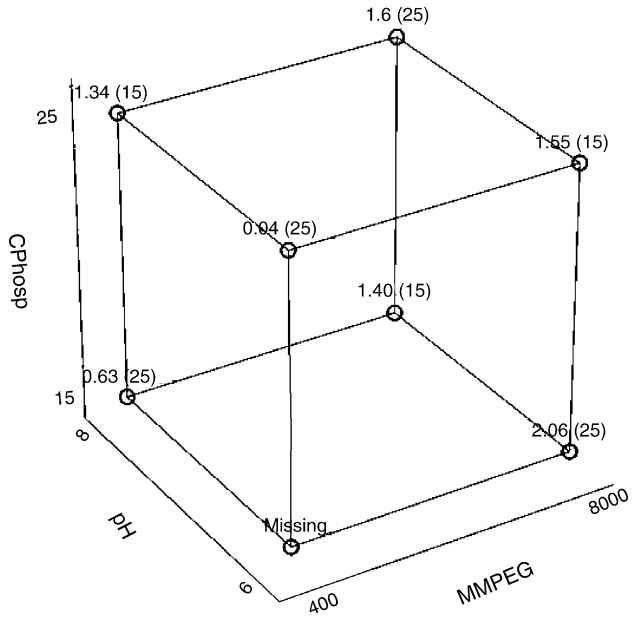

Fig. 2. Cubic plot of the purification values obtained in the design of Table 1. The values in parentheses are the PEG concentration values.

charges in the neutral pH region, like the alpha toxin, move to the top positive phase and the partition coefficient increases around pH 7 by increasing the concentration of phosphate ions [18]. With an increase in CPHOSP, negatively charged proteins prefer the PEG-rich phase, because of the repulsion force caused by salt anions. This phenomenon was observed by Han and Lee [18], in a study of bovine serum albumin partition in PEG-phosphate ATPS. They also observed that neutral protease from *Bacillus subtilis* moved to the bottom phase as phosphate concentration increased, because of its positive charge at neutral pH.

Several authors, such as Fernandez Lahore [19], who studied partition of the acid protease produced by *Mucor bacilliformis* in PEG – phosphate ATPS, and Farrugia [20], who studied bovine serum albumin – PEG interaction to predict the protein partitioning in ATPS, agree that there seems to be a relation between the partition coefficient (K) and MMPEG, with higher molar masses leading to an increase of the exclusion effect and a decrease in K. A possible explanation is that an increase in polymer molar mass decreases the available amount of solvent in the PEG-rich phase needed to solubilize the enzyme and the salt, as argued by Li and Peeples [21] in a study of amylolytic (MJA1) enzyme purification using PEG-phosphate ATPS. Moreover, in aqueous solution PEG acts as a highly mobile molecule with a large exclusion volume. Although this exclusion effect increases with molar mass, PEG of higher molar mass are able to form intramolecular bonds, thus acquiring a more compact conformation [8].

These results for the partition coefficient are of course not negligible, but the purification factor (P_f) is considered a far more important response for this study, because the toxin is being purified for vaccine production purposes. Though the calculated contrasts for P_f have not been found statistically significant, an interpretation of the patterns of the purification results themselves may be attempted, with the help of the cubic plot shown in Fig. 2. Considering first the MMPEG effect, it is clear that

Table 5
Results of the second 2^{4-1} design

Run	MMPEG	CPEG%	pH	CPHOSPH %	K	Y	P_f
1	6000	10	7.5	15	28.0	173.6	4.35
2	6000	10	8.5	25	114.0	146.8	2.7
3	6000	20	8.5	15	200.8	250.0	4.7
4	6000	20	7.5	25	102.7	128.0	2.0
5	10000	10	8.5	15	16.3	156.53	4.5
6	10000	10	7.5	25	178.5	258.4	3.2
7	10000	20	7.5	15	236.3	199.6	3.9
8	10000	20	8.5	25	18.4	59.1	1.0
9	8000	15	8.0	20	119.2	215.7	4.5
10	8000	15	8.0	20	104.1	195.4	3.6
11	8000	15	8.0	20	117.4	212.5	5.5
12	8000	15	8.0	20	115.0	297.0	4.7

K, partition coefficient; Y, activity yield; P_f, purification factor as responses; MMPEG, PEG molar mass; CPEG%, PEG concentration; CPHOSPH%, phosphate concentration as variables studied.

the P_f values on the right face of the cube, which corresponds to MMPEG = 8000 g/mol, are all larger than the corresponding values on the left face (MMPEG = 400 g/mol), indicating that raising MMPEG is likely to improve purification. For the other factors the patterns are less clearcut, and there is indication of considerable interaction effects. The only thing the four largest P_f values, which are those on the right face, have in common is a high MMPEG value. For the other variables, they correspond to very different level combinations: (25%, 15%, 6), (15%, 15%, 8), (15%, 25%, 6) and (25%, 25%, 8), for CPEG, CPHOSPH, and pH, respectively. Also, a purification value almost as large (1.34) is found on the left face (that is, with MMPEG = 400), and with (CPEG, CPHOSPH, pH) = (15%, 25%, 8).

