J Ind Microbiol Biotechnol (2007) 34:547–552
DOI 10.1007/s10295-007-0230-8

ORIGINAL PAPER

Removal of proteases from *Clostridium perfringens* fermented broth by aqueous two-phase systems (PEG/citrate)

Tatiana Souza Porto · Pedro Alcântara Pessôa-Filho · Benício Barros Neto · José Luiz Lima Filho · Attilio Converti · Ana Lúcia Figueiredo Porto · Adalberto Pessoa Jr

Received: 20 October 2006 / Accepted: 16 May 2007 / Published online: 14 June 2007

Abstract In order to reduce the toxicity of *Clostridium perfringens* fermentation broths used in vaccine preparation, we developed two-phase aqueous systems for removal of toxin-activating proteases. Removal of the proteases inhibits the conversion of protoxin to active toxin. In order to establish the conditions under which the phase separation occurs, binodal curves, formed by poly(ethylene glycol) (PEG) and sodium citrate, were investigated at different values of pH and PEG molar mass. A 2^4-experimental design was used to evaluate the influence of PEG molar mass and concentration, citrate concentration and pH on protease partition coefficient, removal factor and protease removal yield. It has been found that simultaneous increase in PEG molar mass and decrease in citrate concentration remarkably improved the removal factor, whereas the protease removal yield showed an opposite trend. The best conditions for the system under consideration (removal factor of 2.69 and yield of 116%) were obtained at pH 8.0 using PEG molar mass of 8000 g mol^{-1} and concentrations of PEG and citrate of 24 and 15%, respectively.

Keywords Extraction · ATPS · Experimental design · *C. perfringens* · Protease · Detoxification

T. S. Porto (✉) · A. Pessoa Jr
Department of Biochemical and Pharmaceutical Technology, University of São Paulo, 05508-000 São Paulo, SP, Brazil
e-mail: portots@usp.br

P. A. Pessôa-Filho
Department of Chemical Engineering, University of São Paulo, 05508-000 São Paulo, SP, Brazil

B. B. Neto
Department of Fundamental Chemistry, UFPE, 50740-540 Recife, PE, Brazil

J. L. L. Filho · A. L. F. Porto
Laboratory of Immunopathology Keizo Asami (LIKA), UFPE, 50670-901 Recife, PE, Brazil

A. Converti
Department of Chemical and Process Engineering ''G. B. Bonino'', via Opera Pia 15, 16145 Genoa, Italy

Introduction

Anti-clostridial vaccines are considered to be important and cost-effective tools for animal welfare and health. Among these, those used against human diseases such as tetanus are of foremost importance to Public Health [5, 27]. At the moment, anti-clostridial vaccines for veterinary use are produced without any purification of the fermented broth, thus producing a low immunogenic response [2, 16]. They are mainly produced in countries with large cattle-breeding areas. In 1999, no less than 55 million doses of polyvalent vaccines combining antigens from different pathogenic clostridial species were produced in Brazil [16].

Many attempts were made to purify toxins of *Clostridium perfringens* for vaccine preparation [4, 9, 26, 28]. This microorganism does in fact produce a variety of toxins, 12 of which are currently known and whose separation is difficult [9]. Some proteases secreted in the fermented broth activate powerful protoxins produced by the same microorganism such as the epsilon- and iota-toxins [12]. This activation takes place by removal of N and C terminal peptides from the corresponding inactive protoxins [13] and leads to toxins, which are likely to be partly active at the end of fermentation. Therefore, there is a great deal of current interest in new, easy and cheap methodologies able to reduce the concentration of these contaminants in fermented media so as to decrease their toxicity.

A possible way to extract proteases from fermented broths is to use aqueous two-phase system (ATPS), which are made up of two aqueous solutions of two water-soluble polymers or a polymer and a salt [1, 15]. They have recently been used to separate biomolecules such as enzymes, other proteins and antibiotics, mainly because of the possibility of being tailored to a specific separation by varying their features and/or those of the extraction systems [10, 11, 18, 24, 25]. Their technical simplicity, easiness of scaling-up and suitability for continuous operation make this process a promising alternative for large-scale operation [17].

