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Effect of biological suspensions on the position of the binodal curve in aqueous two-phase systems

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Abstract

This study is concerned with the influence of biological suspension on the position of the binodal curve in aqueous two-phase systems (ATPSs). Three different biological suspensions (i.e., disrupted yeast, *E. coli* homogenate and fermentation broth from *Trichoderma harzianum*) were selected and their impact upon ATPS performance was evaluated on the basis of changing volume ratio (V_r) and the position of the binodal curve of biological ATPSs (added with biomass). Biological ATPSs with initial V_r greater than 1 and long (>40%, w/w) tie-line length (TLL), exhibited significant changes in V_r when compared with that from non-biological systems. Such behaviour was associated with the top phase biomass accumulation. It was shown that the addition of the biological suspensions used in this study to ATPSs caused the binodal curve to displace towards the origin, which was associated with the critical contribution of the bio-polymer (present in the systems) to the phase formation. The practical implementation of ATPSs for the purification of biological materials exploiting the information reported in this study is discussed.

Keywords: Aqueous two-phase systems; Binodal curve

1. Introduction

In the recovery of macromolecules from fermentation broth and biological extracts, a practical approach using aqueous two-phase systems (ATPSs) comprising mixtures of poly(ethylene glycol) (PEG)–phosphate has been successfully used for particle and solute handling [1–5]. However, the generic application of extraction ATPS processes for the recovery of value products from biological suspensions requires understanding of the process disadvantages associated with the characterisation of loaded working ATPSs [6].

In process development, the effect of added biological suspensions on partitioning characteristic of a system must be considered. Fractionation of biological suspensions in ATPSs for the recovery of proteins, potentially has implications on the performance of the ATPS [7,8]. This behaviour potentially influences the recovery of target proteins in the extraction step. It has been reported that the presence of biomass in ATPSs, has a strong modifying influence on the position of the binodal curve of the phase diagram [9,10]. In these reports, displacements toward the origin of the binodal curve were attributed to the presence of cell debris and intracellular polymer, which reduced the amount of chemical required to form two phases. Such behaviour has

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been exploited to eliminate cell debris during protein recovery. In this context, we have shown [11] that the binodal curve moved away from the origin at high concentration of PEG in the presence of whey. Such behaviour was attributed to the presence of residual fat of the cheese whey. From the process point of view, the reported behaviour of the binodal curve with the presence of whey implies that for an ATPS process for the recovery of whey proteins, more chemicals (i.e., PEG and phosphate) need to be used to form two phases.

The present study is concerned with the influence of biological suspension on the position of the binodal curve in ATPSs. It can be established that current explanation of the mechanism governing the effect of biological suspensions on the phase formation of the ATPS is not available. Herein, we extended the existing knowledge on this area of research to correlate the presence of biological suspensions in ATPSs with the modified phase diagram of working loaded systems.

2. Experimental

2.1. Biological suspensions

Three experimental models; (i) disrupted yeast, (ii) *E. coli* homogenate and (iii) *Trichoderma harzianum* micelial culture were selected for the present study. These experimental models are referred as ‘‘biological suspensions’’ throughout the paper. Brewery yeast was slurried (30%, wet w/v) in 20 m*M* phosphate buffer, pH 7.0 and disrupted in a APV-Gaulin type homogeniser (APV, Crawley, UK) at operating conditions described previously [12]. Fermentation of *E. coli* and disruption were carried out according to Cueto [13]. Fermentation broth of *Trichoderma harzianum* was obtained as described Serrano-Carreon et al. [14]. The biological suspensions so generated were stored at 4°C until use.

2.2. Aqueous two-phase experiments

The binodal curves for non-biological systems were estimated by the cloud-point method [15] using PEG (Sigma, St. Louis, MO, USA) of nominal molecular mass, 1450 g/gmol (50%, w/w, stock solution) and dipotassium orthophosphate (Sigma) (30%, w/w). The experimental aqueous two-phase systems comprised pre-determined quantities of solid PEG 1450 and phosphate were mixed with the corresponding biological suspension to give a final weight of 15 g. In all the experiments the biomass concentration of biological suspensions was fixed at 20% (wet w/w). Complete phase separation was achieved by low-speed batch centrifugation at 1500 *g* for 20 min at 25°C. Tie-line length (TLL) for all the ATPSs was estimated as described previously [11].