No contrast for the yield activity (Y) is statistically significant, at the 95% confidence level. Nevertheless, the values indicate that decreasing MMPEG (1), CPEG (2), CPHOSPH (3) and increasing pH (4) might result in a higher yield activity value.

Taking all these considerations into account, we decided to further investigate the experimental region in the vicinity of the first design, but shifting the MMPEG to higher levels, and modifying slightly the range spanned by the other factors according to a second 2^{4-1} design, whose levels are specified in Table 2.

3.2. Second 2^{4-1} experimental design

The results obtained in the second design are shown in Table 5. The contrasts calculated from them are given in Table 6. In this design they can be interpreted in the usual way, as no response is missing.

For the partition coefficient, all calculated contrasts are highly significant, except those corresponding to the MMPEG main effect and to the sum of interactions 13 and 24. Assuming that third- and higher-order interactions are negligible, these results mean that, on average, the value of K increases when CPEG is raised, but decreases when the same is done to pH and phosphate concentration. However, these factors act in a highly interdependent way, as indicated by the values of the contrasts 12 + 34 and 14 + 23, the last one being the most significant value of all. Since these interactions are both negative, the effect of a given factor

Table 6
Contrast values calculated for the second 2^{4-1} design

Variables	Contrast estimate		
	K	P_f	Y
(1) MMPEG	1.00 ± 4.79	−0.29 ± 0.55	−6.19 ± 32.14
(2) CPEG	**55.35 ± 4.79**	−0.79 ± 0.55	−24.66 ± 32.14
(3) CPHOSPH	**−16.95 ± 4.79**	**−2.14 ± 0.55**	−46.86 ± 32.14
(4) pH	**−49.00 ± 4.79**	−0.14 ± 0.55	−36.79 ± 32.14
12 + 34	**−25.40 ± 4.79**	−0.61 ± 0.55	−53.46 ± 32.14
13 + 24	−10.90 ± 4.79	0.04 ± 0.55	27.54 ± 32.14
14 + 23	**−141.05 ± 4.79**	−0.66 ± 0.55	−84.39 ± 32.14

K, partition coefficient; Y, activity yield; P_f, purification factor as responses; MMPEG, PEG molar mass; CPEG%, PEG concentration; CPHOSPH%, phosphate concentration as variables studied. Three-factor interactions are not shown. The errors are estimated from the replicate runs at the central point. Statistically significant values (95% confidence level) are shown in boldface. The error is descriptive around of the contrast estimate, after the symbol ±.

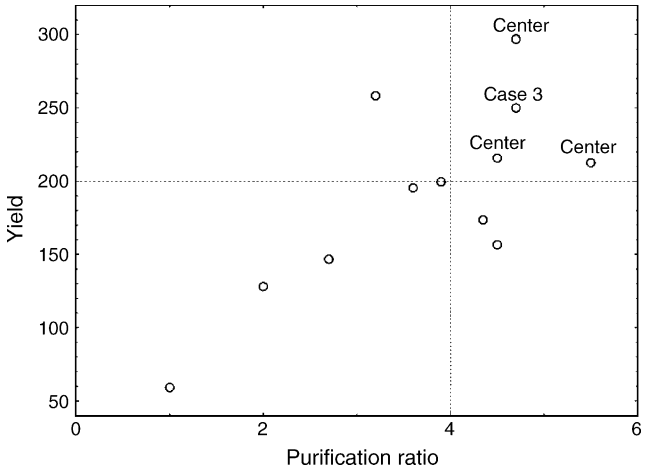

Fig. 4. Yields as a function of purification factor for the second design.

