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Process integration using aqueous two-phase partition for the recovery of intracellular proteins

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Abstract

This paper presents an integrated process for the primary recovery of intracellular glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and other proteins from bakers' yeast wherein cell disruption and aqueous two-phase systems (ATPS) were operated simultaneously. Polyethylene glycol and potassium phosphate were added to the cell suspension before disruption such that the bead mill was exploited as both cell disrupter and product extractor. The partition behaviour of both bulk protein and G3PDH for the integrated process was similar to that for a conventional process in which cell disruption and ATPS extraction were operated as discrete unit operations. The target products concentrated in the upper phase for all the systems evaluated. However, the cell debris generated during the integrated process also concentrated in the upper phase which limited the potential application of the system. Subsequent evaluation of the variation of system volume ratio (V_r) with the partition behaviour of cell debris recommended process conditions (i.e. PEG 12% (w/w), phosphate 28% (w/w), $V_r = 0.45$, pH = 7.0) suitable for the primary recovery of intracellular proteins from debris. The results are discussed in the context of the practical potential of the direct integration of ATPS with cell disruption processes.

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1. Introduction

The production of intracellular proteins presents an interesting set of practical problems for the downstream process engineer. Processes for the separation of such products generally involve the release of the products by mechanical or chemical disruption, followed by removal of cell debris and some contaminants by high speed centrifugation or cross-flow membrane filtration. Quantitative elimination of cell debris with such unit operations may be difficult to achieve on large scales. In addition, the complex nature of the products and contaminants present inside the cell may have a negative impact on the recovery process. For example, product denaturation may occur in the harsh environments of mechanical or chemical cell disruption and/or in the extended periods dedicated to cell debris clarification.

Currently, the achievement of process integration of the upstream operations of fermentation and downstream recovery processes required for the manufacture of bioproducts is of great practical and economic interest. Process integration, wherein two unit operations are combined into one in order to achieve specific goals not effectively met by discrete processes, offers considerable potential benefit for the recovery of intracellular protein products. Direct process integration of cell disruption with primary recovery unit operations could enhance both the yield and molecular quality of protein products. Direct product capture has been achieved by integrating cell disruption with fluidised bed adsorption [1]. Alternatively, as proposed in the present work, cell disruption could be operated in aqueous two-phase systems (ATPS) to achieve the goals of process integration for the recovery of intracellular proteins. Although, significant advances have been made in the development of suitably robust ATPS processes [2,3], their adoption in integrated processes is not widely reported. However, one report [4] has presented the potential integration of ATPS with cell disruption but the work focused largely on the impact of phase-forming chemicals upon the efficiency and kinetics of cell disruption. In contrast, the research presented herein has attempted

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to address the generic process applicability of the strategy. The recovery of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) from bakers' yeast is reported as a representative model system by which to compare the performance of conventional discrete processes and a fully integrated operation.

2. Materials and methods

2.1. Comparison of conventional and integrated cell disruption—ATPS process

Fig. 1 comparatively depicts two process flow diagrams where (i) the upper represents the conventional approach of discrete mechanical cell disruption followed by aqueous two-phase extraction; and (ii) the lower one represents the integrated process adopted for this study. For the conventional process, the suspension made of bakers' yeast (50 wt.% (w/v) in 10 mM Tris–HCl buffer at pH 7.5) was disrupted in a horizontal Dyno-mill, type KDL (Willy A Bachhofen AG Maschinenfabrik). The chamber (0.6 l capacity) was filled to 85% of the total volume with lead-free ballotini glass beads (0.2–0.5 mm diameter). The rotor speed was 3200 rpm. The bakers' yeast suspension was fed at 6 l/h and the temperature was controlled to 4 °C with cooling water. The disrupted biological suspension obtained was used to assemble ATPS as described below. In the case of the integrated process, the yeast cell suspension (50 wt.% (w/v) in 10 mM Tris–HCl buffer at pH 7.5) was fed to the mill with PEG and phosphate solutions (described in Table 1) to achieve simultaneous cell disruption and aqueous two-phase extraction. The disrupted effluent from the bead mill was immediately centrifuged (at 1500 g for 20 min at 25 °C) to achieve full separation of phases which were sampled and diluted for biochemical analyses (see Section 2.3).

Table 1
Systems selected for the evaluation of the direct integration of cell disruption and ATPS

System	Molecular weight of PEG (g/gmol)	PEG (% (w/w))	Phosphate (%(w/w))	TLL (%(w/w))
1	1000	18.2	15.0	38.0
2		18.9	16.0	40.0
3		22.2	19.0	50.0
4		24.1	20.1	55.0
5	1450	15.1	13.0	30.0
6		17.5	14.3	40.0
7		21.9	18.0	52.0
8		23.0	19.8	55.0

PEG 1000– phosphate ATPS (systems 1–4) and PEG 1450– phosphate ATPS (systems 5–8) were selected to evaluate the direct impact of the integration of cell disruption and ATPS upon process performance. The volume ratio (V_r estimated from blank systems) of the selected systems was maintained at a constant value of one. All systems were assembled at pH 7.0 as described in Section 2.

