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On the kinetics of phase separation in aqueous two-phase systems

M.H. Salamanca[a], J.C. Merchuk[b], B.A. Andrews[a], J.A. Asenjo[a,*]

[a]*Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering, University of Chile, Beauchef 861, Santiago, Chile*

[b]*Ben Gurion University of the Negev, Beer-Sheva, Israel*

Abstract

The effect of the tie-line location (phase volume ratio) on the kinetics of phase separation in batch PEG/salt aqueous two-phase systems (ATPS) has been investigated. PEG/sulphate systems with a stability ratio (sr) of 0.34 and 0.37 and relative tie-line lengths in the range 0.1 to 0.6 for a continuous top phase and in the range 0.03 to 0.15 for a continuous bottom phase were used in the batch studies. A continuous settler was designed with three different inlet geometries. Phase separation is much faster when the bottom phase is continuous and in this case the location on the tie-line and the presence or absence of *Bacillus subtilis* extract makes little difference. When the top phase is continuous the relative sizes of the phases (phase ratio, *R*, relative distance on tie-line, rd) has an important effect, the larger the top phase (larger *R* and rd) the slower the phase separation. The presence of *Bacillus* extract also makes the operation slower which is more marked at the largest values of *R* (and rd). At the largest volume ratios (*R* or rd) three different settling regions have been recognised, a region of coalescence, a region of drops moving to the interphase and a region where drops queue at the interphase to coalesce into the large phase. A modified correlation that takes into account the location on the tie-line and thus volume ratio (*R*) and relative distance (rd) has been proposed and successfully tested. The behavior of batch and continuous systems in the presence and absence of *Bacillus subtilis* extract in systems with continuous bottom phase was also studied. The settling velocity was lower in the continuous than in the batch systems, and in both cases the initial rate was lower in the presence of *Bacillus* extract.

Keywords: Aqueous two-phase systems; Kinetics; Phase separation; Poly(ethylene glycol)

1. Introduction

The use of aqueous two-phase systems (ATPS) for the separation of biomolecules is gaining importance in biotechnology. In recent years partition coefficients have been optimized by manipulation of the properties of the system. For the large scale, industrial implementation of ATPS it is necessary to study fundamental aspects of these systems to gain some insight into their stability and for the design of appropriate equipment. The kinetics of phase separation in ATPS has been studied in terms of the physico–chemical properties of the phases (density, viscosity, interfacial tension) by measuring dispersion height as a function of separation time. Kaul [1] and Kaul et al. [2] found that the kinetic behavior depends greatly on which of the phases is continuous and that the properties of the continuous phase strongly influence the movement of the drops of the dispersed phase and hence phase separation.

Mistry et al. [3] developed a mathematical model

*Corresponding author. Tel.: +56 2 6784288; fax: +56 2 6991084; e-mail: juasenjo@cec.uchile.cl

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to describe the continuous, steady-state operation of an aqueous two-phase system for protein extraction. The model is based on steady state mass balances of the main system components and phase equilibrium data. Experimental data on the separation of α-amylase from *B. subtilis* supernatant in a PEG 4000/phosphate system was used [4]. This model has now been extended to account for phase separation and some aspects of continuous processing [5]. The rate of phase separation was correlated as a function of the density, surface tension and viscosity in both the continuous top and bottom phase regions. This model can be used to follow the effects of changes in key parameters which are essential to continuous operation.

In this paper we have investigated the effect of the location on the tie-line on the kinetics of phase separation in batch systems and the behavior of batch and continuous systems in the absence and presence of *Bacillus subtilis* extract.

2. Experimental

2.1. Reagents and chemicals

PEG 4000 with an average molecular mass of 4000 Da was obtained from Fluka Chemika (Seelza, Germany). PEG 1500 was obtained from Sigma Chemical Company (St. Louis, MO, USA). $MgSO_4 \cdot 7H_2O$ was obtained from Riedel-Haën (Seelza, Germany).

Stock solutions were prepared as follows: PEG 4000 50% w/w, PEG 1500 50% w/w, SO_4^{2-} 11.7% w/w prepared from $MgSO_4 \cdot 7H_2O$.

An extract of *B. subtilis* was prepared by centrifugation and crossflow filtration of fermentation supernatant. This extract had a total protein concentration of 0.55 mg/ml measured using the method of Bradford [6].

2.2. Batch systems

PEG 1500/sulphate systems were used. Phase diagrams of these systems have been published elsewhere [7]. Each experiment was done with and without extract of *B. subtilis*. Each experiment was done in duplicate. In those with *B. subtilis*, 25 μl of extract per 1 ml of system was added. Systems were mixed for 2 min in a Vortex mixer. The kinetics of phase separation was measured using the dispersion height as a function of time. The volume ratio and stability ratio of each system was also measured. The batch settlers were 3 cm high and 5 g systems were used.

2.3. Continuous systems

A mixer–settler (1400 g capacity) was used with PEG 4000/sulphate systems. Fifteen μl of extract of *B. subtilis* was added per each ml of system. The well mixed ATPS was injected into the settler using a peristaltic pump. The dispersion separated along the length of the equipment and the dispersion height was measured.

Three inlet geometries were built for the settler, Fig. 1 shows the three different geometries used. The dimensions of the square settlers were 60 cm long, 4 cm wide and 4 cm high. The baffle was 2 cm high and 3 cm wide. The prismatic inlet was 10 cm long, and the narrow end was 2 cm square, the other end was 4 cm by 4 cm and was attached to the basic settler which was 50 cm long, 4 cm wide and 4 cm high.

