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Partial purification of penicillin acylase from *Escherichia coli* in poly(ethylene glycol)–sodium citrate aqueous two-phase systems

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Abstract

Studies on the partition and purification of penicillin acylase from *Escherichia coli* osmotic shock extract were performed in poly(ethylene glycol)–sodium citrate systems. Partition coefficient behavior of the enzyme and total protein are similar to those described in other reports, increasing with pH and tie line length and decreasing with PEG molecular weight. However, some selectivity could be attained with PEG 1000 systems and long tie line at pH 6.9. Under these conditions 2.6-fold purification with 83% yield were achieved. Influence of pH on partition shows that is the composition of the system and not the net charge of the enzyme that determines the behaviour in these conditions. Addition of NaCl to PEG 3350 systems significantly increases the partition of the enzyme. Although protein partition also increased, purification conditions were possible with 1.5 *M* NaCl where 5.7-fold purification and 85% yield was obtained. This was possible due to the higher hydrophobicity of the enzyme compared to that of most contaminants proteins. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Aqueous two-phase systems; Partitioning; Poly(ethylene glycol)–sodium citrate; Penicillin acylase

1. Introduction

Aqueous two-phase systems had been widely and successfully used on the extraction and purification of biological macromolecules. They are formed by mixing two polymers or a polymer and a salt above some threshold concentration. Separation is achieved by the different distribution, between the two phases, of the target compound and the contaminants. Due to the high content of water in both phases and low interfacial tension, they provide mild conditions especially suited for biological macromolecules separation. The mechanism of partition is not well understood and separation of compounds is usually attained by a systematic variation of system composition. This includes type, molecular weight and concentration of polymer, type and concentration of salt and pH. Although most studies are empirical, significant purification of proteins [1], nucleic acids [2] and antibiotics [3,4] have been achieved with this method. Furthermore the ease of scale-up and suitability for continuous operation makes this technique very interesting for large-scale application.

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is an enzyme present in several bacteria and fungi with relevance in the antibiotic industry. Although its biological role is not clearly understood, genetic studies suggest that it should be involved in

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the metabolism of aromatic compounds [5,6]. In terms of substrate specificity it could be divided in penicillin G acylase (PGA) and penicillin V acylase (PVA). Both exist in bacteria and fungi, but the former is more common in bacteria and the latter in fungi. PGA is the most studied and although it is able to catalyse the hydrolysis of amide bonds in compounds containing a phenylacetyl moiety, it is best known by its ability to hydrolyse penicillin G yielding 6-aminopenicillanic acid (6-APA) and phenylacetic acid. Due to this capability the enzyme is used by the pharmaceutical industries for the production of 6-APA, the starting material for the synthesis of semi-synthetic β-lactam antibiotics, such as ampicillin and amoxicillin. The enzyme is usually used in immobilised form to allow reutilisation. Immobilisation of the enzyme requires a relatively pure preparation to get a good yield. Enzyme purification is, therefore, a determinant step on the preparation of the active catalyst. Since most purification protocols described involve several chromatographic steps which increase the cost of the process and reduce the yield, simpler and more efficient process of purification are needed. Aqueous two-phase systems could be a good alternative to a first step purification, as these allow removal of several contaminants by a simple and economic process.

In a previous paper [7] we reported that in opposition to what should be expected the partition coefficient of penicillin acylase in PEG–salt systems varies with phase volume ratio. That study was an attempt to improve previous attained purification conditions. In this work we report the detailed studies of partition and purification of penicillin G acylase from *Escherichia coli* in aqueous two-phase systems which led to the selection of those conditions. As the enzyme is located on the periplasmic space, the studies were performed on an osmotic shock extract of the bacteria, which is significantly enriched on the enzyme. The chosen system was polyethyleneglycol (PEG)–sodium citrate. A PEG–salt system was selected because this favours one-sided partition of the compounds due to larger differences in the physicochemical properties of the two phases. This is a valuable feature in a protein first step purification where most of the contaminants should be separated. Although the PEG–phosphate systems are better studied, there is an increased interest in the utilisation of PEG–citrate systems [8–10] due to the lower environmental toxicity of citrate compared to phosphate.