Although the use of citrate salts in ATPS, as an alternative to sulfate and phosphate salts, started in the earlier nineties [29], they have only recently been applied with success [17, 20, 30]. Because these salts are biodegradable and non-toxic, they can be discharged into common biological wastewater treatment plants. However, only a few information is available on PEG/citrate aqueous two-phase systems [30, 31]; therefore, additional basic work is needed to effectively use them for enzyme separation.

In order to assess the effect of some parameters on protease extraction, statistical design of experiments and analysis of results have been utilized in this work. Such an approach was successfully used to investigate the different effects of PEG molar mass and concentration, citrate concentration and pH, which were revealed to be the variables most significantly influencing the purification process (preliminary unpublished data). Statistical design of experiments was widely utilized for process optimization and control as well [3, 17, 18].

To the best of our knowledge, ATPS has not yet been applied to the removal of *C. perfringens* proteases, on which this study is focused. In this sense, it represents a novel application with respect to previous work where the ATPS was used to purify α-toxin of the same microorganism [8].

Material and methods

Fermented medium

Fermented media were kindly supplied by a pharmaceutical industry engaged in the preparation of vaccines. Anaerobic fermentations were performed in industrial fermenters in a typical medium for *C. perfringens* cultivation containing meat extract and 1–2% glucose at pH ≅ 7. Although the production of both proteases and toxins took place during growth, the former reached a maximum level after a period of 5–12 h, after which cells were removed by centrifugation. The supernatant was then used for protease removal tests. After protease removal, toxins were deactivated by the addition of formaldehyde and then utilized for industrial vaccine preparation.

Binodal curves

Stock solutions of 50.0% (w w^{-1}) polyethylene glycol (PEG) with different molar mass (400–8000 g mol^{-1}) (Sigma, St. Louis, MO), 30.0% (w w^{-1}) and tri-sodium citrate dihydrate and 30% (w w^{-1}) citric acid monohydrate (Merck, Darmstadt, Germany), both expressed as anhydrous citric acid, were prepared. The solution of 30.0% (w w^{-1}) citrate/citric acid employed for both binodal curves and extraction was prepared by mixing suitable volumes of the above tri-sodium citrate and citric acid solutions at different pH values (6.0–8.0) and 25 ± 1°C. Binodal curves were obtained by the titration method, as described by Albertson [1].

Preparation of aqueous two-phase systems

Aqueous two-phase systems were set up by mixing stock solutions and water up to the desired PEG and citrate concentrations and by adding the fermentation broth up to a mass percentage of 20% (w w^{-1}). A total amount of 10 g of each system was prepared in 15 ml-graduated tubes with conical tips. After vortex shaking for 1.0 min, phases were separated by settling (40 min) at 25 ± 1°C. After measurement of phase volumes, top and bottom aliquots were withdrawn separately with pipettes and assayed for protein concentration and protease activity.

Analytical techniques

Total protein concentration was determined by the method of Bradford using bovine serum albumin as a standard [7]. To minimize interferences of PEG and citrate, samples were diluted at least tenfold with distilled water before dye addition. The controls of each phase were prepared without protein extract and diluted in the same way.

Enzyme activity was determined by the azocasein method, measuring the absorbance at 440 nm [14]. One unit of enzyme activity (U) was defined as the amount of enzyme that produced an increase in the optical density of 1.0 after 1 h at this wavelength. The activity was then expressed as U ml^{-1}. Total protein and enzyme activity determinations were made using a spectrophotometer.

Experimental design and statistical analysis

The influence of PEG molar mass and concentration, concentration of citrate and pH on the extraction was

Table 1 Factor levels of the 2^4-experimental design used for the study of protease removal by ATPS

Variables	Levels		
	Low (–1)	Central (0)	High (+1)
PEG molar mass (g mol^{-1})	400	3350	8000
PEG concentration (% w w^{-1})	20.0	22.0	24.0
pH	6.0	7.0	8.0
Citrate concentration (% w w^{-1})	15.0	17.5	20.0

evaluated following a 2^4 factorial design with 4 repetitions at the central point (Table 1). This is in fact usually the case when the simultaneous effects of 4 independent variables have to be investigated, especially in the phases preceding the final optimization [6, 19]. The region of the independent variables to be investigated in this work was selected according to preliminary tests as well as the results available in the literature for ATPS [17, 20]. The software ''Statistica'' (version 6.1) (Statsoft Inc) was used for graphical and variance analysis (ANOVA) of the results.