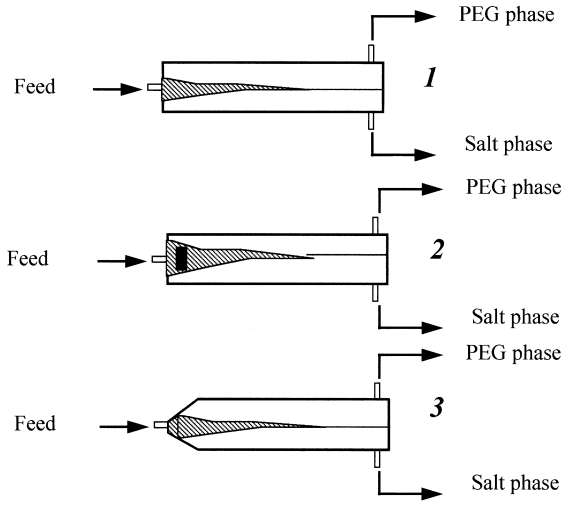

Fig. 1. Settler inlet geometries: (1) Square; (2) square with baffle; (3) prismatic.

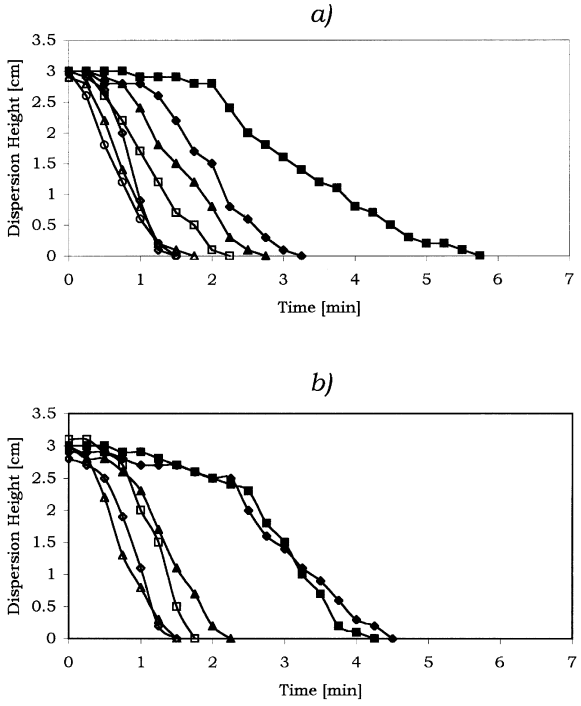

Fig. 2. Dispersion height vs. time for PEG 1500/sulphate systems with different mass ratios (R). The full (dark) symbols are systems with a continuous top phase, the open (empty) symbols are systems with a continuous bottom phase; (a) stability ratio of 0.34: (■) $R=9$, (◆) $R=5$, (▲) $R=3.28$, (□) $R=2.76$, (◇) $R=1.5$, (△) $R=1$, (○) $R=0.66$; (b) stability ratio of 0.37: (■) $R=9$, (◆) $R=6.25$, (▲) $R=3$, (□) $R=2.33$, (◇) $R=1.54$, (△) $R=0.875$.

3. Results aud discussion

3.1. Batch systems

The kinetics of phase separation was measured using dispersion height as a function of separation time with and without *B. subtilis* supernatant. When the top phase is continuous, observation of the dispersion height is more difficult than with a continuous bottom phase. The size of the drops is also much smaller when the top phase is continuous, which is important when trying to follow the movement of the drops. Another observation which is useful for the design of a mixer–settler is that phase separation is much more rapid when the bottom phase is continuous. This phenomenon has also been observed by Mistry [5]. Fig. 2 shows the kinetics of phase separation for PEG 1500/sulphate systems at two stability ratios (sr, Fig. 3) and with different volume ratios. Kaul et al. [2] used stability ratios of 0.18 and 0.50 whereas Mistry et al. [3] used a range of different ones. The highest were 0.37 and 0.34 in order to avoid the formation of another phase. In our case, small values of the stability ratio could not be used as sufficiently long tie-lines were needed. Also, in order to work in a sufficiently stable region of the phase diagram stability ratios of 0.34 and 0.37 were chosen, which showed quite different behavior. The

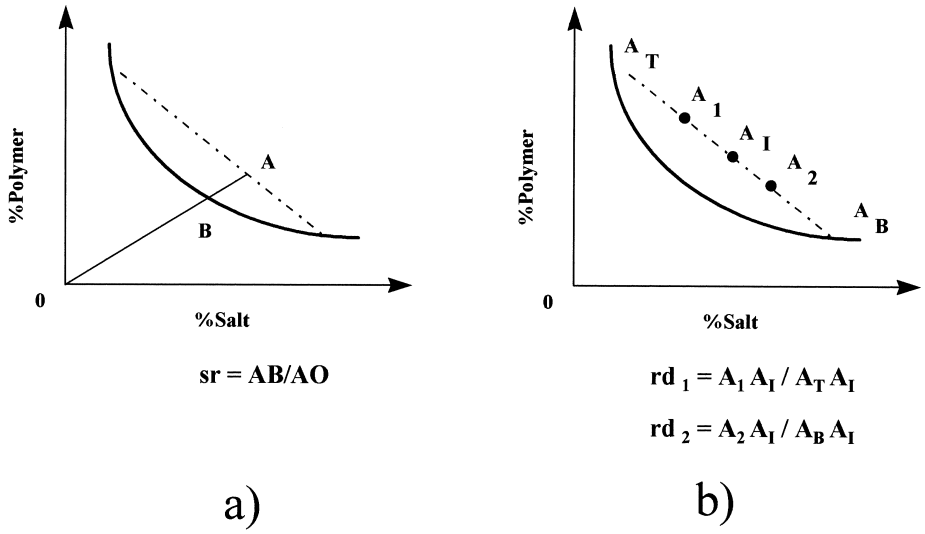

Fig. 3. Definition of stability ratio (sr) associated with a tie-line (a) and the relative distance (rd) in (b). B represents the point where the tie-line is 0 and A_I is the inversion point (to the left continuous top phase, to the right continuous bottom phase), the phase inversion line cuts the binodial at B.

dark (full) symbols represent systems with a continuous top phase. It is evident that as the volume ratio (R) decreases, the phase separation time is shorter and, that at the larger volume ratios there is an apparent ''lag'' time during which there is almost no decrease in the dispersion height.

