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# Impact of cell disruption and polymer recycling upon aqueous two-phase processes for protein recovery

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**Abstract**

A practical study is presented of the influence of cell debris and polymer recycling upon the operation of two-stage aqueous two-phase systems (ATPS) for the recovery of yeast bulk protein, pyruvate kinase and fumarase. Brewers' yeast was disrupted using one of two types of high-pressure homogenisers or a bead mill. The different cell debris suspensions were partitioned in a single PEG–phosphate ATPS extraction and the efficiency of solid–liquid separation was examined. A continuously operated two-stage ATPS process, using spray columns, is presented and practical problems of polymer recycling are discussed. Conclusions are drawn concerning the generic implementation and operational stability of ATPS in practical protein recoveries.

*Keywords:* Aqueous two-phase systems; Proteins; Pyruvate kinase; Fumarase

## 1. Introduction

The production of intracellular enzymes and other high value proteins from microbial sources requires initial cell disruption [1–3]. The location of different target proteins varies between discrete cell compartments and requires rupture of both whole cell and constituent components (eg. mitochondria, chloroplasts, etc.). In order to achieve economic yields, high percentages (>95%) of total cell (and organelle) disruption are necessary, which commonly generate a wide size distribution of particulate debris.

After mechanical cell disruption, subsequent microfiltration or centrifugal separation of cell debris is conventionally applied but such processes are not always dependable [4]. For example, wet-milled Brewers' yeast has proved especially obdurate in centrifugation processes operated as a first stage in the recovery of bulk protein [5,6]. This work also concluded that microfiltration was not an adequate alternative because of low permeate fluxes. Potential solutions to problems of centrifugation and microfiltration lie with a technique that has not been sufficiently exploited for the explicit removal of cell debris i.e. aqueous two-phase separation (ATPS) [7].

Aqueous two-phase partition in mixtures of poly-(ethylene glycol) (PEG) and potassium phosphate has been widely used for the recovery of macromolecules from fermentation broths and biological extracts. Previous work [5,6] has demonstrated aqueous two-phase partition as an alternative to conventional processes for concomitant particle (debris) and solute (macromolecule) handling. However, adoption of ATPS for other than the highest value products

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necessarily requires economic re-use of phase components in subsequent operational cycles.

The practical implementation of ATPS for the recovery of products will most simply involve the development of robust two-stage processes. In a typical two-stage aqueous PEG–phosphate process (Fig. 1), the first stage or loading extraction yields a bottom phase containing cell debris and contaminants, and a top phase containing the intracellular protein [8,9]. The partition of solutes between phases is characterized by the partition coefficient ($K_p$) where: $K_p = C_t / C_b$ and $C_t$ and $C_b$ are the concentration of target solute in the top and bottom phase respectively. In the second stage or stripping extraction, manipulation of salt addition, system pH, volume ratio ($V_r$) and tie-line length (TLL, representing the composition of PEG and phosphate in the system [8]), concentrates protein products in the bottom phase. Further processing of that phase by ultrafiltration yields a protein product concentrate and salt solution as waste or recyclable permeate [5,6,8].

The generic application of two-stage ATPS to a variety of product solids requires systems that remain stable in performance in the face of varied feedstocks and process fluctuations. Operational perturbation of any disruption processes, or the adoption of different methods to break cells, may influence the performance of solids and protein fractionation in subsequent stages of downstream processing. In addition, re-use of phase-forming chemicals which represents an attractive economic option [9,10] may also affect the stability of processes by recycling solutes antagonistic to subsequent cycles [9].

The impact of debris generation upon subsequent conventional solid–liquid separations has been previously examined [11]. However, no reports are known to the authors which discuss the effect of cell debris upon ATPS processes. On the other hand, polymer recycling has been demonstrated in batch systems by Hustedt [12] and in more detail by Rito-Palomares et al. [9,13], but the impact of continuous re-use of the polymer-rich phase upon the recovery of products and stability of processes, requires further study. In the present work, an ATPS process using spray columns [14,15] has been studied as a tool to evaluate polymer recycling through 20 two-stage cycles. The research addresses the overall impact of different cell disruption methods and polymer recycle upon the protein productivity of ATPS.