2.3. Effect of biological suspensions on the ATPS volume ratio

In order to examine the impact of added biological suspensions upon ATPS volume ratio a total of 15 (A to O in Fig. 1) systems were selected (refer to Table 1 for their composition). These ATPSs were characterised by changing volume ratio (V_r) and three different TLLs (i.e., 32, 40 and 58%, w/w), that defined their positions in the phase diagram with respect to the binodal curve. The systems were assembled as described above. For each ATPS selected two set of experiments were carried out; one defined by the sole presence of PEG, phosphate and deionised water (i.e., blank systems) and the other

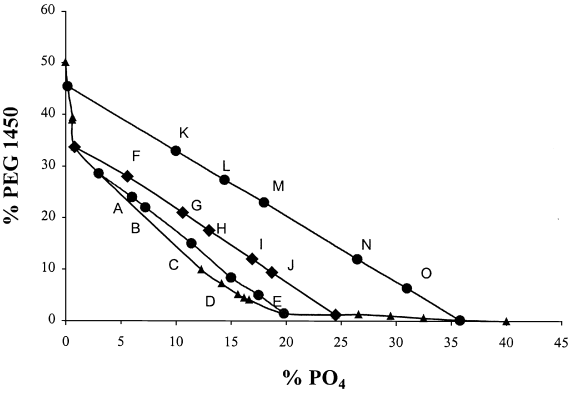

Fig. 1. ATPSs selected for the evaluation of the effect of added biological suspensions on the volume ratio. The 15 systems (A to O; refer to Table 1 for their composition) were selected close to the binodal (A to E), distant to the binodal (F to J) and further distant to the binodal curve (K to O) to evaluate the impact of the addition of the biological suspensions on the volume ratio. All systems were assembled as described in Section 2.

Table 1

Composition of the ATPSs selected for the evaluation of changes on the volume ratio (V_r)[a]

System	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)
A	24.0	6.0	32
B	22.0	7.2	32
C	15.0	11.4	32
D	8.4	15.0	32
E	5.0	17.5	32
F	24.0	8.5	40
G	21.0	10.6	40
H	17.5	13.0	40
I	12.0	16.9	40
J	9.4	18.7	40
K	33.0	10.0	58
L	27.4	14.4	58
M	23.0	18.0	58
N	12.0	26.5	58
O	6.4	31.0	58

[a] The position in the phase diagram of the ATPS used (A to O) is depicted in Fig. 1. The systems were selected close to the binodal (A to E), distant to the binodal (F to J) and further distant to the binodal curve (K to O) to evaluate the impact of biological suspensions on the volume ratio. All systems were assembled and characterised as described in Section 2.

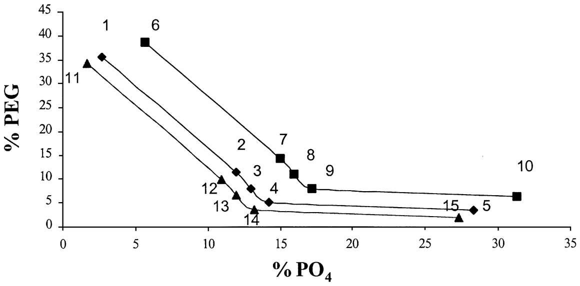

Fig. 2. ATPSs selected for the evaluation of the effect of added biological suspensions on the phase formation. The 15 systems (1 to 15; refer to Table 2 for their composition) were selected on the binodal, (1 to 5), above the binodal (6 to 10) and below the binodal curve (11 to 15) to evaluate the impact of the addition of the biological suspensions on the phase formation. All systems were assembled as described in Section 2.

with the corresponding biological suspensions instead of deionised water. After mixing for 30 min, the phases were separated by batch centrifugation (25°C for 20 min at 1500 *g*). The volume ratio of the top and bottom phase from biological and non-biological (blank) systems was estimated in graduated centrifuge tubes.