Fig. 4 shows the kinetics of phase separation in PEG 1500/sulphate systems in the presence of extract of *B. subtilis*. As in the systems without extract, systems with a continuous bottom phase separate more quickly than those with a continuous top phase.

On comparison of Figs. 2 and 4, it is evident that the presence of cell extract has an important effect on phase separation time. For the stability ratio of 0.34 (a) at a volume ratio of 9 with extract it is ca. 35 min and without extract it is ca. 6 min. All systems with a continuous top phase take longer to separate in the presence of *Bacillus* supernatant. This is clearly shown in Fig. 4a,b. Fig. 2a,b and Fig. 4a,b show that systems with a continuous bottom phase show little difference in their settling times in the absence or presence of *Bacillus* supernatant.

This difference in separation time can be due to a number of factors caused by the material added (*Bacillus* supernatant) which include properties of the proteins and other molecules added and their interaction with the phase system which will affect the charge and size of the drops and the hydrophobicity, and thus their surface properties.

Why do systems with a continuous top phase take longer to separate than systems with a continuous bottom phase? It appears that the balance of forces on the drops during coalescence is different in each case. In general, three forces are acting on a drop during coalescence: gravitational, flotation and frictional, as shown in Fig. 5. The movement of a drop depends on the balance between these forces. The gravitational force depends on the density of the drops, flotation or frictional forces depend on the rheological properties of the phases. The frictional force always impedes drop movement. These forces together with the interfacial tension, determine the coalescence behavior and the characteristics of the dispersed phase. In ATPS the densities of phases are very similar so it is the flotation forces which determine the behavior of the drops. The ratio of the viscosities of the polymer and the salt phase can be

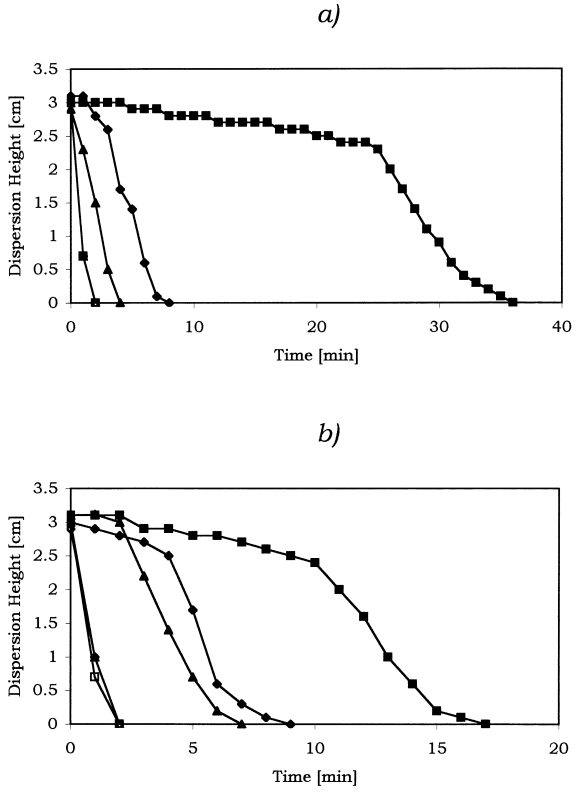

Fig. 4. Dispersion height vs. time for PEG 1500/sulphate systems with different mass ratios (R) with extract of *B. subtilis*. The full (dark) symbols are systems with a continuous top phase, the open (empty) symbols are systems with a continuous bottom phase. (a) stability ratio of 0.34: (■) $R=9$, (◆) $R=5.2$, (▲) $R=3.14$, (□) $R=1.3$, (◇) $R=1$, (△) $R=0.7$; (b) stability ratio of 0.37: (■) $R=5$, (◆) $R=4.16$, (▲) $R=2.75$, (□) $R=1.72$, (◇) $R=1.23$, (△) $R=0.875$.

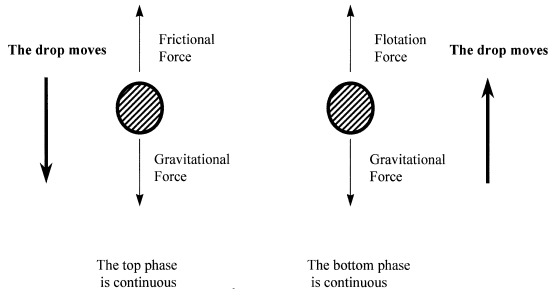

Fig. 5. Diagram showing the different forces acting on a drop depending on which phase is continuous.

very large (5 to 50 times), the salt phase being much less viscous than the polymer phase. When the bottom phase is discontinuous, the coalescing drops must descend through the polymer phase. As the polymer phase has a higher viscosity, the friction between the drops and the phase is high, hence the separation time is longer. When the bottom phase is continuous, drops of the top phase move through the bottom phase which has a much lower viscosity, favoring coalescence.

When changing from a continuous top phase to a continuous bottom phase (crossing the phase inversion point) the size of the drops increases [2,3]. In the case of a continuous top phase (small drops), the surface charge density of the drops is high and coalescence is slower due to electrical repulsion. This phenomena has been clearly described in aqueous–organic two-phase systems [8–10]. When the bottom phase is continuous, the drops are larger and have a lower surface charge density and hence, less resistance to coalescence.

The process of phase separation appears to be composed of several stages as shown in Fig. 6. In this figure, three stages are shown. Stage I is the region of coalescence of the small drops into larger drops [11]. During this stage the dispersion height decreases very little. In systems with a continuous top phase this stage tends to be longer compared with systems with a continuous bottom phase. In zone II, the coalesced drops move to the interface and phase separation occurs as seen by the decrease in dispersion height. Zone III is a region of inertia where very large drops (3–5 mm diameter) do not coalesce and stay ''resting'' at the interface thus slowing down the process of phase separation. Eventually they coalesce into the phase. This is more marked in systems with a continuous bottom phase.