Determination of partition coefficient, removal factor and yield

The protease partition coefficient (K) is defined as the ratio of the volumetric activity in the top phase (A_t) to that in the bottom phase (A_b):

$$K = \frac{A_t}{A_b} \quad (1)$$

The removal factor (RF) was calculated as the ratio of the specific activity in the top phase to the specific activity in the cell extract before partition (A_i):

$$RF = \frac{A_t/C_t}{A_i/C_i} \quad (2)$$

where C_t and C_i are total protein concentrations (expressed as μg ml^{-1}) in the top phase and the starting extract, respectively.

The protease removal yield (Y) was determined as the ratio of total activity in the top phase to that in the starting extract and expressed as a percentage:

$$Y = \left(\frac{A_t V_t}{A_i V_i}\right) 100 \quad (3)$$

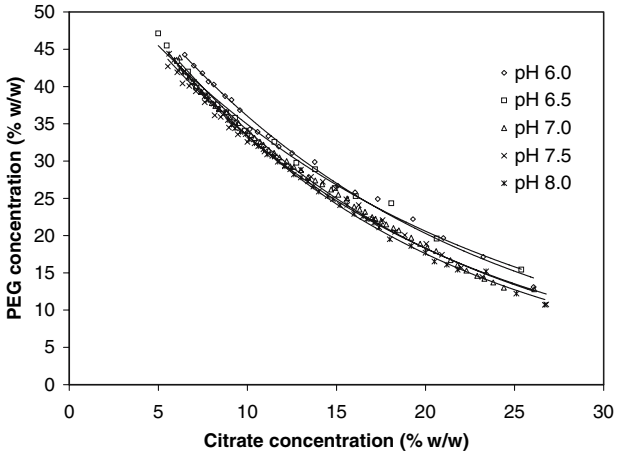

Fig. 1 Binodal curves of PEG/citrate systems obtained at different pH values. M_{PEG} = 400 g mol^{-1}; C_C = 30.0% w w^{-1}; C_{PEG} = 50.0% w w^{-1}

where V_t and V_i are the volumes of the top phase and the starting extract, respectively. It should be noticed that this parameter can exceed 100% when the activity in the extract is lowered by the presence of inhibitors or the one in the top phase increased by positive enzyme/system interactions.

Results and discussion

Binodal curves of the PEG/citrate system

In order to establish the best extraction conditions, the binodal curves of the PEG/citrate system were determined at different values of pH and PEG molar mass.

Figure 1 shows that pH did not exert any appreciable effect on the equilibrium, the different responses of the system being of the same order of magnitude as the experimental error. On the other hand, the concentration of citrate needed to induce phase separation decreased as the PEG molar mass was increased (Fig. 2). This finding agrees with most of the data reported in the literature for similar systems [1, 20, 29].

Liquid–liquid extraction with PEG/citrate system

Once the binodal curves had been determined, it was possible to establish a concentration range of phase separation in every system that allowed us to select the domain of conditions to be investigated through the experimental design. The experimental points below the binodal curve obtained with PEG 400 (Table 2, tests 1, 3, 5 and 7) were taken into account as well, because this curve was positioned at much higher PEG concentrations with respect to