2.4. Effect of biological suspensions on the position of the binodal curve

To study the effect of added biological suspensions to ATPSs on the position of the binodal curve, two different approaches were adopted. A total of 15 ATPSs were selected (see Fig. 2 and refer to Table 2 for their composition). The systems selected were characterised by (i) those located on the binodal curve (1 to 5), (ii) those located above the binodal curve (6 to 10) and (iii) those located below the binodal curve (11 to 15). The position of the systems was selected to examine the degree of displacement of the binodal curve in the presence of biological suspensions. The biological and non-biological ATPSs were assembled as described before, with this approach the displacement of the binodal curve was

Table 2

Composition of the ATPSs selected to evaluate the effect of the biological suspensions on the position of the binodal curve in the phase diagram[a]

System	PEG (%, w/w)	Phosphate (%, w/w)
1	35.6	2.7
2	11.3	12.0
3	8.0	13.0
4	5.0	14.2
5	3.2	28.3
6	38.6	5.7
7	14.3	15.0
8	11.0	16.0
9	8.0	17.2
10	6.2	31.3
11	33.1	0.7
12	8.8	10.0
13	5.5	11.0
14	2.5	12.2
15	0.7	26.3

[a] The position of the ATPS used (1 to 15) with respect to the binodal curve is depicted in Fig. 2. The systems were selected on the binodal (1 to 5), above the binodal (6 to 10) and below the binodal curve (11 to 15) to evaluate the impact of biological suspensions on the phase formation. All systems were assembled as described in Section 2.

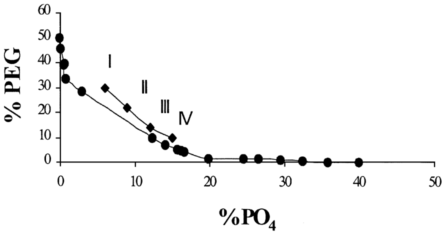

Fig. 3. ATPSs selected for the evaluation of the effect of added biological suspensions on the position of the binodal curve. The four systems (I to IV; refer to Table 3 for their composition) were selected close to the binodal curve to evaluate the impact of the addition of the biological suspensions on the position of the binodal curve. All systems were assembled as described in Section 2.

evaluated on the basis of the formation or not of two phases. An alternative approach was also used to examine the impact of the added biological suspensions on the position of the binodal curve. Four systems close to the binodal were selected (I to IV in Fig. 3 and refer to Table 3 for their compositions). Once the systems were carefully constructed (as described above), addition of the corresponding biological suspension (i.e., disrupted yeast, *E. coli* homogenate and fermentation broth from *T. harzianum* culture) was started. This was repeated for each systems, until the existence of a biphasic system was not observed (monophasic region). The total amount of the each biological suspension used was recorded and used to estimated the composition (PEG and phosphate concentration) of the resulting systems.

Table 3
Composition of the ATPSs selected to evaluate the effect of biological suspensions on the position of the binodal curve on the phase diagram[a]

System	PEG (%, w/w)	Phosphate (%, w/w)
I	30	6
II	22	9
III	14	12
IV	10	15

[a] The position in the phase diagram of the ATPS used (I to IV) is depicted in Fig. 3. All systems were assembled as described in Section 2.

3. Results and discussion

3.1. Effect of biological suspensions on the ATPS volume ratio

Aqueous two-phase systems are formed above critical concentrations of polymer and salt as described by the binodal curve. Commonly, the characterisation (e.g., estimation of; TLL, V_r, top and bottom phase concentration, etc.) of the ATPS is performed in non-biological systems. However, it is known that when complex systems are used, the presence of the biological suspensions have an impact on the final characteristics of the ATPS (e.g., TLL, V_r, position of the binodal curve, etc.). In the particular case of the volume ratio, added biological suspensions have an interesting effect. The accumulation of the biomass to either phase caused the volume of that phase to increase. Thus, the volume ratio is increased by top-phase biomass accumulation or decreased by bottom-phase biomass accumulation. This phenomenon may also affect the position of the binodal curve, which is discussed later in this paper. In the current study, ATPSs both close and distant to the binodal curve were selected to examine the behaviour of the volume ratio of biological systems. For these experiments, pH and temperature of the systems was kept constant at 7.0 and 25°C, respectively. In addition, the amount of the corresponding biological suspension (with a concentration of 20%, wet w/w) loaded to the ATPS was fixed at 7.0 g for the 15 g experiments. It was expected that any changes in the volume ratio of the ATPSs could be attributed to the different nature of the biological suspensions used.