2.2. Characterisation of ATPS

ATPS were compounded for convenience on a fixed mass basis on a top-loading balance. Predetermined quantities (see Table 1) of solid poly(ethylene glycol) (PEG, Sigma) of nominal molecular mass of 1000 and 1450 g/gmol, potassium phosphate (Sigma) were mixed with deionised or disrupted bakers' yeast to give a final weight of 15 g and a system pH value of 7.0. Adjustment of pH was made by addition of orthophosphoric acid (1 M) or sodium hydroxide (2 M). Solids components (PEG and salts) were dissolved and phases dispersed by gentle mixing for 30 min at 25 °C. Complete phase separation was achieved by low speed batch centrifugation at 1500 g for 20 min at 25 °C. Visual estimates of the volumes of top and bottom phases and solids, were made in graduated centrifuge tubes and used to estimate the

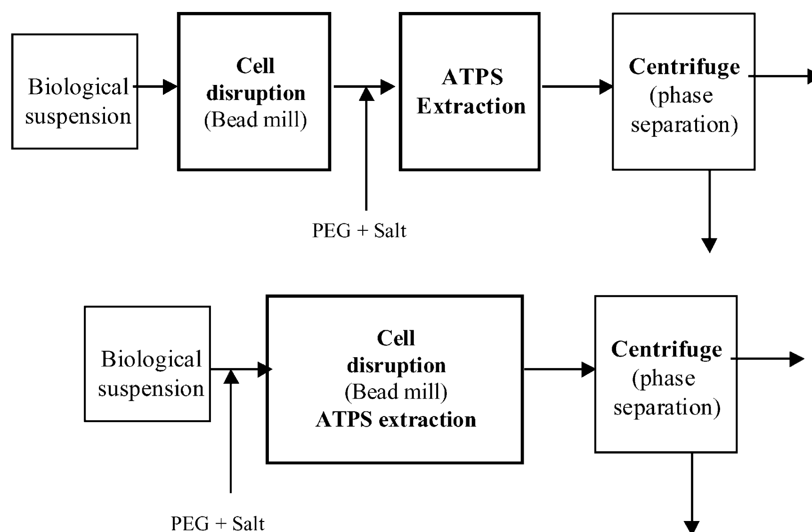

Fig. 1. Simplified representation of the comparison of two flow diagram. The upper flow diagram represents the conventional process in which mechanical cell disruption is followed by aqueous two-phase extraction. The lower diagram represents the integrated process proposed in this study.

volume ratio (V_r = volume of the top phase/volume of the bottom phase). In the cases when interface was formed, such phase was not considered for the estimation of V_r. Samples were taken from the phases and diluted for biochemical estimation of bulk protein and G3PDH partition coefficients (K, K_E = concentration of solute in the top phase/concentration of solute in the bottom phase). Tie-line length (TLL) of ATPS was estimated as described by Huddleston et al. [5] and the results recorded as the mean of three independent experiments having errors of ±5%.

2.3. Analytical procedures

Protein content and G3PDH activity in the disrupted suspension and in phase samples was estimated, following appropriate dilution and centrifugal clarification, using methods described by Gilchrist [6].

3. Results and discussion

3.1. Partition behaviour of protein and G3PDH in conventional and integrated process

Eight ATPS were selected (see Table 1) for the direct comparison of the performance of the conventional and integrated processes for the recovery of intracellular protein and G3PDH. Based upon previous work [3,4,7], these ATPS were characterised by (i) increasing TLL; (ii) two different polymer molecular weights (i.e. PEG 1000 and PEG 1450 Da); and (iii) constant volume ratio ($V_r = 1.0$). Fig. 2a and b illustrates the observed variation of protein partition coefficient with increasing TLL in these systems. It has been previously reported [5] that increasing TLL causes the partition coefficient of yeast proteins to increase in a manner attributed to changes in the free volume [8], surface tension at the interface and density of the phases [9,10]. In the case of PEG 1000, the decreasing trend observed at high TLL values (i.e. >50% (w/w)) in Fig. 2a might be attributed to the saturation of the volume available for protein solutes to be accommodated in the upper phase [8]. Overall, the data in Fig. 2 confirm the similar partition behaviour of bulk intracellular protein in both conventional and integrated processes, and invites the conclusion that the efficiency of the latter is not altered by the direct addition of phase-forming reagents at disruption.