2. Experimental

2.1. Materials

All reagents used were of analytical grade. Polyethyleneglycol of various molecular weights used were obtained from Sigma (St. Louis, MO, USA).

2.2. Production and extraction of penicillin acylase

A mutant strain of *E. coli* ATCC 9637 was grown in 1000-cm^3 shake flasks with 250 cm^3 of medium, containing 1% (w/v) yeast extract and 0.3% (w/v) phenylacetic acid, at pH 7.0, 300 rpm and 25°C. Cells were harvested by centrifugation at 12 000 *g* for 10 min, at the end of the exponential phase (40 h), washed with 200 m*M* phosphate buffer, pH 7.5, and stored at 4°C until used.

Enzymatic extract was obtained by cold osmotic shock rupture of the bacterial cells. *E. coli* cells were suspended in 250 m*M* Tris–HCl (pH 8.0)+12.5 m*M* EDTA+20% (w/w) sucrose, stirred for 15 min at 4°C, and collected by centrifugation. They were then resuspended in cold distilled water for 30 min with stirring at 4°C. Intact cell and cell debris were removed by centrifugation and the extract was stored at 4°C.

2.3. Characterisation of aqueous two-phase systems

Binodal curves were determined by titration according to Albertsson [11]. Small amounts of water were added to several biphasic systems of defined composition until turbidity disappeared. The final composition of the system was then calculated and taken as a binodal point. Tie lines were defined by determining the composition of top and bottom phases for selected systems. Tie line lengths (TLL) were calculated by the following formula:

$$\mathrm{TLL} = \sqrt{\Delta P^2 + \Delta C^2}$$

where ΔP is the difference between the PEG concentrations of the two phases; and ΔC is the difference between the citrate concentrations of the two phases.

Citrate was quantified by isocratic elution on a Merck 5-μm LiChrospher 100 RP-18, 250×4 mm I.D. column, with 200 m*M* phosphoric acid–methanol (90:10, v/v) as eluent, at a flow-rate of 1.0 ml/min. Detection was at 220 nm and the concentration was calculated from the calibration curve with standard concentrations of citrate. Chloride concentration was determined by the Volhard titration method according to Ref. [12]. In systems containing sodium chloride, its concentration was estimated by assuming that it was equal to chloride concentration. PEG concentration was determined by refractometry after correcting for the contribution of the citrate and NaCl, if present.

2.4. Preparation of aqueous two-phase systems

Stock solutions of 50% (w/w) PEG of molecular weights 1000, 3350 and 8000 were prepared and stored at 4°C. PEG 400 was used directly as the 100% (w/w) commercial liquid form. Concentrated (35.3%, w/w) sodium citrate solutions at the required pH were prepared by mixing appropriate amounts of equimolar solutions of tri-sodium citrate dihydrate and citric acid monohydrate. Systems were prepared at 20±1°C by mixing suitable amounts of PEG and citrate solution, with enzymatic extract, in 15-ml graduated tubes with conical tips. Solid NaCl was added when needed. The final weight was adjusted to 8 g by addition of water. After Vortex mixing for 1 min the two phases were separated by centrifugation and assayed for protein concentration and penicillin acylase activity.

Protein concentration was determined by the method of Bradford [13]. To correct for the interference of PEG and citrate the samples were diluted and read against blanks with the same composition but without enzymatic extract.

Penicillin acylase activity was assayed by the method of Kutzbach and Rauenbusch [14]. The hydrolysis of 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) was followed spectrophotometrically by the increase in absorbance at 410 nm. The reaction was performed at 37°C in 100 m*M* phosphate buffer, pH 7.5, in stirred cells. Under these conditions neither PEG nor citrate interfere with the enzymatic activity. Enzymatic activity (Act.) was calculated from the following expression:

$$\text{Act. (U/ml)} = \frac{\Delta \text{Abs}}{\Delta t \times v \times 4.49}$$

where v is the volume of the analysed sample.