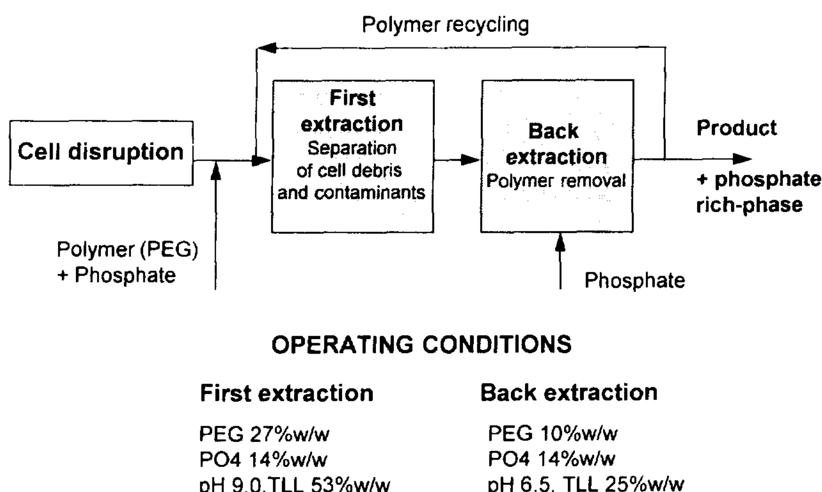


Fig. 1. Simplified representation of a two-stage aqueous two-phase process. The first two-phase system is formed by the addition of solid PEG and phosphate to the disrupted material. Bottom phase is then discarded and the top phase is mixed with fresh phosphate solution to form the second two-phase system. The second top PEG-rich phase so generated is recycled as described in Section 2.

## 2. Experimental

### 2.1. Cell disruption experiments

Waste yeast (the gift of Bass Brewery, Birmingham, UK) was slurried (30% wet w/v) in 20 m*M* phosphate buffer pH 7.0 and disrupted in three different devices. An APV-Gaulin type homogeniser 15M-8BA, equipped with a cooling system to control the temperature was operated at a fixed flow-rate of 54 l/h, a maximum pressure (55.2 MPa, equivalent to 8000 p.s.i.) and a maximum temperature of 10°C for six passes of feedstock. Disruption of the cells held initially at 4°C was also performed at high pressure (up to 276 MPa) in a single-pass of feedstock using a bench-top Z-series homogeniser from Constant Systems, Warwick, UK. Wet-milling of cells was also performed in a Dynomill (KDL 0.61) at 15 l/h operated at 3200 rpm with 0.2–0.5 mm glass beads filled to 85% chamber capacity. In this case the temperature was controlled at maximum of 10°C between feedstock passes by cooling the milling chamber with chilled water (8°C) and the effluent with a jacket of ice.

### 2.2. Analyses

The release of protein was estimated [16] in samples taken and clarified by bench-top centrifugation after each pass through individual disruption devices. Enzymatic activity of pyruvate kinase and fumarase was estimated as described by Hess and Wieker [17] and Massey [18], respectively. Changes in particle size distributions of disrupted cell suspensions were estimated using laser analysis in a Coulter LS 130 instrument.

### 2.3. Aqueous two-phase experiments

The partition experiments were performed using PEG (Sigma) of nominal molecular mass of 1000 and di-potassium phosphate (BDH). Systems were assembled with a fixed mass on a top-loading balance. Complete phase separation was achieved by low speed centrifugation at 1200 *g* for 15 min at 20°C. The volume ratio of the top and bottom phase from biological and non-biological (blank) systems was estimated in graduated centrifuge tubes. Batch systems were assembled and sampled to evaluate the effect of protein and enzyme concentrations upon solute partition coefficients. Concentrations of PEG and phosphate in the phases were estimated from the graphical interception of system tie-line length with the binodal curve as described elsewhere [5,7,13].