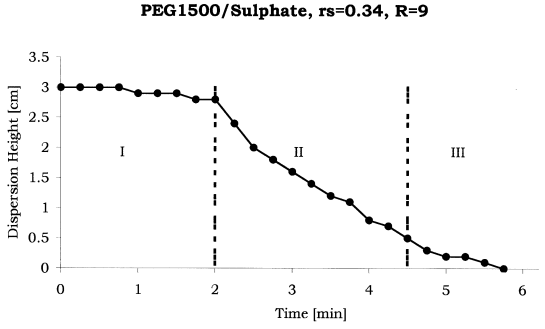

Fig. 6. Stages of phase separation.

3.2. Correlations for the kinetics of phase separation

In order to predict the phase separation rate of a system it is necessary to establish mathematical correlations for the kinetics of phase separation.

Mistry et al. [3] and Mistry [5] correlated the rate of phase separation with the physical properties of the system using the expression proposed by Golob and Modic [12] for aqueous–organic systems:

$$V = a \times \left(\frac{\Delta\rho}{\rho_C}\right)^b \times \left(\frac{\mu_C}{\mu_D}\right)^c \times \left(\frac{\sigma}{\sigma_W}\right)^d, \quad (1)$$

where V is the rate of phase separation, ρ the density, μ the viscosity, σ the interfacial tension, and σ_W corresponds to the surface tension of water. $\Delta\rho$ corresponds to the density difference between the phases, subscripts C and D refer to the continuous and discontinuous phase; a, b, c and d are constants to be determined. This correlation was tested in detail and correlated extremely well the points on each side of the phase inversion point, but the effect of the location on different positions on the tie-line was not investigated. The results shown in the previous section clearly show that, particularly when the top phase is continuous, the actual location on

Table 1
Parameters fitted for Eqs. (4) and (5)

Continuous phase		k	N	R^2
Without supernatant				
rd1: sr=0.37	Top	0.428	−0.595	0.560
rd1: sr=0.34	Top	1.017	−0.349	0.944
R: sr=0.37	Top	4.326	−0.877	0.928
R: sr=0.34	Top	1.483	−0.240	0.705
rd2: sr=0.37	Bottom	2.437	0.074	0.822
rd2: sr=0.34	Bottom	3.104	0.073	0.999
R: sr=0.37	Bottom	2.194	−0.054	0.667
R: sr=0.34	Bottom	2.543	−0.140	0.799
With supernatant				
rd1: sr=0.37	Top	0.161	−1.128	0.900
rd1: sr=0.34	Top	0.221	−0.793	0.971
R: sr=0.37	Top	3.033	−1.181	0.988
R: sr=0.34	Top	2.211	−1.233	0.938

the tie line, and thus relative mass of top and bottom phase (R), has an effect on the rate of phase separation.

To better represent the settling behavior of ATPS, the expression was modified to include a term to take this effect into account. Either, the volume (mass) ratio (R) or relative distance to the phase inversion point (rd) can be used for this purpose. The relative distance (rd) is defined in Fig. 3. rd is a new concept related to a tie-line. It represents for each phase, in a scale from 0 (phase inversion point) to 1 (transition to one phase only), how far that particular system is from phase inversion, which does not happen with the variable R. It treats both phases in the same way