3. Results and discussion

3.1. Effect of polymer molecular weight and tie line length

The development of a purification procedure using aqueous two-phase systems involves the variation of several factors until a good result is achieved. In the present work we started by studying the influence of polymer molecular weight and phase composition on enzyme partition and purification. The studies were conducted at pH 6.9, the isoelectric point (p*I*) of the enzyme, in order to minimise the influence of possible electrostatic interactions thus allowing an easier correlation between the observed effects and the examined factors. As PEG–citrate systems are not very well documented in the literature, phase diagrams were determined for different PEG molecular weights (Fig. 1). It can be seen in this figure that the binodal lines became more asymmetric and close to the origin with the increase in polymer molecular weight. This happens because as the polymer molecular weight increases, the components of the system become more different and so lower concentrations are required for incompatibility and, therefore, phase separation. The above-mentioned effect is more pronounced for higher concentrations of sodium citrate.

For each PEG size, partition and purification studies were carried out in systems of three different composition corresponding to different tie line lengths. For all these systems the phase volume ratio was close to one. Results are presented in Table 1. Protein (K_p) and enzyme (K_e) partition coefficients followed the same trend, decreasing with the increase in PEG molecular weight. This has also been observed for other proteins [15–17] and could be attributed to two different effects. The most obvious

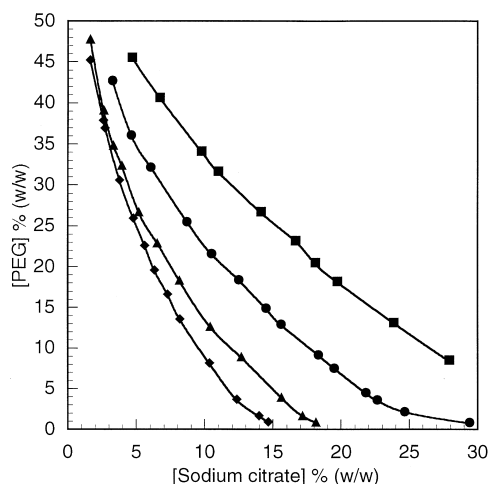


Fig. 1. Phase diagrams for poly(ethylene glycol)–citrate systems with different PEG molecular weight: (■) PEG 400; (●) PEG 1000; (▲) PEG 3350; and (♦) PEG 8000.

is the increase in the upper phase hydrophobicity. In fact as PEG chain length increases there will be less hydroxyl groups for the same concentration of the polymer and so the polymer-richer upper phase will increase in hydrophobicity. On the other hand, the chain length increase will also cause the reduction of the excluded volume, meaning less space available for the protein. However it is important to note that although the trend is similar for total protein and enzyme, the magnitude of the decrease is more pronounced in the case of the enzyme and some selectivity is achieved for PEG 1000.

The tie line length for systems containing PEG 400 and 1000 also influences enzyme partition. For PEG of higher molecular weight no significant influence was observed. The tie line length only affected total protein partition with PEG 400.

Best selectivity was achieved with PEG 1000 and long tie line. The purification obtained was 2.6-fold with 83% yield.