### 2.4. Construction and assembly of the ATPS continuous system

A two-stage aqueous two-phase process was assembled using two spray columns, each having a total height of 600 mm, an inner diameter of 25 mm and a total volume of 250 ml. The process was assembled (Fig. 2) using seven Watson–Marlow peristaltic pumps (two of Type 501U and five of Type 101U) calibrated for flows of PEG and phosphate solutions with tubing of 1.6 and 3.2 mm in diameter, respectively. The relation between the flow-rates of the phases (Table 1) was adjusted to maintain values of the volume ratio previously obtained in batch experiments i.e. 1.65 and 0.50 for first and back extractions, respectively.

### 2.5. ATPS spray column experiments

The defined aqueous two-phase system (Fig. 1) was prepared using PEG, phosphate and wet-milled yeast suspensions. Phases were separated by centrifugation and cell debris discarded with the bottom phase and interface. Protein-rich top phase (PEG) was contacted in the first spray column (first extraction), with fresh bottom phosphate-rich phase at a flow-rate appropriate to a global residence time of 28.5 min (Table 1). The top phase obtained from this first extraction was contacted with a phosphate solution (18% w/w, pH 7.0) to obtain the operating conditions (appropriate PEG and phosphate compositions) for the back extraction. The pH was adjusted to 6.50 using orthophosphoric acid and the mixture pumped to the second column (back extraction). The phases separated under gravity and were withdrawn continuously so that the bottom phosphate-rich phase and products could be collected.

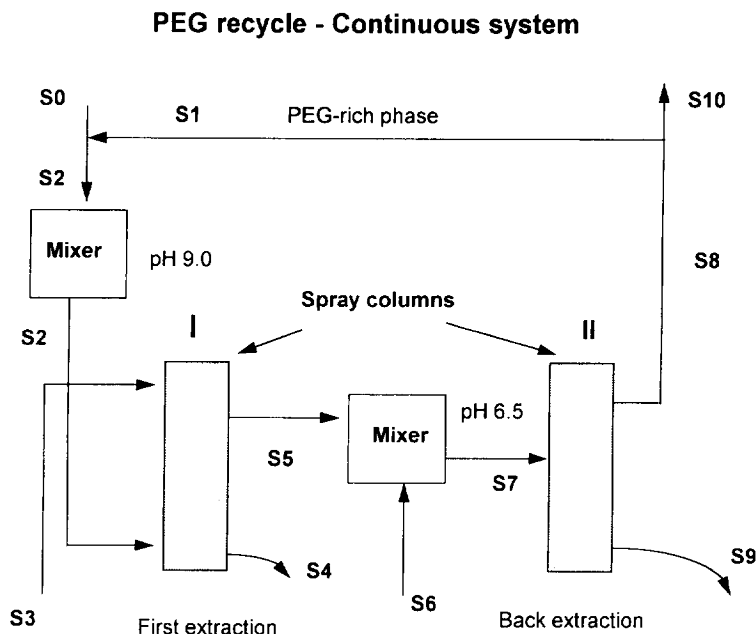


Fig. 2. Process scheme of the ATPS continuous system. The streams of the process are identified by S0 to S10, and their flow-rates and composition are detailed in Table 1. Part of the recycling stream generated (S8) is then recycled at different flow-rate (S1), to achieve the necessary residence time, and the rest is discarded (S10). The dimensions of the spray columns (I and II) are described in Section 2.

Table 1
Stream flow-rates and compositions within the ATPS continuous process

| Stream | Flow-rate (ml/min) | Composition (%, w/w) | | System pH |
|---|---|---|---|---|
| | | PEG | Phosphate | |
| *First extraction* | | | | |
| S0 | 3.08 | 52.0 | 5.60 | 9.0 |
| S1 | 3.08 | 32.0 | 2.20 | 6.5 |
| S2 | 6.12 | 42.0 | 3.90 | 9.0 |
| S3 | 3.70 | 1.00 | 33.0 | 9.0 |
| S4 | 3.70 | 1.00 | 33.0 | 9.0 |
| S5 | 6.12 | 42.0 | 3.90 | 9.0 |
| *Back extraction* | | | | |
| S6 | 19.0 | 0.0 | 18.0 | 7.0 |
| S7 | 25.0 | 10.0 | 14.0 | 6.5 |
| S8 | 8.33 | 32.0 | 2.20 | 6.5 |
| S9 | 16.67 | 2.80 | 18.2 | 6.5 |
| S10 | 5.25 | 32.0 | 2.20 | 6.5 |