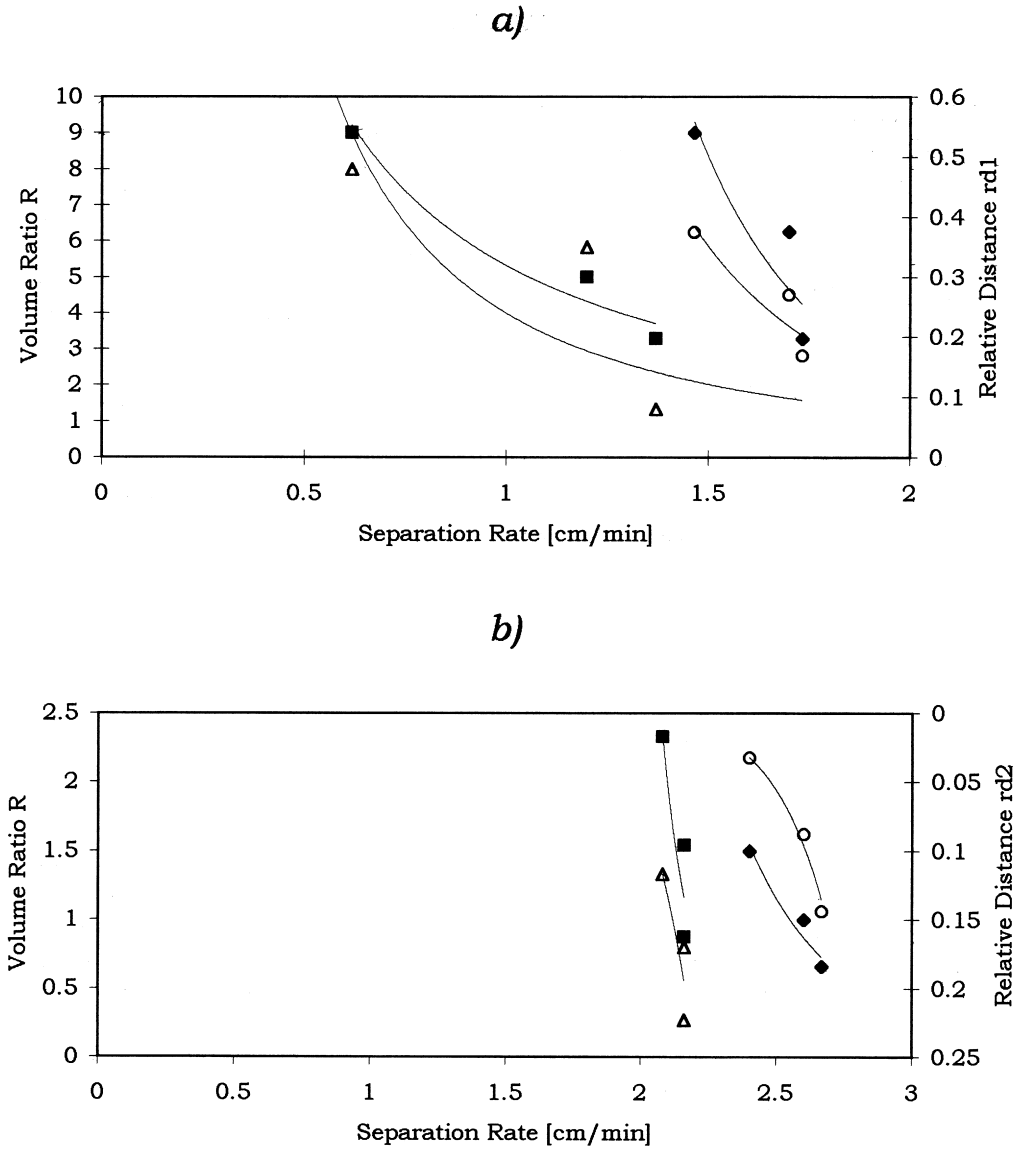

Fig. 7. Comparison of experimental data and the proposed model for the rate of phase separation in the absence of extract of *B. subtilis* in PEG 1500/sulphate. The points represent experimental data and the dotted lines the correlation. (a) Continuous top phase: (■) R: sr=0.37, (◆) R: sr=0.34, (△) rd1: sr=0.37, (○) rd1: sr=0.34; (b) continuous bottom phase: (■) R: sr=0.37, (◆) R: sr=0.34, (△) rd2: sr=0.37, (○) rd2: sr=0.34.

as it is independent of which phase is the top one and which is the bottom one. It also gives an idea of the phase stability of a particular system. Those with small values of rd (near the phase inversion point) will be thermodynamically less stable as has been recently found by experiment [13].

The modified equation in both cases is given by:

$$V = a \times \left(\frac{\Delta\rho}{\rho_C}\right)^b \times \left(\frac{\mu_C}{\mu_D}\right)^c \times \left(\frac{\sigma}{\sigma_W}\right)^d \times (\mathrm{rd})^N \quad (2)$$

$$V = a' \times \left(\frac{\Delta\rho}{\rho_C}\right)^{b'} \times \left(\frac{\mu_C}{\mu_D}\right)^{c'} \times \left(\frac{\sigma}{\sigma_W}\right)^{d'} \times (\mathrm{R})^{N'}. \quad (3)$$

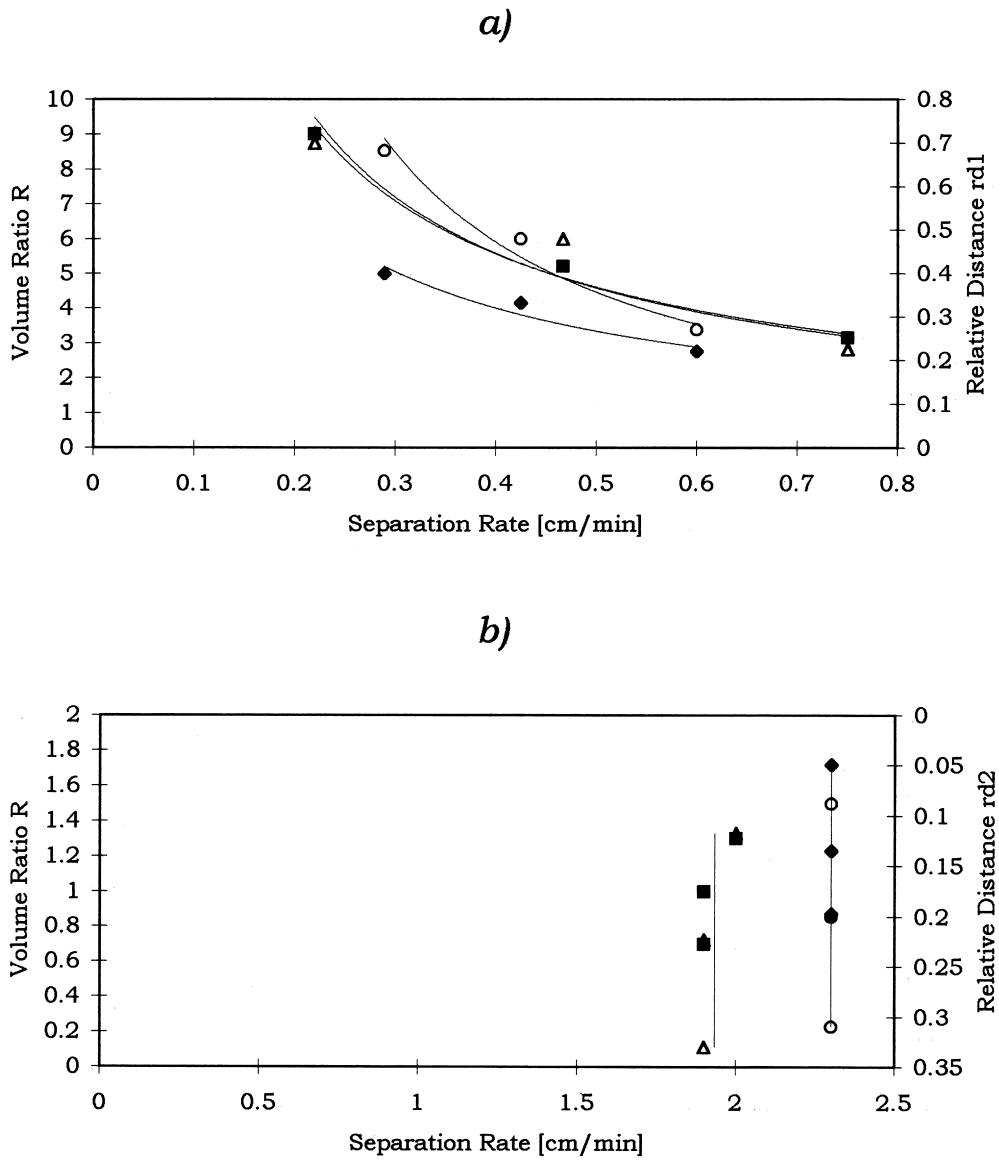

Fig. 8. Comparison of experimental data and the proposed model for the rate of phase separation with extract of *B. subtilis* in PEG 1500/sulphate. The points represent experimental data and the dotted lines the correlation as in Fig. 7; (a) continuous top phase; (b) continuous bottom phase.