Table 1
Influence of PEG molecular weight and phase composition on the partition coefficients of penicillin acylase (K_e) and total protein (K_p), purification factor and yield, in PEG–sodium citrate systems

PEG MW	[PEG] (%)	[Na citrate] (%, w/w)	Log K_e	Log K_p	Yield (%)	Purif. fact.
400	21	18.23	2.33	0.40	85	1.1
400	23	19.41	>3.00	0.44	86	1.1
400	24	20.59	2.90	>2.32	86	1.2
1000	16	14.71	0.24	−0.33	62	1.6
1000	19	15.88	1.21	−0.35	83	2.3
1000	21	17.06	2.59	−0.41	83	2.6
3350	10	12.65	−1.02	−0.78	90	1.1
3350	12	13.53	−0.92	−0.67	90	1.1
3350	14	14.12	−2.10	−0.92	100	1.1
8000	10	10.59	−2.30	−1.20	108	1.0
8000	12	11.76	−2.70	−1.26	93	1.0
8000	14	12.94	−2.52	−1.16	101	1.1

3.2. Effect of pH

The effect of pH on partition and purification was also evaluated. The phase diagrams for PEG 1000–sodium citrate, pH 5.9, 6.9 and 7.6, were determined (Fig. 2). Although the shape of the binodal lines is similar for the several pH values, the tie line length for systems with the same composition increases with increasing pH (Table 2). This change is more pronounced for the short tie line, the one closer to the critical point. The ratio between trivalent and divalent citrate ions increases with pH and since trivalent ions are more effective in phase separation a smaller concentration of citrate is needed for two-phase formation. Such effect is more clearly seen for lower PEG concentration.

Partition and purification studies were performed at the previous pH values for three different tie line lengths (Fig. 3). Although to different extents, partition coefficients of both enzyme and total protein increase with pH for all tie lines. However, whereas the differences in enzyme partition for different tie lines increase with pH, total protein partition for different tie lines remains approximately constant, irrespective of the pH. Since tie line length differences become smaller with pH increase (Table 2), the observed effect on enzyme partition should not be related with total system composition but with citrate anion electrostatic effects. In fact the pH could affect the partition, either by changing the charge of the solute or by altering the ratio of the charged species present. Other studies have referred that negatively charged proteins prefer the upper phase in PEG–salt systems [15,18,19]. However current results show that the major increase in partition coefficient is observed when the protein charge changes from positive to neutral. Only a

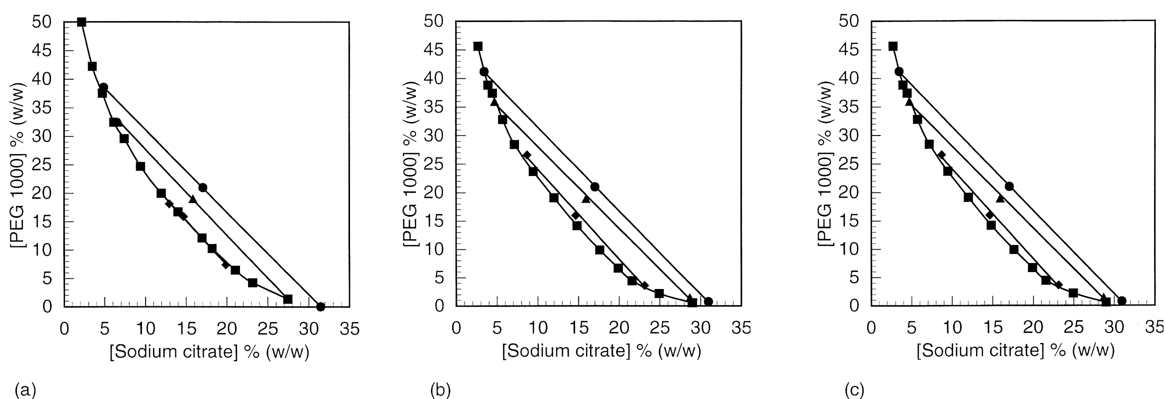


Fig. 2. Phase diagram for PEG 1000–sodium citrate systems at pH: 5.9 (a), 6.9 (b), 7.6 (c). (■) Binodal line; (♦) short tie line; (▲) medium tie line; and (●) long tie line.