Numbered streams refer to the flows indicated in Fig. 2. Compositions of the phases were estimated by graphical interception of the tie-line length with the binodal curve in batch experiments as described in Section 2.

### 2.6. Recycling experiments

The recycling of total (100%) polymer-rich phase generated by the back extraction would clearly dilute the process and adversely affect the system residence time, and thus only 40% of the phase generated was recycled. The top polymer-rich phase was recycled to the first extraction and contacted with the necessary fresh top phase to re-achieve the initial operating conditions. The pH was adjusted to pH 9.0 by using sodium hydroxide and the mixture pumped to the column where it was contacted with fresh phosphate solution (see Fig. 2). The residence time was estimated as the time necessary for the phases (PEG and phosphate) to separate in the presence of debris-free material, and designated as one single cycle. In order to evaluate the effect of polymer recycle for up to 20 cycles, the system was operated for approximately 600 min during which each cycle was sampled from the top and bottom phase of first and back extractions. The protein concentration and enzymatic activity (fumarase and pyruvate kinase) were estimated as described above.

## 3. Results and discussion

### 3.1. Impact of cell disruption upon ATPS

Three different biological suspensions were generated to evaluate their impact upon solid–liquid separation in ATPS. The optimum operating conditions to obtain maximum intracellular protein release were established in preliminary experiments with the different disruption devices. The APV homogeniser required more than four discrete passes of yeast feedstock (Fig. 3a) before constant concentrations of products were achieved. On the other hand, the bead mill (Fig. 3b) released the majority of available soluble products in a single pass. The Constant Systems high-pressure homogeniser required an operating pressure of more than 207 MPa (Fig. 3c) to achieve similar results. Subsequent passes in the bead mill, and at pressures greater than 207 MPa in the bench-top homogeniser, apparently diminished product release, but this was attributed to proteolytic degradation and/or enzymatic inactivation in the face of localised heating.

The performance of the three methods was compared on the basis of one single pass from the bead mill, four passes from the APV-Gaulin homogeniser and one single-pass at 207 MPa from the Z-series homogeniser (Fig. 3) which individually resulted in approximately equal release of target products (total protein, fumarase and pyruvate kinase). The cell debris generated under these operating conditions was distinguished by apparent differences in particle size. That from the bead mill (Fig. 4) exhibited the smallest average size which was attributed to the effects of solid shear phenomena. On the contrary, the liquid shear attributed to both homogenisers