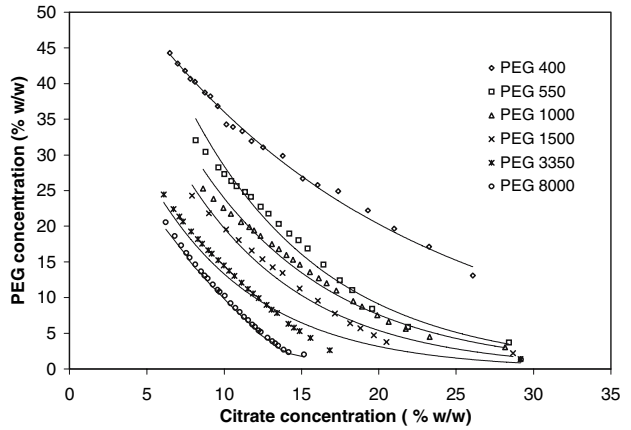

Fig. 2 Binodal curves of PEG/citrate systems obtained using different values of M_{PEG}. pH = 6.0; C_C = 30.0% w w^{-1}; C_{PEG} = 50.0% w w^{-1}

the other molar masses. Otherwise, the system would have been too concentrated, thus surely affecting the extraction. The narrow ranges of PEG and citrate concentrations were selected on the basis of the results of previous experiments (not shown).

The results of protease partition coefficient, removal factor and yield as functions of PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), citrate concentration (C_C) and pH are summarized in Table 2 as well. Statistical analysis of these responses revealed that all of them were significant (Table 3). A negative effect of PEG molar mass and a positive effect of citrate concentration on the partition coefficient were observed, which means that such a parameter was improved by simultaneous decrease in M_{PEG} and increase in C_C (Tables 2 and 3).

The maximum value of the partition coefficient (K = 29.2) was obtained using M_{PEG} = 400 g mol^{-1}, C_{PEG} = 24.0% and C_C = 20.0%. Low PEG molar mass along with high concentration of salt favored protease partition to the top phase. Two different effects may have taken place under these conditions: a little effect of volume exclusion, because of low M_{PEG}, and a more remarkable effect of salting out promoted by high salt concentration in the bottom phase. This behavior was similar to that shown by other proteins. For instance, Oliveira et al. [20] demonstrated that low PEG molar mass (400 g mol^{-1}) and high citrate concentration (20%) effectively increased the partition coefficient of *S. cerevisiae* hexokinase in PEG/citrate ATPS.

The same evaluation can be made for the removal yield as the response. Table 3 shows that only two variables, citrate concentration and pH, had significant positive effects at the 95% confidence level. The negative interaction between M_{PEG} and C_C (A.D) indicates that an increase in citrate concentration along with a decrease in M_{PEG} enhanced the yield.

Table 2 Experimental schedule and results of protease removal from *C. perfringens* fermentation broth by ATPS

Test	M^a_{PEG} (g mol^{-1})	C^b_{PEG} (%)	pH	C^c_C (%)	K^d	Y^e (%)	RF^f
1[g]	400	20.0	6.0	15.0	–	–	–
2	8000	20.0	6.0	15.0	0.62	37.0	2.60
3[g]	400	24.0	6.0	15.0	–	–	–
4	8000	24.0	6.0	15.0	1.11	65.9	1.49
5[g]	400	20.0	8.0	15.0	–	–	–
6	8000	20.0	8.0	15.0	0.87	115.0	2.59
7[g]	400	24.0	8.0	15.0	–	–	–
8	8000	24.0	8.0	15.0	1.08	115.9	2.69
9	400	20.0	6.0	20.0	6.41	153.1	1.67
10	8000	20.0	6.0	20.0	2.87	82.6	1.28
11	400	24.0	6.0	20.0	29.25	145.4	1.57
12	8000	24.0	6.0	20.0	1.97	57.0	0.91
13	400	20.0	8.0	20.0	17.93	193.7	1.73
14	8000	20.0	8.0	20.0	3.14	109.0	1.73
15	400	24.0	8.0	20.0	16.35	206.3	2.16
16	8000	24.0	8.0	20.0	2.54	117.4	2.51
17[h]	3350	22.0	7.0	17.5	2.34	97.5	2.33
18[h]	3350	22.0	7.0	17.5	2.38	122.6	2.30
19[h]	3350	22.0	7.0	17.5	1.82	114.2	2.13
20[h]	3350	22.0	7.0	17.5	2.55	123.9	2.48

[a] PEG molar mass
[b] PEG concentration
[c] Citrate concentration
[d] Partition coefficient
[e] Protease removal yield
[f] Removal factor referred to the top phase
[g] No formation of any aqueous two-phase system
[h] Central point