Table 2
Tie line length for PEG 1000–sodium citrate systems at different pH values

pH	Tie line (%, w/w)		
	Short	Medium	Long
5.9	12.7	37.3	46.8
6.9	21.1	40.1	47.9
7.6	27.1	42.0	48.9

slight increase is observed when the protein charge changes from neutral to negative. Since the pK_a for divalent citrate is 5.82, these results suggest that the affinity of the enzyme for the upper phase increases when the trivalent to divalent citrate ion ratio increases. The determinant factor in partition of the enzyme seems to be the relative amount of component anions on the phases and not the net charge of the enzyme. This agrees with the results of Ref. [20], which found that four modified taumatins with different p*I* values show small differences on partition on each of the several systems studied. The results were most impressive in PEG–citrate systems, where almost no correlation was found between log *K* and charge density. Therefore, it could be concluded that the enhanced affinity for the upper phase with pH increase is due to an increase of the salting-out effect in the lower phase, and to a lesser extent to the increase of hydrophobic interaction between protein and PEG in the upper phase. Both effects are due to an increased ratio of trivalent to divalent citrate ions with pH increase. It is a well-

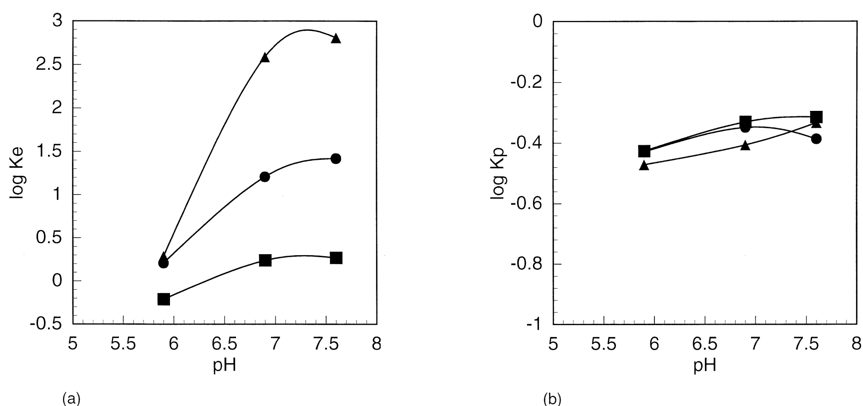


Fig. 3. Influence of pH on partition coeficient of penicillin acylase (a) and protein (b) in PEG 1000–sodium citrate systems. (■) Short tie line; (●) medium tie line; and (▲) long tie line.

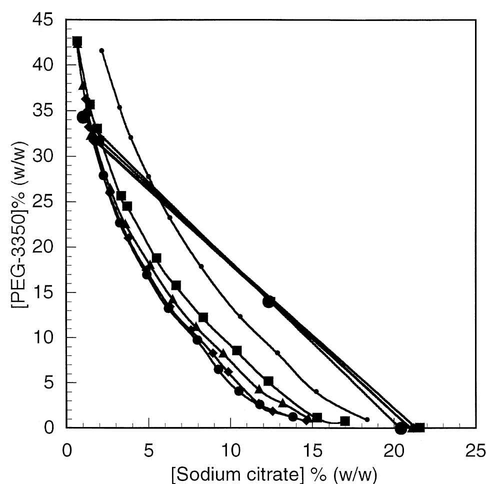


Fig. 4. Phase diagram of PEG 3350–sodium citrate in the presence of different concentrations of NaCl as follows: (●) no NaCl; (■) 0.5 mol/kg; (▲) 1.0 mol/kg; (♦) 1.5 mol/kg; and (●) 2.0 mol/kg. Tie lines were determined for 12.35% (w/w) sodium citrate and 14% (w/w) PEG.

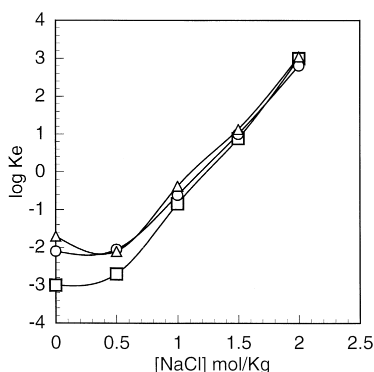
known fact that trivalent ions are more efficient than divalent ions in promoting the previous effects.