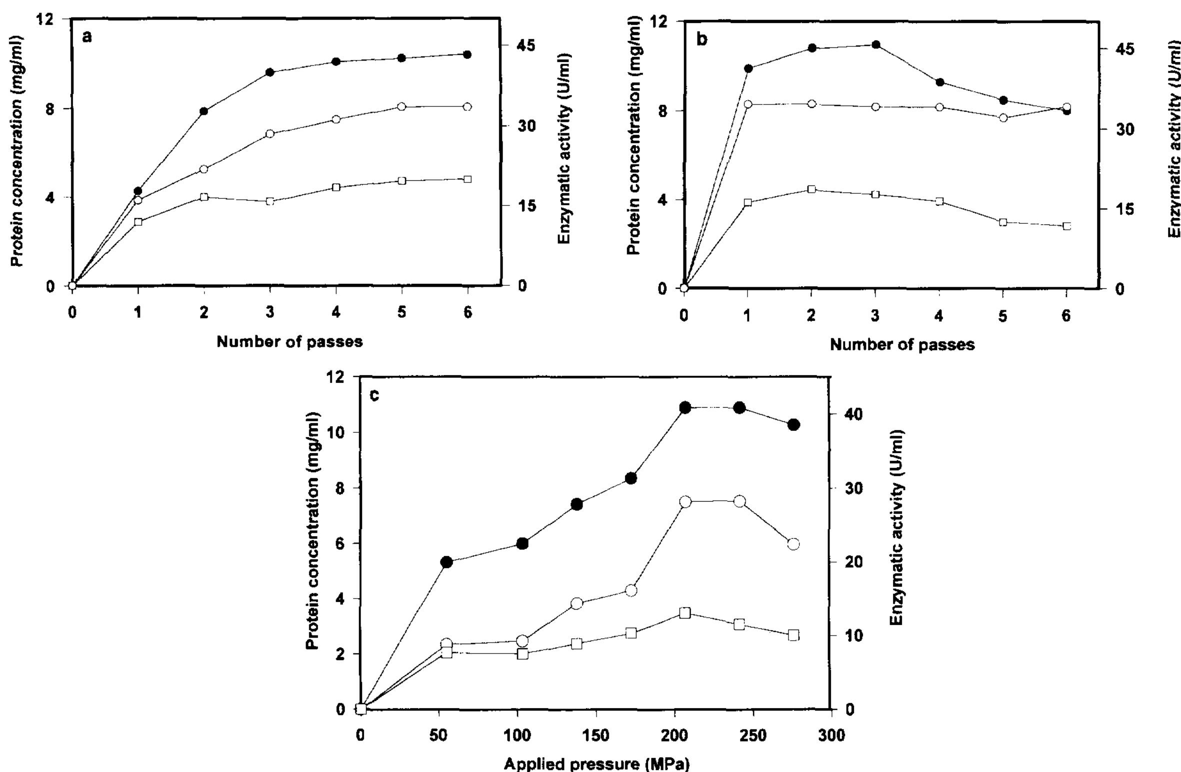


Fig. 3. Release of intracellular products from Brewers' yeast. The concentrations of bulk protein (●), and the enzyme activity of pyruvate kinase (○) and fumarase (□) are expressed relative to the number of passes (a) in a APV-Gaulin type homogeniser 15M-8BA, (b) in a Dynomill (KDL 0.61) and (c) relative to the pressure applied in the Z-series bench-top homogeniser from Constant Systems as described in Section 2.

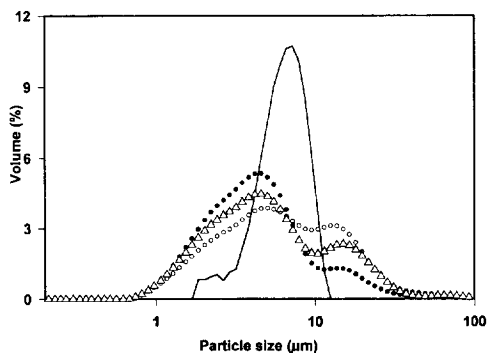


Fig. 4. Apparent particle size distribution from homogenised and milled brewers' yeast. The particle size distributions estimated from a Coulter LS 130 instrument for intact cells (□), and the products of a single pass in the bead mill (●), four passes in homogeniser at 55.2 MPa (○) and one pass at 207 MPa (△). Product release from the different disruption methods is depicted in Fig. 3.

produced an apparent broader size distribution of debris, which may be due to particle agglomeration or the characteristic shape of debris so generated. Both may benefit the operation of subsequent centrifugal solid–liquid separations [11]. However, in the present work, high-performance batch centrifugation (up to 40 000 $g$) could not sufficiently clarify either homogenised or wet-milled yeast to meet operating specifications of downstream chromatographic processes [5]. Solid–liquid separation of cell debris exploiting a single aqueous two-phase extraction was therefore investigated as an alternative, to better handle apparent different size distributions of cellular debris arising from selected methods of cell disruption.