A further comparison of the variation of G3PDH partition coefficient (K_E) with increasing TLL is shown in Fig. 3. Similar trends for K_E were observed for both conventional and integrated processes, but these displayed different forms for PEG 1000 and PEG 1450 ATPS (compare Fig. 3a and b). It is likely, that as in the case of bulk protein partition behaviour, saturation of the free volume of the upper phase of PEG 1000 systems was achieved for G3PDH at high values of TLL (>50% (w/w)). In this preliminary scouting study, the maximum enzyme partition coefficient (i.e. $K_E = 8.0$) was achieved in a PEG 1000 ATPS characterised by operating parameters in the integrated process of 22.2% (w/w) PEG 1000, 19.0% potassium phosphate, TLL = 50% (w/w) and $V_r = 1.0$ at pH 7.0. Such a system yielded a recovery of G3PDH from the PEG-rich top phase of 66.5% of the system load (see Table 2), corresponding to the best enzyme recovery obtained in studies of the integrated process. The results summarised in Table 2 indicate that no substantial effect impact was inflicted upon primary recovery of either bulk protein or G3PDH by the direct integration of ATPS with cell disruption. It is important to note that, although, the residence time in the bead mill is short ($\ll$10 min), the process is sufficient to achieve simultaneous disruption and extraction. In this context, it has been reported [11] that interfacial tension of ATPS is low and thus the extractive equilibrium would readily be achieved in the mixing regime of a horizontally agitated bead mill.

Table 2
Protein and G3PDH recovery from the top phase from the conventional and integrated processes

System	Protein recovery (%)		G3PDH recovery (%)	
	Conventional process	Integrated process	Conventional process	Integrated process
1	46.3	36.5	5.3	2.3
2	60.0	51.2	39.8	30.0
3	80.8	79.8	55.9	66.5
4	66.0	54.7	67.5	61.5
5	55.2	58.3	nd	nd
6	27.5	21.0	nd	nd
7	27.7	31.0	14.9	17.1
8	44.8	47.2	35.1	32.4

The composition of systems 1–8 is defined in Table 1. For both conventional and integrated processes the recovery of protein and G3PDH from the top phase is expressed relative to the initial values determined for disrupted yeast feedstocks (nd: values not determined).

3.2. Effect of the direct integration of ATPS and cell disruption upon process performance

An important contaminant generated during the recovery of microbial intracellular products is the cell debris. It has long been recognised that cell debris, and particularly intracellular contents (referred herein collectively as biomass) in microbial disruptates, impact upon ATPS performance (discussed in [12]). The nature of such influences can be qualitatively assessed by studying the variation of volume ratio (V_r) and phase preference of biomass and cell debris across a spectrum of candidate systems. The data recorded in Table 3 indicates that, for selected systems, the volume ratio and biomass phase preference recorded for integrated processes exhibited a uniquely, different behaviour to that seen with conventional processes.

Systems with PEG molecular weight of 1450 Da and high TLL values (i.e. 52 and 55% (w/w)), exhibited common volume ratios and biomass bottom phase preferences in both