In general the determination of the volume ratio for loaded ATPSs requires a clear definition of top, bottom and any other phase which develop. The latter clearly depends on the nature and complexity of the biological suspensions. To simplify the estimation of the volume ratio in the biological ATPSs, when the formation of an interface was observed, this was considered as a part of the top or bottom phase according to the initial volume ratio of the non-biological ATPSs. Fig. 4 illustrates the effect of added biological suspensions on the volume ratio for systems close and distant to the binodal curve. For ATPSs “A” to “E” close to the binodal curve (Fig. 4a) it is clear that changes in the volume ratio occurred at short TLL (i.e., 32%, w/w) for the systems added with disrupted yeast and *E. coli* homogenate. In the latter case, at high volume ratio (greater than 5.0) a decreases in the volume ratio of the biological systems was observed compared to that from the blank systems. This implied that biomass was accumulated in the bottom phase. In contrast, systems added with disrupted yeast exhibited a significant increase in the volume ratio, which can be associated with an accumulation of biomass in the top phase. Although a clear explanation for such opposite behaviour is not available, the sensitivity of the ATPSs close to the binodal curve can be used for a possible explanation. Albertsson [1] reported that ATPSs located close to the binodal curve exhibited certain sensitivity to changes in the systems composition. Small changes in the composition of PEG and phosphate caused by different factors (as in this case by the addition of biological suspensions), resulted in great changes in the phase composition of the systems and, as a result, in the final characteristics (V_r in this particular case) of the ATPS.

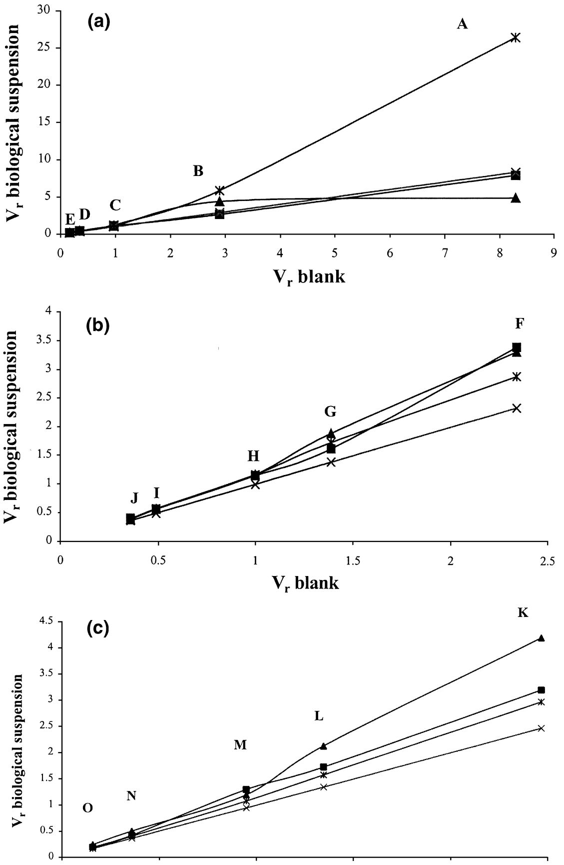

Fig. 4. Effect of added biological suspensions on the volume ratio. The position in the phase diagram of the ATPS used (A to O; refer to Table 1 for their composition) is depicted in Fig. 1. The impact of the addition of disrupted yeast (*), *E. coli* homogenate (▲) and *T. harzianum* fermentation broth (■) on the resulting volume ratio was compared with that from the blank (×) systems. ATPSs close to the binodal (a), distant to the binodal (b) and further distant to the binodal curve (c) were assembled as described in Section 2.