Selected PEG–phosphate ATPS can efficiently fractionate protein from cell debris generated by either wet-milling or homogenisation [5,6,13]. The partition of debris to either phase serves to modify the volume ratio from values obtained with a "blank" system (i.e. a two-phase system without biological suspension). Thus, the volume ratio is increased by top-phase debris or decreased by bottom-phase debris, with the latter being most appropriate condition for two-stage extractions. This phenomena may also affect the position of the binodal curve [19]. The fractionation of debris in ATPS (Table 2) from homogenised, milled and high-pressured yeast shows that the maximum effect upon the volume ratio occurs with systems having initial values (taken from blank systems) of less than 1.0. No significant difference was observed between systems close to, or distant from the binodal on tie-line lengths of 25 and 60% w/w, respectively. The debris suspension generated by high-pressure homogenisation, produced the maximum change of

Table 2
Volume ratio and phase preference of cell debris in an ATPS

| Composition of: | PEG (% w/w) | 7 | 9 | 14 | 26 | 22 | 42 |
|---|---|---|---|---|---|---|---|
| | Phosphate (% w/w) | 16 | 15 | 12 | 16 | 8 | 7 |
| Tie-line length (%, w/w) | | 25.0 | 60.0 | 25.0 | 60.0 | 25.0 | 60.0 |
| Volume ratio of: | blank | 0.25 | 0.24 | 1.28 | 1.31 | 4.74 | 6.84 |
| | homogenised yeast | 0.40 | 0.45 | 0.84 | 1.13 | 3.34 | 3.52 |
| | milled yeast | 0.46 | 0.43 | 0.94 | 1.13 | 2.84 | 3.92 |
| | high-pressured yeast | 0.49 | 0.52 | 1.0 | 1.90 | 2.94 | 3.67 |
| Changes in $V_r$ (%): | homogenised yeast | 60.0 | 88.0 | 34.0 | 14.0 | 30.0 | 49.0 |
| | milled yeast | 84.0 | 80.0 | 27.0 | 14.0 | 41.0 | 43.0 |
| | high-pressured yeast | 96.0 | 117 | 22.0 | 45.0 | 38.0 | 46.0 |
| Debris in B/T phase:[a] | homogenised yeast | T | T | B | B | B | B |
| | milled yeast | T | T | B | B | B | B |
| | high-pressured yeast | T | T | B | T | B | B |

ATPS comprising PEG 1000 and potassium phosphate. The volume ratio of top and bottom phases for cell debris was estimated after phase separation in graduated centrifuge tubes.
[a] T and B denote top and bottom phase majority preferences.

volume ratio in the majority of the selected systems. This may be related to the apparent size distribution observed (see Fig. 4). However, differences between the change in the volume ratio produced by introduction of different debris suspensions, reduced significantly in systems having both high values of volume ratio and tie-line length (see last column of Table 2). In these systems, the difference between the percent change of volume ratio observed within the three methods was only 3–6%. This indicates that, regardless of origins in wet-milling or homogenisation, differences in particle size distribution and/or shape apparent in Fig. 4 do not significantly influence the overall efficiency of solid and protein fractionation in ATPS with volume ratios greater than one at long tie-line lengths.

### *3.2. Impact of polymer recycling upon ATPS*

The recycling of phases has been investigated before [10,12] in studies illustrating the re-use of PEG in batch systems up to four [12] and five times [9,13] without detriment to the stability of operations. Further research required a different approach to more accurately study the recycling of phases in longer term experiments without encountering problems of time-based decay of enzyme activity.

Recycling for more than five cycles using batch protocols requires time-consuming experiments (greater than 14 days) which demand labour-intensive maintenance and analysis [13]. In order to study the impact of polymer recycling upon protein recovery for up to 20 two-stage cycles, two different approaches may be applied. An appropriate simulation may be exploited to evaluate the recycling phenomena. Empirical batch recycling experiments, monitored by sensitive and fast analytical equipment, are required to characterise phase compositions and partition coefficients of different solutes in the experimental feedstocks (e.g. proteins, pigments, RNA, carbohydrates, etc.). An alternative approach involves the operation of two-stage ATPS processes in a continuous manner. This requires very sensitive control and monitoring stages [20], high cost equipment and excess amounts of chemicals [21,22] to maintain systems for only limited periods of time (e.g. less than 60 min). Both approaches were judged to be practically impossible in the present study. However, continuous liquid–liquid separation processes can be simply operated by using extraction columns which present several advantages including ease of construction and operation.