This correlation has been tested with the systems on the same tie-lines investigated in the previous section (one corresponding to a stability ratio of 0.34 and the other to 0.37), which, by definition, have the same top and bottom phase composition and thus the same physical properties in Eqs. (2) and (3). The equations are thus reduced to:

$$V = k \times (\mathrm{rd})^{N} \quad (4)$$

$$V = k' \times (R)^{N'}. \quad (5)$$

These equations were used to correlate the data for PEG 1500/sulphate systems in the presence and absence of extract of *B. subtilis* as shown in Figs. 7 and 8. The values obtained for the parameters are shown in Table 1.

It is clear that the location on the tie-line has very little effect when the bottom phase is the continuous

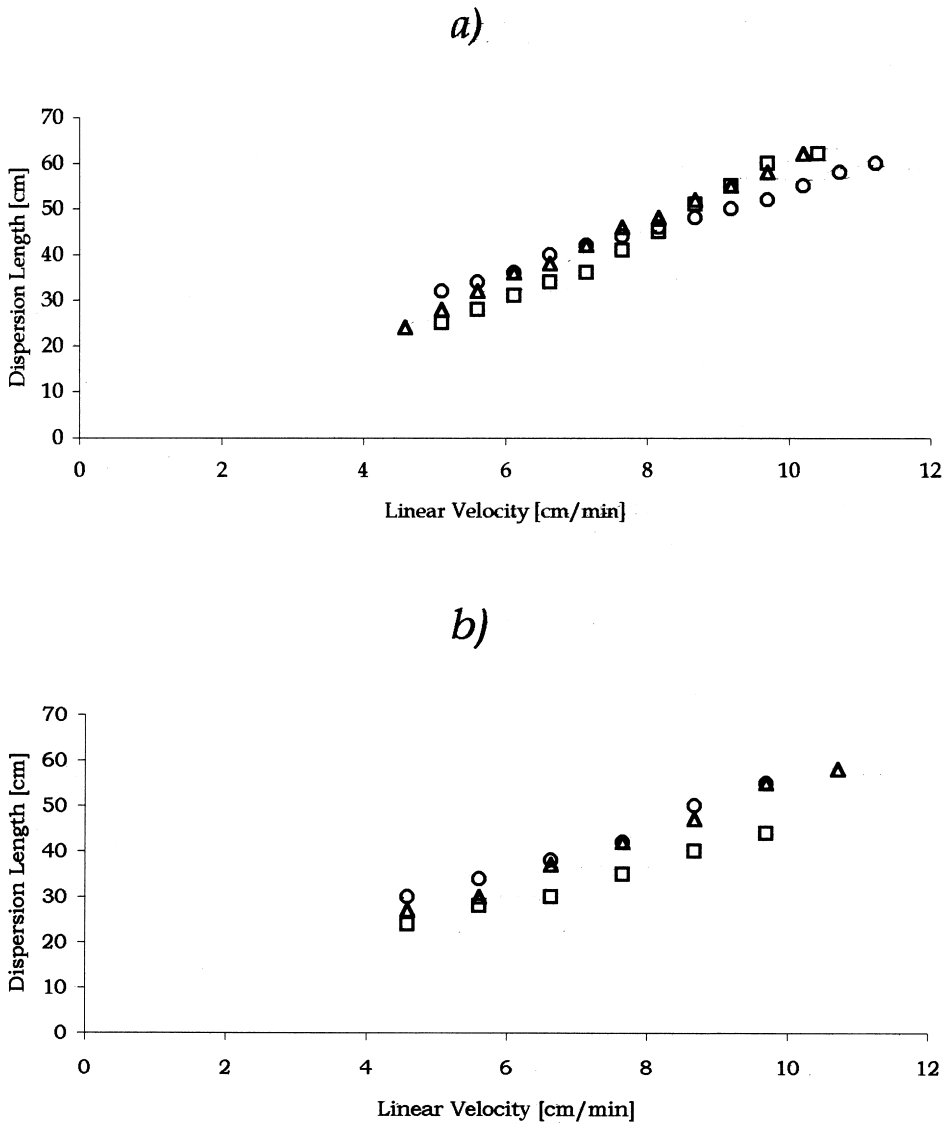

Fig. 9. Dispersion height as a function of linear velocity in PEG 4000/sulphate systems (sr = 0.34, $R = 0.66$) in a continuous settler with three inlet geometries; (a) without extract of *B. subtilis*; (b) with extract of *B. subtilis*; (□) square with baffle, (△) square, (○) prismatic.

one, but a strong effect when the top phase is continuous; the larger the top phase (larger *R* or rd1) the slower the separation rate. In the presence of *Bacillus*, settling times are larger than in its absence particularly when the top phase is very large. This is not the case when the bottom phase is continuous.