In the case of ATPSs comprising TLLs of 40% and 58% (w/w) (distant to the binodal curve) and volume ratio greater than 1 (as estimated from the blank systems) an increase in the volume ratio of the biological suspension ATPSs was observed (see Fig. 4b and c). Such a situation may be associated with the increase of the chemical phase forming components (as TLL increases) and to the position of the systems in the phase diagram (upper left part of the diagram; which it has been reported that favours the accumulation of biomass in the top phase; [16]). As a consequence the effect of the biomass accumulation in the top phase on the V_r was more significant (in comparison to the system close to the binodal; Fig. 4a). In these systems, a decrease in V_r for all the ATPSs studied, was not observed (such as in the case of systems close to the binodal; see ATPSs loaded with *E. coli* homogenate in Fig. 4a). Such behaviour can be associated to the robustness of the ATPSs distant to the binodal curve. The increased volume ratio of the ATPSs added with disrupted yeast differs only slightly from that of the ATPSs added with *E. coli* homogenate or fermentation broth of *T. harzianum*. In contrast, ATPSs with an initial volume ratio less than 1, exhibited a minimum effect of the added biomass on the volume ratio (see ‘‘G’’ to ‘‘J’’ ATPS in Fig. 4b and ‘‘M’’ to ‘‘O’’ ATPS in Fig. 4c). Here, it seems that the accumulation of the biomass in the large bottom salt-rich phase had a small effect on the volume of such phase and as a consequence in the final V_r.

3.2. *Effect of biological suspensions on the position of the binodal curve*

The position of the binodal curve in the phase diagram can be affected by several system parameters (e.g., system pH, molecular mass of PEG, type of salt, temperature, etc.). As an example of such phenomenon, recently it was shown [17] that the binodal curve moved to higher concentrations of PEG and salt or dextran in the presence of high concentration of urea (i.e., 10–30%). This knowledge is currently being exploited for the recovery of recombinant proteins using ATPSs. Additionally, the exploitation of ATPSs in the fractionation and recovery of compounds from biological suspensions have revealed problems associated with the addition of biomass to the ATPSs [11,12]. However, reports discussing the effect of the biological suspension in the position of the binodal curve are not common. In this context, it has been addressed that the presence of biomass has a strong influence on the position of the binodal curve [9,11]. In the present research to extend this type of work, three different biological suspensions were selected to examine their effect on the position of the binodal curve. Such selection was made in order to correlate the diversity of the biological suspensions with the modified phase diagram of working (biological) loaded systems.

The high content of biomass prevented the use of the turbidity method described by, for example, Albertsson [1]. Alternative methods involve the determination of the concentration of phase components chemicals in the top and bottom phase of ATPSs exploiting chromatography analysis [17–19]. However, such methods require extensive sample preparation when applied to complex systems. To study the effect of biological suspensions on the position of the binodal curve, the phase diagram was estimated using selected points in the presence of different biological suspensions (see Experimental). Furthermore, a comparison between the formation of selected ATPSs with the presence of biomass and those with the absence of biomass was made.

A first attempt to examine the effect of added biological suspensions to ATPSs on the position of the phase diagram was carried out using selected ATPSs distributed between the mono- and the biphasic region (see Fig. 2). The results showed that at a high concentration of salt (i.e., 26%, w/w; system 15 in Fig. 2) the biological ATPSs exhibited two-phase formation compared with the blank systems (which under the same conditions did not exhibited two-phase formation; data not shown). Such a situation implied that a displacement of the binodal curve toward the *x*-axis occurred, which can be explained by the contribution of the biological suspensions to phase formation in the presence of high concentration of salt. To further pursue these studies, selected points above the binodal curve were used to estimate the position of the binodal curve in the presence of biomass.