Although the magnitude of partition coefficient increase is much higher for enzyme than for total protein no significant improvement in purification was achieved at the higher pH tested.

3.3. Effect of NaCl addition

Partition in the presence of NaCl was studied in PEG 3350 systems. Phase diagrams were determined for different pH values as before. Fig. 4 shows the phase diagram at pH 8.2. Deviation of the binodal to the origin was observed with addition of NaCl. However increase of NaCl concentration does not cause much difference in binodal shape. For 1.5 and 2.0 *M* NaCl the binodals almost overlap. A slight increase in tie line length is also observed from 37.2% (w/w) at 0.5 *M* NaCl to 39.4% (w/w) at 2.0 *M* NaCl.

Partition coefficients of both enzyme and protein increase with the addition of NaCl (Fig. 5). The increase is more pronounced for the enzyme and almost independent of pH. Total protein revealed decreased influence of pH with the increase on NaCl concentration. Several authors [9,15,17,18,21,22] found that addition of NaCl to PEG–salt systems increases the difference in hydrophobicity of the phases, promoting the partition of the more hydrophobic proteins to the upper phase. Resolution of the system is also increased with NaCl concentration [18,22]. This means that the difference between partition coefficient of two proteins increases with NaCl concentration. From our results we could conclude that penicillin acylase hydrophobicity is higher than that of the majority of the other proteins



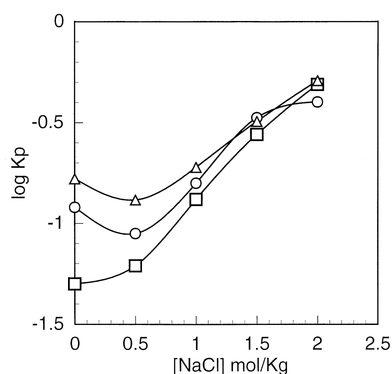


Fig. 5. Influence of NaCl addition on partition coeficient of penicillin acylase (a) and protein (b) in PEG 3350–sodium citrate systems: (□) pH 5.9; (○) pH 6.9; and (△) pH 8.2.

present in the extract, because its partition coefficient increases more than the partition coefficient for total protein with NaCl concentration. Other studies showed that penicillin acylase could be significantly purified by hydrophobic interaction chromatography with matrices containing different hydrophobic side chains in the presence of ammonium sulfate [23]. This indicates the presence of hydrophobic regions on the surface of enzyme other than substrate binding site. Partition is determined, in this case, by hydrophobic interaction of the enzyme with PEG. This is promoted by the high concentration of chloride in the upper phase. In fact, as the partition coefficient of chloride ranges from 0.7 to 0.8 for the concentrations tested, its concentration in the upper phase will be 5–20 times that of total citrate. According to this the variation of the trivalent/divalent citrate ions ratio with pH has almost no effect on the partition. The small differences observed for total protein at low NaCl concentration must be due to some contaminant proteins very sensitive to citrate salting-out.

Although the separation factor, given by the ratio K_e/K_p, increased with NaCl concentration the best selectivity was obtained with 1.5 *M* NaCl. A purification factor of 5.7 with a 85% yield was achieved.

The effect of NaCl addition on enzyme partition and purification was also studied in PEG 8000–citrate systems at pH 6.9. The behaviour was similar to that observed previously. However, higher concentrations of NaCl were needed to achieve similar partition coefficients for both protein and enzyme. The best purification factor obtained at 2.0 *M* NaCl was only 4.8-fold due to the lower selectivity of this system compared with the previous one.