The use of spray columns in ATPS has been reported previously [14,15] for semi-continuous processes exploiting passive phase separation in a liquid–liquid extraction under normal gravity. The important parameters involved include solute and solvent mass transfer and the system residence time which define the necessary periods for phase contact and separation. Unfortunately, the solids concentration that can be efficiently handled is very limited. Initial experiments with disrupted yeast cells were attempted, but solids accumulation in the interface severely limited mass transfer and efficient performance of solute separations. However, it was concluded that the system had inherent potential for the evaluation of repetitive re-use of the polymer rich-phase in a two-stage process with clarified feedstock. Any conclusion drawn from such studies was judged to be broadly applicable to processes handling disrupted cell suspensions.

The operation of two-stage spray columns ATPS indicated that the impact of polymer recycling upon the partition coefficient of the bulk protein and fumarase in the first extraction (Fig. 5a) was negligible in terms of separation efficiency. A moderate increase in the partition coefficient of the pyruvate kinase was observed in the first extraction after the equivalent of twelve operating cycles. In the back extraction, although the partition coefficient of the target products (bulk protein, fumarase and pyruvate kinase), remained relatively constant for up to five cycles a more pronounced increase was subsequently observed (Fig. 5b). This may be explained by inherent accumulation of top-phase products (protein, pigments, etc.), which displaced the system position in the phase diagram from their exact starting composition (PEG 27% w/w–phosphate 14% w/w for the first extraction and PEG 10% w/w–phosphate 14% w/w for the back extraction). In addition, continuous addition of the low molecular weight products (orthophosphoric acid or sodium hydroxide) may affect on the position of the binodal [19,22]. Such phenomena, individually or collectively, result in an increase in the tie-line length and associated increase in the partition coefficient.

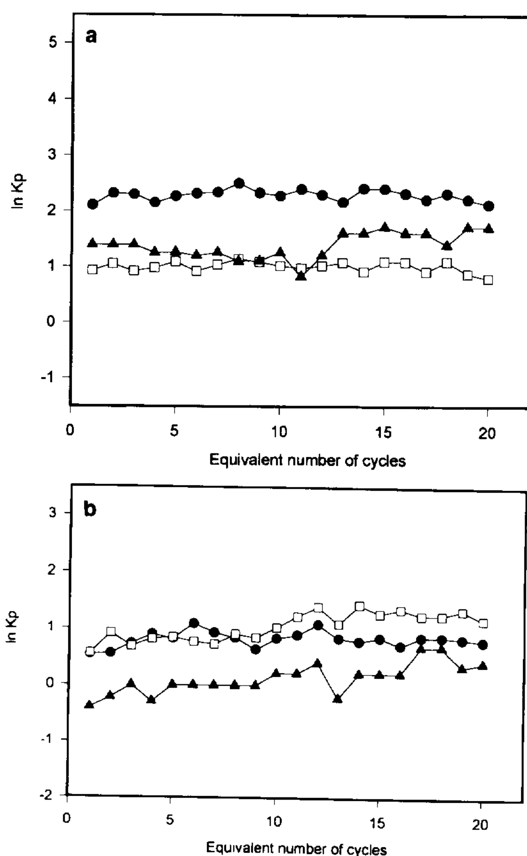


Fig. 5. The impact of polymer recyling upon the partition coefficient The partition coefficient of bulk protein (●), fumarase (□) and pyruvate kinase (▲) from the first extraction (a) and back extraction (b) in spray column experiments (Fig. 2) are represented relative to the numer of equivalent operational cycles. The partition coefficients were estimated from samples taken from the continuous system fed with clarified feedstock as described in Section 2.