The effect of biological suspensions on the position of the phase diagram is illustrated in Fig. 5. A strong displacement of the binodal curve towards the origin was observed at the critical point area of the phase diagram constructed in the presence of fermentation broth of *T. harzianum* (Fig. 5a). Such behaviour of the binodal curve can be explained by the nature of the micelial culture used. The fermentation broth from *T. harzianum* is characterised by a high viscosity caused by the presence of bio-polymers produced during the fermentation [14], which make a critical contribution to the systems and reduce the amount of chemical needed to form two phases. From the process point of view a type of behaviour exhibited by the ATPSs added with *T. harzianum* fermentation broth implied that the TLL of the systems increased and as a result a sensitive ATPS may become robust. In the case of the ATPSs added with disrupted yeast and *E. coli* homogenate, a change in the position of the binodal curve was observed. However, those changes were not significant (see Fig. 5b and c) which, although appeared to question previous result [9,10], the observed differences may be attributed to the disruption conditions used. From the results presented here and those previously reported [11], it has been shown that added biomass affects the position of the phase diagram, either moving the binodal curve towards

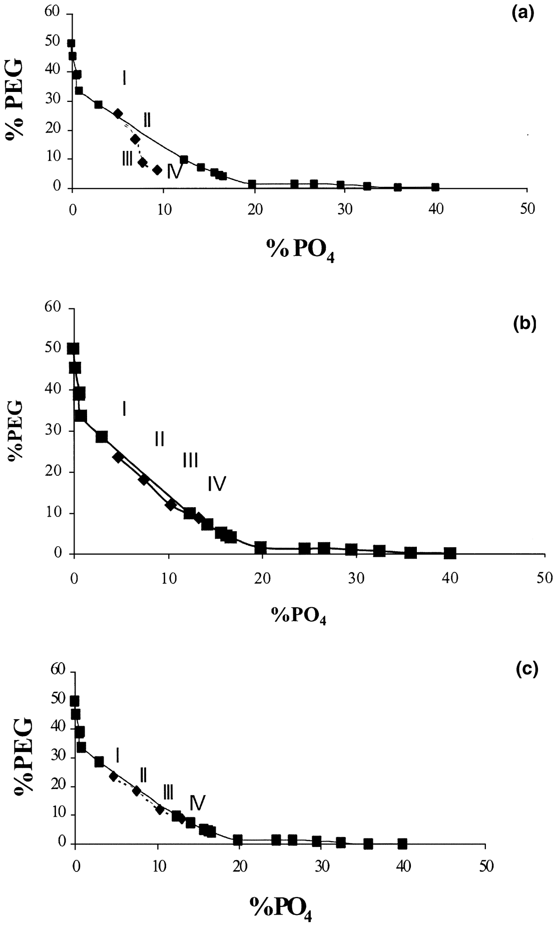

Fig. 5. Effect of added biological suspensions on the position of the binodal curve. The original position of the four systems is depicted in Fig. 3. The new position of the binodal with the addition of *T. harzianum* fermentation broth (a), *E. coli* homogenate (b) and disrupted yeast (c) was estimated as described in Section 2.

the origin (associated to the contribution of the biopolymer presence in the biological suspensions) or away to the origin (caused by the presence of residual fat; [11]). Such findings implied that biomass loading could facilitate the stabilisation of sensitive ATPSs with respect to external changes and that they can be exploited for the design of robust ATPS extraction stages for the recovery of compounds from biological suspensions. It is important to point out that practical studies of the type described here produces information of the way biological suspensions affect the position of the binodal curve in the ATPS phase diagram. However, the mechanism governing the phase formation in the presence of biological suspensions remain unclear, which raise the need for the development of methods that allow the generation of data required to understand such phenomenon.

4. Conclusion

ATPSs are affected by the loading of biological suspensions (i.e., fermentation broth of *T. harzianum*, disrupted yeast and *E. coli*), which it was shown in this study by changing in systems V_r and in the position of the binodal curve. Biological ATPSs with V_r greater than 1 and long TLL, exhibited significant changes in V_r when compared with that from non-biological systems. Such behaviour was associated with the top phase biomass accumulation. It was shown that the three biological suspensions used in this study caused the binodal curve to displace towards the origin, which was associated with the critical contribution of the bio-polymer (present in the systems) to the phase formation. It was concluded that the addition of biological suspensions affects the phase formation of ATPSs in a manner that at present cannot be fully explained. However, the practical study presented here produced information to facilitate the practical implementation of ATPSs for the purification of biological materials.

Acknowledgements

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