The use of the lowest PEG molar mass (400 g mol^{-1}) and the highest citrate concentration (20.0%) allowed excellent recovery, as demonstrated by Y values always higher than 100%. Yields above 100% are frequently reported for liquid–liquid extraction of enzymes. According to Meyerhoff et al. [18], this could be the result of removal of inhibitors from the PEG phase during extraction. Alternatively, as proposed by Pancera et al. [22], PEG could have altered the structure of the enzyme active site, hence enhancing the relative activity. For example, Oliveira et al. [21], using a PEG/cashew-nut tree gum ATPS, obtained a maximum yield of 129%. Yields of about 230% were even reported for ascorbate oxidase [23] and for phospholipase α-toxin [8] using PEG/phosphate ATPS.

The removal factor was another important parameter for the extraction. As one can observe in Table 3, M_{PEG}, C_C and pH exerted significant positive effects on this

Table 3 Effects calculated from the responses of Table 2. The four-factor interaction was assumed to be negligible

	K[a]	Y[b]	RF[c]
A[d]	–4.35*	0.02	6.58*
B[e]	5.37*	0.36	–0.21
C[f]	1.84	6.50*	3.23*
D[g]	14.36*	15.02*	3.48*
A.B	–4.87*	0.15	–0.75
A.C	–0.24	2.33	2.16
A.D	–7.44*	–13.70*	–8.39*
B.C	–4.77*	0.53	2.40
B.D	–3.36*	–0.86	1.45
C.D	0.06	1.23	1.24
A.B.C	4.29*	–0.29	1.52
A.B.D	0.00	–1.06	0.91
A.C.D	0.00	–2.93	0.16
B.C.D	1.28	1.69	0.37

[a] Partition coefficient

[b] Protease removal yield

[c] Removal factor

[d] PEG molar mass (M_{PEG})

[e] PEG concentration (C_{PEG})

[f] pH

[g] Citrate concentration (C_C)

*Statistically significant values (at the 95% confidence level)

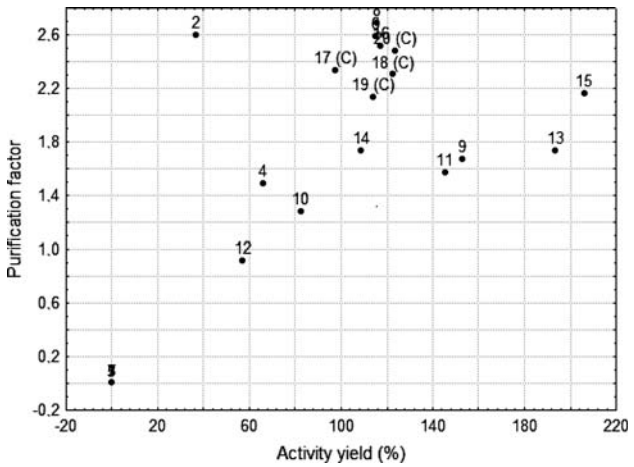

Fig. 3 Purification factor versus the activity yield. Numbers refer to tests according to the experimental schedule of Table 2

response. The negative effect of the interaction between the first two variables (A.D) means that an increase in M_{PEG} along with a simultaneous decrease in C_C resulted in an improvement in the removal factor. This behavior is just opposite to that observed for K and Y. The highest value of this parameter (2.69) was obtained with M_{PEG} = 8000 g mol^{-1}, C_{PEG} = 24.0%, C_C = 15.0% and pH 8.0 (test 8). Under these conditions, which favor RF, the activity yield was 116% (Fig. 3) and the partition coefficient 1.08. This promising result was due to protease partition to the two phases almost to the same extent, while the other proteins preferably partitioned to the bottom phase. Similar significant effect of M_{PEG} was observed by Mayerhoff et al. [18], who obtained, for xylose reductase of *Candida mogii*, a purification factor of 1.24 and a yield of 105% using a 2^4-experimental design.

Such a behavior allowed successful extraction of proteases by the selected aqueous two-phase system. Notwithstanding a relatively low selectivity, the contaminants were in fact almost totally transferred to the bottom phase, and the main aim of this study was reached.