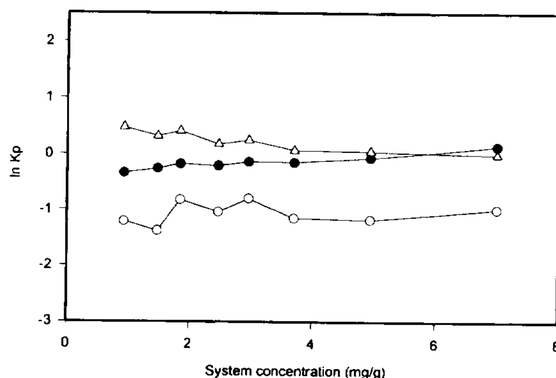


Fig. 6. The effect of system concentration upon the partition coefficient. The partition coefficient of bulk protein (●), fumarase (△) and pyruvate kinase (○) are represented relative to the total concentration of protein in the system. Batch systems were assembled on a top loading balance with PEG and di-potassium phosphate and incremental increases in the protein concentration for the wet-milled yeast feedstock. Phases were separated by centrifugation at 1200 *g*, 15 min and 20°C and samples were taken from the phases to estimate protein concentration and enzymatic activities, as described in Section 2.

The increase in the partition coefficient of first extraction products reflects an associated increase in the mass of protein to be further partitioned in the back extraction. The effect of continuous accumulation of products in the first extraction top phase upon partition coefficients was studied in selected batch experiments (Fig. 6) characterised by incremental increases of the initial protein concentration in the first extraction top phase to be processed under back extraction conditions. No significant effect was observed (Fig. 6) for bulk protein, fumarase and pyruvate kinase. This implies that for any observed changes in partition coefficient in recycling experiments (Fig. 5), the contribution from product accumulation in the process is negligible or counter balanced by indeterminate drift in the overall phase composition.

The increase of the partition coefficient for products in the first extraction is associated with an increase in the recovery of the top-phase products. This is balanced by a decrease for the back extraction which confers a stable behaviour upon the two-stage processes and maintains repetitive recovery of the products within a fixed range i.e.; 35.4 ± 6%, 34.6 ± 6% and 52.74 ± 10% respectively for bulk protein, fumarase and pyruvate kinase. It might be anticipated that in extended experiments a significant decrease in the recovery and possible collapse of processes would be observed at a point defining the maximum number of achievable cycles. This situation was not approached or indicated in the current work which serves to confirm the robustness of these practical systems.

## 4. Conclusion

Wet-milling and homogenisation of waste brewers' yeast under appropriate operating conditions

released similar levels of intracellular products, but generated debris suspensions characterised by different size distributions. Biological suspensions can be readily clarified in single-stage ATPS but size distribution of debris (and biochemical characteristics) have an impact upon ATPS, particularly those comprising either small volume ratios and/or short tie-line lengths. More robust ATPS systems, distant from the binodal curve with volume ratios greater than one, were found to significantly minimise the impact of different cell disruption methods, particularly when integrated with a two-stage ATPS. In addition, although the bead mill generated the most intractable debris suspension, the practical simplicity of single-pass and continuous operation recommended the general adoption of wet-milling processes for recovering the products identified in this work.

A continuous ATPS process operated with spray columns was used to evaluate the impact of polymer recycling upon the recovery of products in two-stage processes. Polymer recycling had a minimal effect upon the stability and productivity of the process with limited recycling (up to 5 cycles). An increase in the partition coefficient for bulk protein, fumarase and pyruvate kinase from the back extraction after an increased number of two-stage cycles (from 6 to 20) was observed and attributed to the inherent accumulation of top-phase preference products (proteins) and other solutes (e.g. pigments). The negative effect upon the recovery of bottom-phase products was in part compensated by the enhanced recovery of top-phase products from the first extraction.

We conclude that the robust qualities of ATPS in multiple cycle operations with partial polymer recycle deserves recognition as a process aid to the solution of problems of solid–liquid separation and solute isolation associated with the primary extraction of protein products.

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