4. Conclusions

The study of several factors influencing total protein and enzyme partitioning in PEG–sodium citrate systems allowed the definition of selective conditions for the degree of partial purification of penicillin acylase. The results, which attained almost 6-fold purification and 85% yield, are better than what is usually obtained by similar procedures, and very interesting for a first step purification. Although the partition behaviour of penicillin acylase is very similar to that observed for most proteins described in the literature, separation from contaminants was possible by exploring the hydrophobicity of the enzyme. In addition, these experiments support the idea that protein charge does not strongly influence its partition in polymer–salt systems.

Theoretically in systems with $K_e > K_p$ the purification factor increases with decreasing phase volume ratio, whereas the yield decreases. The purification conditions obtained here could be, therefore, optimised by manipulating the phase volume ratio, in a search for a good compromise between these two parameters. However, this is only possible if the partition coefficient of both total protein and enzyme remains constant with the variation of phase volume ratio. As was observed in the previous report [7], this condition is not fulfilled for this system and so no further optimisation of purification conditions could be achieved.

References

[1] B.Y. Zaslavsky, Aqueous two-phase partitioning, in: Physical Chemistry and Bioanalytical Applications, Marcel Dekker, New York, 1995.

[2] K. Kimura, H. Kobayashi, Biotechnol. Tech. 8 (1984) 473.

[3] C.-K. Lee, S.I. Sandler, Biotechnol. Bioeng. 35 (1990) 408.

[4] G. Yixin, M. Lehe, Z. Ziqiang, Biotechnol. Tech. 8 (1994) 473.

[5] M.A. Prieto, A. Perez-Aranda, J.L. Garcia, J. Bacteriol. 175 (7) (1993) 2162.

[6] M.A. Prieto, E. Díaz, J.L. Garcia, J. Bacteriol. 178 (1) (1996) 111.

[7] J.C. Marcos, L.P. Fonseca, M.T. Ramalho, J.M.S. Cabral, J. Chromatogr. B 711 (1998) 295.

[8] J.M.R. Vernau, Kula, Biotechnol. Appl. Biochem. 12 (1990) 397.

[9] M.A. Eitman, Aqueous biphasic separation, in: R.D. Rogers, M.A. Eitman (Eds.), Biomolecules to Metal Ions, Plenum Press, New York, 1995, p. 31.

[10] M.A. Eitman, Sep. Sci. Technol. 30 (12) (1995) 2509.

[11] P.-A. Albertsson, in: Partition of Cell Particles and Macromolecules, 3rd ed, Wiley, New York, 1985.

[12] J.R. Caldwell, H.G. Moyer, Ind. Eng. Chem. Anal. Edu. 7 (1935) 38.

[13] M.M. Bradford, Anal. Biochem. 72 (1976) 248.

[14] C. Kutzbac, E. Rauenbusch, Hoppe-Seylers Z. Physiol. Chem. 354 (1974) 45.

[15] A.S. Schmidt, A.M. Ventom, J.A. Asenjo, Enzyme Microb. Technol. 16 (1994) 131.
[16] D.P. Harris, A.T. Andrews, G. Wright, D.L. Pyle, J.A. Asenjo, Bioseparation 7 (1997) 31.
[17] M.V. Miranda, O. Cascone, Biotechnol. Tech. 8 (4) (1994) 275.
[18] J.A. Asenjo, A.S. Schmidt, F. Hachem, B.A. Andrews, J. Chromatogr. A 668 (1994) 47.
[19] R.A. Hart, J.E. Bailey, Enzyme Microb. Technol. 13 (1991) 788.
[20] T.T. Franco, A.T. Andrews, J.A. Asenjo, Biotechnol. Bioeng. 49 (1996) 309.
[21] F. Hachem, B.A. Andrews, J.A. Asenjo, Enzyme Microb. Technol. 19 (1996) 507.
[22] T.T. Franco, A.T. Andrews, J.A. Asenjo, Biotechnol. Bioeng. 49 (1996) 300.
[23] V.K. Sudhakaran, J.G. Shewale, Biotech. Lett. 8 (1987) 539.