Conclusion

A complete 2^4-factorial design has been used to investigate the main effects of PEG molar mass (M_{PEG}), concentrations of PEG (C_{PEG}) and citrate (C_C) and pH on the removal of proteases from *C. perfringens* fermentation broth by PEG/citrate ATPS.

The main effects of these independent variables on the partition coefficient (K), the protease removal yield (Y) and the removal factor (RF) can be summarized as follows: K was improved by an increase in C_C and a decrease in M_{PEG}, Y was improved by increases in both pH and C_C, and RF was effectively improved by an increase in M_{PEG} and pH along with a decrease in C_C.

Because of the different behaviors exhibited by the responses, RF was selected as the most interesting of them. Under the best conditions for this parameter (M_{PEG} = 8000 g mol^{-1}, C_{PEG} = 24.0%, C_C = 15.0% and pH 8.0), it was found RF = 2.69, Y = 116% and K = 1.08. Although the performance of the proposed ATPS has to be improved by further investigation to meet the requirements of possible application, the present results clearly demonstrate the potential of such a technique to reduce the toxicity of fermented broths for vaccine preparation. In general, it appears to be a powerful preliminary concentration/decontamination step, which could be used for the purification of bioproducts of industrial concern. In particular, it could be successfully exploited in the pharmaceutical industry as the first step of the purification of antibiotics, immunoglobulins, pigments, enzymes, etc.

Acknowledgments The authors wish to acknowledge the financial support of FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, São Paulo State, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasilia, Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasilia, Brazil).