tends to become more negative (or less positive) when the level of another factor is raised at the same time. A similar behavior was observed by Costa [22] in a study of xylanase extraction in PEG – phosphate ATPS. These authors reported that the effect of pH was statistically significant, while the effect of MMPEG was not significant. Once again, due to the complex interaction effects these values would best be interpreted with the help of a cubic plot, but we shall instead proceed to analyze the most important response, the purification factor. In this second design, only the main effect of CPHOSPH is statistically significant. Because its value is negative, raising the phosphate concentration from 15 to 25% decreases the P_f value, on average. This is illustrated in the cubic plot of Fig. 3, where the P_f values on the top face, which correspond to 25% phosphate, are systematically lower than the corresponding values on the bottom face (15% phosphate). As in the first design, purification factor values of the same order

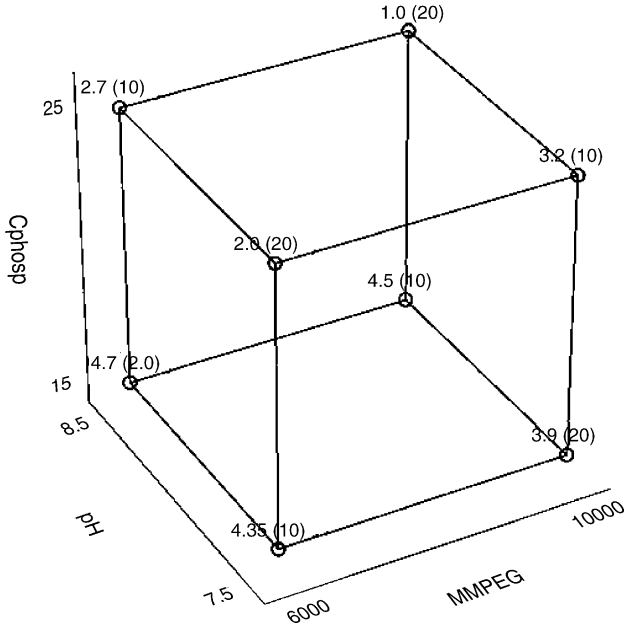

Fig. 3. Cubic plot of the purification values obtained in the second design. The values in parenthesis are the PEG concentration values.

can be obtained at widely differing conditions, as shown by the level combinations at the bottom face.

As for the yield, the large value of the error estimate makes all effects statistically non significant. Since all main effects are negative, however, one may expect higher yields when all factors are set at their lower levels. Several of these yields are in fact quite high. All but one is in excess of 100%.

Similar values have frequently been reported for enzyme extraction using liquid–liquid systems [23–25]. It has been suggested that PEG is essentially an inert polymer that does not interact with protein, due to its steric and hydration properties [8]. However, several authors have found an interaction between PEG and protein, such as, for example, the dissociation of the phosphofructokinase tetrameric enzyme induced by PEG and alteration of the UV absorption spectrum of ribonuclease due the modification of the tyrosine residue in presence of PEG, as cited by Tubio [8]. Farrugia [20] reported that PEG induces a displacement of the fluorescent probe, ANS, from its binding site in human albumin. Grimonprez and Johansson [26] reported high yield values in presence of PEG, which varied with enzyme type and size of chain length, which might lead to an increase in the hydrophobicity, as suggested by Tubio [8], and consequently enhance the activity of the hydrophobic enzyme. Pancera [27] reported that PEG can influence enzyme activity, because the alteration on structure and the type of active site of a particular enzyme in the presence of PEG might affect its relative activity.

Since one would like to obtain not only high purification ratios but also high yield values, a scatter diagram of these two responses helps to decide which is the best factor combination to approach both goals to the maximum possible extent (Fig. 4). The four runs with $P_f > 4$ and $Y > 200$ are labeled in the plot. Three of them are center point replicates, while the fourth, run 3 in Table 5, has the level combination (MMPEG, CPEG, pH, CPHOSP) = (6000, 20%, 8.5, 15%). The wide scatter of the replicates indicates that these responses are affected by a relatively large reproducibility uncertainty.

In short, we may conclude from a Fig. 4 that the best conditions for α-toxin purification, of all investigated in this study, are those corresponding to the central point of the second

experimental design: MMPEG 8000, pH 8,0, CPEG 15% and CPHOSPH 20%.

These results are in agreement with the physico-chemical characteristics of the α-toxin (one small hydrophobic enzyme with 43 kDa and p*I* 5.4 [28]) and a mechanism involving exclusion volume effect, hydrophobic and electrostatic interactions. Apparently these mechanisms are the main phenomena that govern the α-toxin partition in PEG-phosphate ATPS.

4. Conclusion

The fractional experimental designs proved suitable for the study of α-toxin purification in PEG-phosphate ATPS. The findings reported here demonstrate the potential application of the ATPS process for the recovery of α-toxin produced from *C. perfringens* type A, with the possibility of scaling up for industrial vaccine production.

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