3.3. Continuous systems

One of the advantages of using aqueous two-phase systems to separate proteins is the possibility of relatively simple continuous operation. This requires the design of appropriate settlers. An important question would be the design of the inlet geometries of which three different ones were chosen (square, square with baffle and prismatic, Fig. 1). The dispersion height was measured as a function of separator length at different injection velocities, which resulted in different linear velocities in the settler, in the presence and absence of extract of *B. subtilis* (Fig. 9). In the absence of *B. subtilis* extract there is very little difference between all three geometries. The presence of extract makes little difference which would be expected in systems where the bottom phase is continuous. In this case

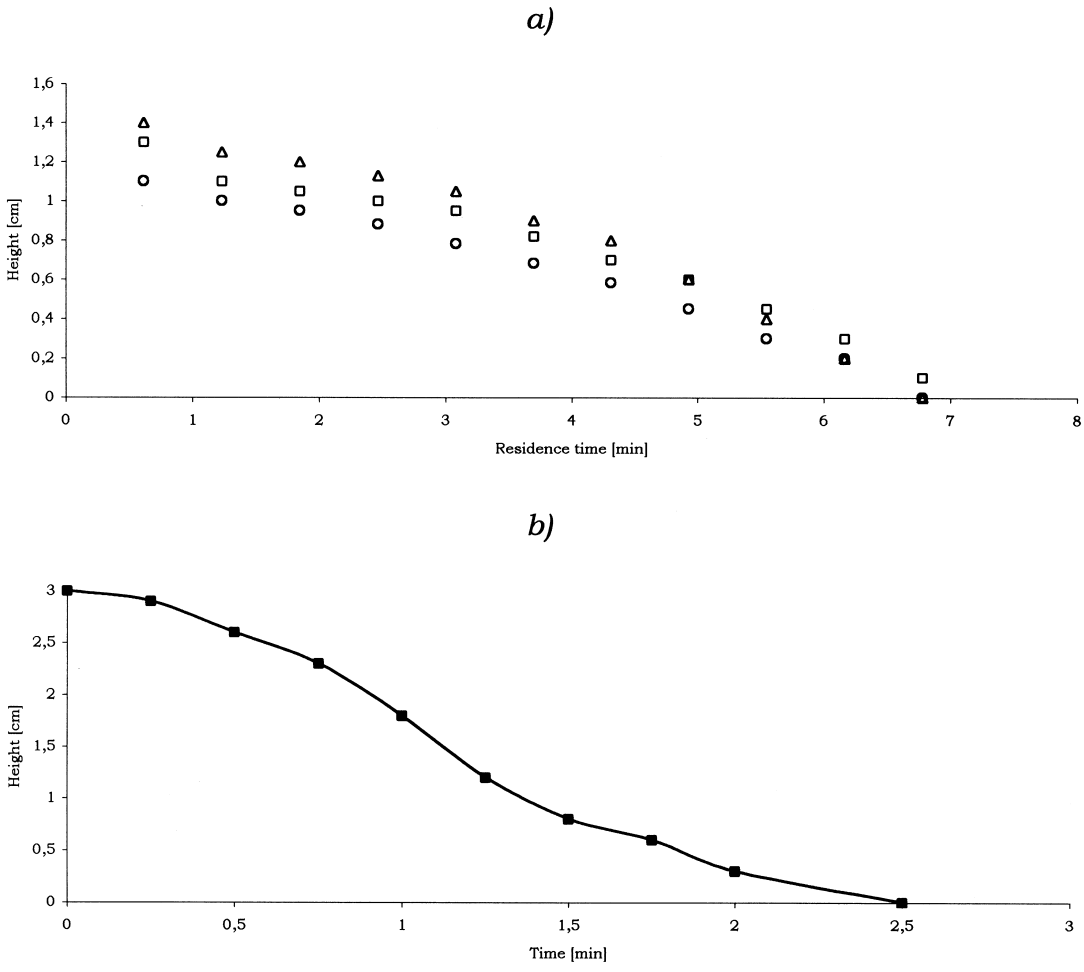

Fig. 10. Dispersion height as a function of (a) residence time in a specific square section of the continuous settler [calculated as (dispersion length×cross sectional area)/(volumetric flow-rate)]; (b) time in the batch system (system characteristics as in Fig. 9); (□) square with baffle, (△) square, (○) prismatic.

however, the higher linear velocities give slightly shorter separation lengths along the settler for the square inlet configuration with baffle. The geometry of the square inlet with a baffle also prevents the accumulation of dispersion in the corners of the settler and stops the formation of ''macrofluids'' which hinder the adequate flow of dispersion into the settler.

Figs. 10 and 11 compare the dispersion height as a function of residence time in a specific square section of the continuous settler to the dispersion height as a function of time in the batch settler in the absence (Fig. 10) and presence (Fig. 11) of *Bacillus subtilis* extract. Both correspond to systems with a continuous bottom phase. Even though in the batch systems the starting height is larger (2 to 3 times), the total phase separation time is much smaller. In both cases (batch and continuous) the initial separation rate is smaller in the presence of *Bacillus subtilis* extract. In batch the total separation time, however, is similar whereas in continuous it is longer in the presence of *Bacillus*.