References

1. Albertsson PA (1986) Partition of cell particles and macromolecules. Wiley-Interscience Press, New York
2. Azevedo EO, Lobato FCF, Abreu VLV (1998) Avaliação de vacinas contra *Clostridium perfringens* tipos C e D. Arq Bras Med Vet Zootec 50:239–242
3. Balasubramaniam D, Wilkinson C, Cott KV, Zhang C (2003) Tobacco protein separation by aqueous two-phase extraction. J Chromatogr A 989:119–129
4. Borrmann E, Schulze F, Cussler K, Hänel I, Diller R (2006) Development of a cell culture assay for the quantitative determination of vaccination-induced antibodies in rabbit sera against *Clostridium perfringens* epsilon toxin and *Clostridium novyi* alpha toxin. Vet Microbiol 114:41–50
5. Boukes FS, Wiersma TJ, Beaujean D, Burgmeijer RJ, Timen A (2004) Tetanus prophylaxis in general practice. Ned Tijdschr Geneeskd 148:2172–2173
6. Box GEP, Hunter WG, Hunter JS (1978) Statistics for experimenters. John Wiley and Sons, New York
7. Bradford MM (1976) A rapid and sensitive method for the quantification of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
8. Cavalcanti MTH, Porto TS, Neto BB, Porto ALF, Lima-Filho JL, Pessoa A Jr (2006) Aqueous two-phase systems extraction of α-toxin from *Clostridium perfringens* type A. J Chromatogr B 833:135–140
9. Cavalcanti MTH, Porto TS, Porto ALF, Lima-Filho JL, Pessoa A Jr (2004) Large scale purification of *Clostridium perfringens* toxin: a review. Braz J Pharm Sci 40:151–164
10. Cisneros M, Benavides J, Brenes CH, Rito-Palomares M (2004) Recovery in aqueous two-phase systems of lutein produced by the green microalga *Chlorella protothecoides*. J Chromatogr B 807:105–110
11. Esmanhoto E, Kilikian BV (2004) ATPS applied to extraction of small molecules—polycetides—and simultaneous clarification of culture media with filamentous microorganisms. J Chromatogr B 807:139–144
12. Hatheway CL, Whaley DN, Dowell VR Jr (1990) Epidemiological aspects of *Clostridium perfringens* in foodborne illness. Food Technol 34:77–90
13. Jin F, Matsushita O, Katayama S-I, Jin S, Matsushita C, Minami J, Okabe A (1996) Purification, characterization, and primary structure of *Clostridium perfringens* lambda-toxin, a thermolysin-like metalloprotease. Infect Immun 64:230–237
14. Leighton TJ, Doi RH, Warren RAJ, Kelln RA (1973) The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis*. J Mol Biol 76:103–122
15. Li M, Peeples TL (2004) Purification of hyperthermophilic archaeal amylolytic enzyme (MJA1) using thermoseparating aqueous two-phase systems. J Chromatogr B 807:69–74
16. Lobato FCF, Moro E, Umehara O (2000) Avaliação da resposta de antitoxinas beta e épsilon de *Clostridium perfringens* induzidas em bovinos e coelhos por seis vacinas comerciais no Brasil. Arq Bras Med Vet Zootec 52:313–318
17. Marcos JC, Fonseca LP, Ramalho MT, Cabral JMS (2002) Application of surface response analysis to the optimization of penicillin acylase purification in aqueous two-phase systems. Enzyme Microbial Technol 31:1006–1014
18. Mayerhoff ZDVL, Roberto IC, Franco TT (2004) Purification of xylose reductase from *Candida mogii* in aqueous two-phase systems. Biochem Eng J 18:217–223
19. Neto BB, Scarminio IC, Bruns RE (2002) Como fazer experimentos: pesquisa e desenvolvimento na ciência e na indústria. 2nd edn. Editora da Universidade de Campinas, Campinas-SP, Brazil
20. Oliveira GGG, Silva DP, Roberto IC, Vitolo M, Pessoa-Jr A (2003) Partition behavior and partial purification of hexokinase in aqueous two-phase polyethylene glycol/citrate systems. Appl Biochem Biotechn 105:787–797
21. Oliveira LA, Sarubbo LA, Porto ALF, Lima-Filho JL, Campos-Takaki GM, Tambourgi EB (2002) Partition of trypsin in aqueous two-phase systems of poly(ethylene glycol) and cashew-nut tree gum. Proc Biochem 38:693–699
22. Pancera SM, Silva LHM, Loh W, Itri R, Pessoa-Jr A, Petri DFS (2002) The effect of poly(ethylene glycol) on the activity and structure of glucose-6-phosphate dehydrogenase in solution. Colloid Surface B 26:291–300
23. Porto ALF, Sarubbo LA, Moreira KA, Melo HJF, Lima-Filho JL, Campos-Takaki GM, Tambourgi EB (2004) Recovery of ascorbic oxidoreductase from crude extract with an aqueous two-phase system in a perforated rotating disc contactor. Braz Arch Biol Technol 47:821–826
24. Rabelo APB, Tambourgi EB, Pessoa-Jr A (2004) Bromelain partitioning in two-phase aqueous systems containing PEO–PPO–PEO block copolymers. J Chromatogr B 807:61–68
25. Rito-Palomares M (2004) Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B 807:3–11
26. Schoepe H, Neubauer A, Schlapp T, Wieler LH, Baljer G (2006) Immunization with an alphatoxin variant 121A/91-R212H protects mice against *Clostridium perfringens* alpha toxin. Anaerobe 12:44–48
27. Téllez S, Casimiro R, Vela AI, Fernández-Garayzábal JF, Ezquerra R, Latre MV, Briones V, Goyache J, Bullido R, Arboix M, Dominguez L (2006) Unexpected inefficiency of the European pharmacopoeia sterility test for detecting contamination in clostridial vaccines. Vaccine 24:1710–1715
28. Thompson DR, Parreira VR, Kulkarni RR, Prescott JF (2006) Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Vet Microbiol 113:25–34
29. Vernau J, Kula MR (1990) Extraction of proteins from biological raw material using aqueous polyethylene glycol-citrate phase systems. Appl Biotechnol Biochem 12:379–404
30. Zafarani-Moattar MT, Sadeghi R, Hamidi AA (2004) Liquid-liquid equilibria of an aqueous two-phase system containing polyethylene glycol and sodium citrate: experiment and correlation. Fluid Phase Equilib 219:149–155
31. Zhi W, Song J, Ouyang F, Bi J (2005) Application of response surface methodology to the modeling of α-amylase purification by aqueous two-phase systems. J Biotechnol 118:157–165