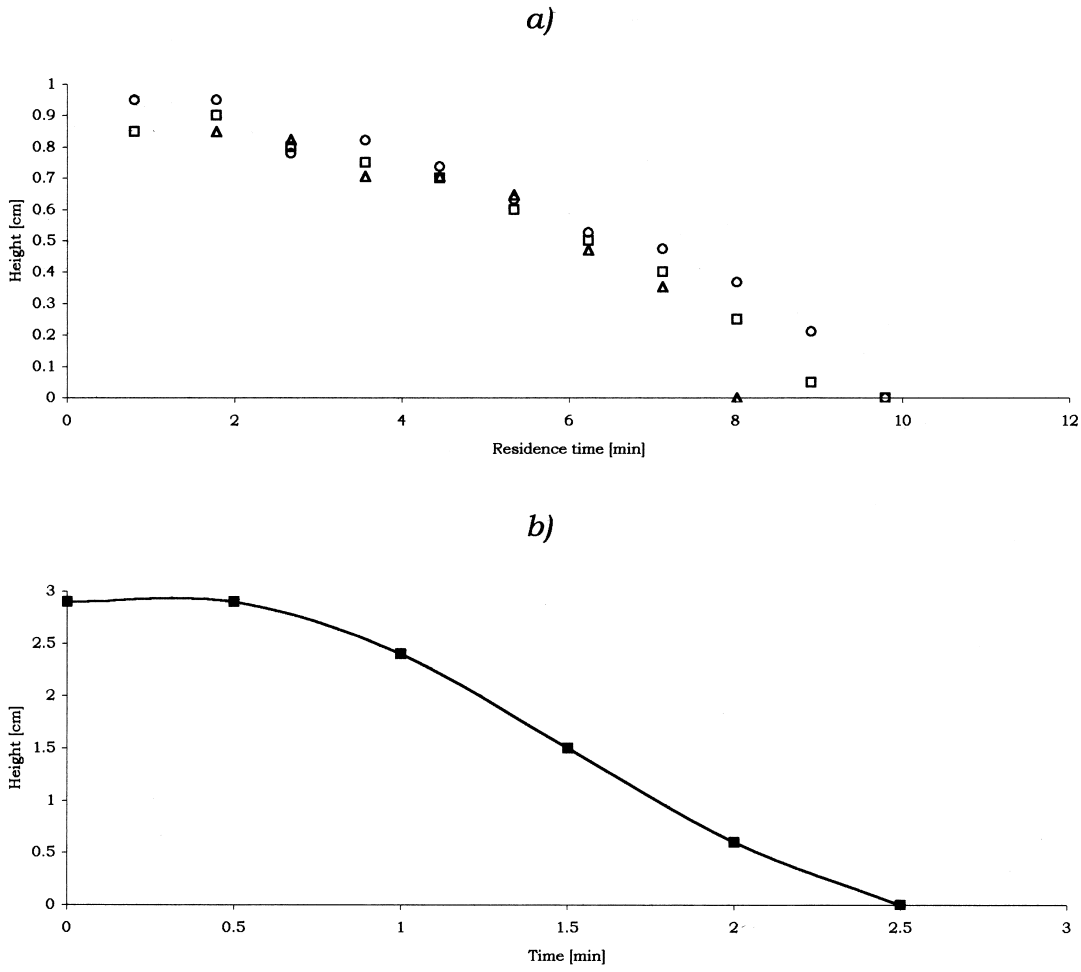

Fig. 11. Dispersion height as a function of (a) residence time in a specific square section of the continuous settler [calculated as (dispersion length×cross sectional area)/(volumetric flow-rate)]; (b) time in the batch system in the presence of extract of *Bacillus subtilis* (system characteristics as in Fig. 9); (□) square with baffle, (△) square, (○) prismatic.

4. Conclusions

The effect of the tie-line location on the kinetics of phase separation in batch PEG/salt ATPS in the absence and presence of *Bacillus subtilis* extract has been investigated. Phase separation is much faster when the bottom phase is continuous and in this case the location on the tie-line and the presence or absence of *Bacillus subtilis* extract makes little difference. When the top phase is continuous the relative sizes of the phases (phase ratio, *R*, relative distance on tie-line, rd) has an important effect, the larger the top phase (larger *R* and rd) the slower the phase separation. The presence of *Bacillus* extract also makes the operation slower which is more marked at the largest values of *R* (and rd).

At the largest volume ratios (*R* or rd) three different settling regions have been recognised (I, II and III in Fig. 6). A region of coalescence of small drops into larger drops with virtually no decrease in the dispersion height (I); a region where the drops move to the interphase and dispersion height drops dramatically (II); and a region of inertia where the drops ''wait'' near the interphase in order to coalesce (III).

A modified correlation that takes into account the location on the tie-line and thus volume ratio (*R*) and relative distance (rd) has been proposed and successfully tested.

The behavior of batch and continuous systems in the presence and absence of *Bacillus subtilis* extract in systems with continuous bottom phase was also studied. The design of the inlet geometry of the continuous settler (square, square with baffle and prismatic) had little effect on the overall settling rate. Also, little difference was found in the settling rates in the batch systems. The settling velocity was lower in the continuous than in the batch systems and in both cases the initial rate was lower in the presence of *Bacillus* extract. In batch the overall settling time was similar in the absence and presence of supernatant, whereas in the continuous settler, the rate was slower in its presence.

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References

[1] A. Kaul, PhD Thesis, Rationale for the selection of aqueous two-phase systems for the purification of recombinant proteins expressed in *Escherichia coli*, The University of Reading, UK, 1996.
[2] A. Kaul, R.A.M. Pereira, J.A. Asenjo, J.C. Merchuk, Biotechnol. Bioeng. 48 (1995) 246.
[3] S.L. Mistry, A. Kaul, J.M. Merchuk, J.A. Asenjo, J. Chromatogr. A 741 (1996) 151.
[4] A.S. Schimdt, A.M. Ventom, J.A. Asenjo, Enzyme Microb. Technol. 16 (1994) 131.
[5] S.L. Mistry, PhD Thesis, Mathematical modelling and computer simulation of aqueous two-phase continuous protein separation, The University of Reading, UK, 1997.
[6] J.J. Sedmak, S.E. Grossberg, Anal. Biochem. 79 (1977) 544.
[7] B. Zaslavsky, Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications, Marcel Dekker, New York, 1994.
[8] C. Hanson, Recent Avances in Liquid–Liquid Extraction, Pergamon Press, Budapest, Hungary, 1971.
[9] V. Levich, Physicochemical Hydrodynamics, Prentice Hall, Englewood, NJ, 1962.
[10] G.S. Ladaha, T.E. Degalelsan, Transport Phenomena in Liquid Extraction, Tata McGraw-Hill, New Delhi, India, 1976.
[11] A.W. Adamson, Physical Chemistry of Surfaces, 3rd Edition, Wiley-Interscience Publications, John Wiley and Sons, NY, (1976).
[12] J. Golob, R. Modic, Coalescence of liquid/liquid dispersions in gravity settlers, Trans IChemE, 55 (1977).
[13] J.C. Merchuk, personal communication.