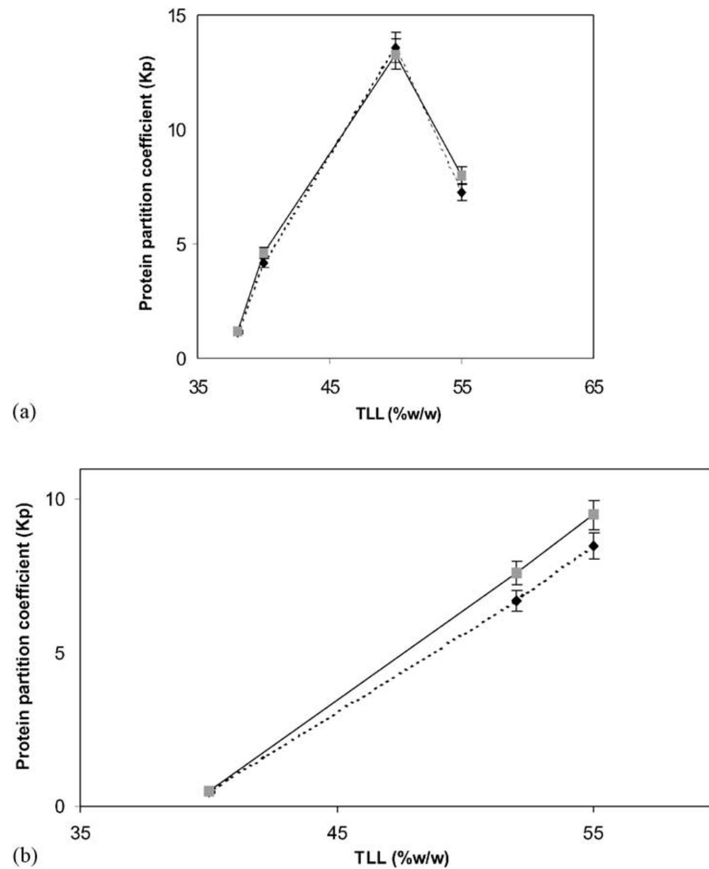

Fig. 2. Effect of system TLL on the bulk protein partition coefficient in (a) PEG 1000–phosphate ATPS and (b) PEG 1450–phosphate ATPS for conventional and integrated processes. The TLL of systems 1–8 were estimated from the composition of PEG and phosphate as described in Section 2 (see Table 1). The protein partition coefficient from the conventional (◆) and integrated (■) processes, represents the ratio of the concentration of protein in the top and bottom phases. The values for system 5 (TLL = 35% (w/w)) proved too low to plot accurately on the scale of Fig. 2b. For all systems, volume ratio (estimated from blank, non-biological systems) and the systems pH were kept constant at 1.0 and 7.0 respectively.

conventional and integrated processes (see systems 7 and 8 in Table 2). This can be attributed to a robustness in the face of added biomass which is characteristic of ATPS distant from the binodal and possessed of long TLL (refer to Albertsson [13]). In other systems, the volume ratio of the integrated process decreased relative to that of the conventional process (see systems 1, 2 and 6 in Table 3). Here, cell debris from the integrated process exhibited a clear bottom phase preference which is the preferred location from a process point of view when considering solids disposal. In contrast, that from the conventional process was distributed between the interface and the bottom phase. A decrease in the volume

Table 3
Change in volume ratio and cell debris phase preference in the conventional and integrated processes

System	V_r		Cell debris phase preference	
	Conventional process	Integrated process	Conventional process	Integrated process
1	0.85	0.79	I–B	B
2	0.85	0.56	I–B	B
3	0.53	1.20	B	T
4	0.83	1.27	B	T
5	0.79	1.78	I–B	T
6	0.78	0.32	I–B	B
7	0.77	0.79	B	B
8	0.76	0.79	B	B

The composition and volume ratio of clean systems 1–8 is defined in Table 1. For both types of process, the equilibrium volume ratio of the top and bottom phase (V_r) was estimated after phase separation in graduated tubes without regard to the small volumes (<5%) occupied by any interface. T, B and I denote the top, bottom and interface phase preference for cell debris (determined visually).

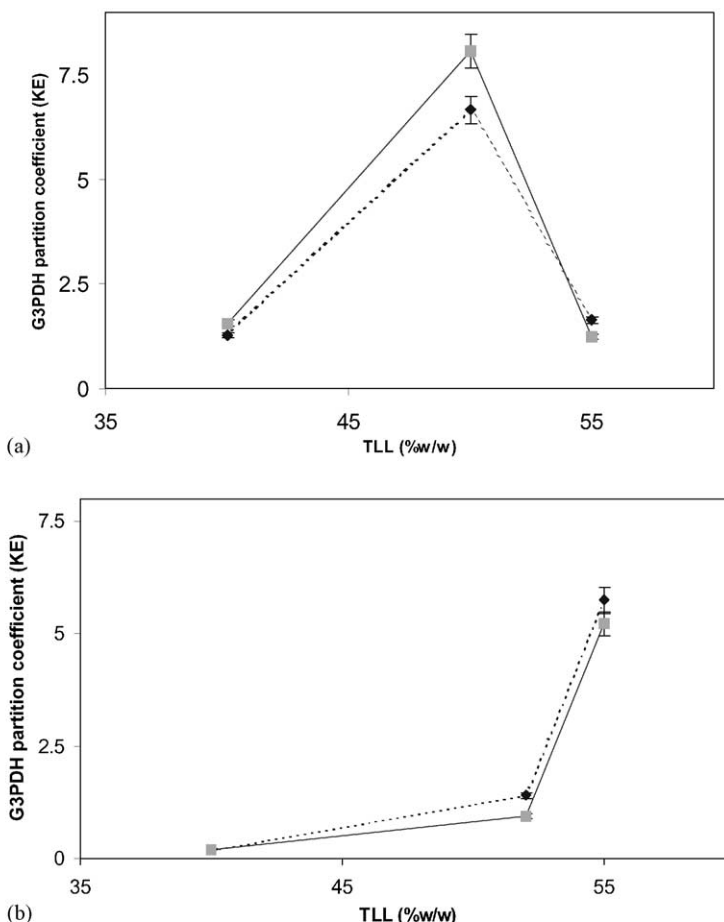

Fig. 3. Effect of system TLL on the G3PDH partition coefficient in (a) PEG 1000–phosphate ATPS and (b) PEG 1450–phosphate ATPS for conventional and integrated processes. The TLL of systems 1–8 were estimated from the composition of PEG and phosphate as described in Section 2 (see Table 1). The G3PDH partition coefficient from the conventional (◆) and from the integrated (■) process, represents the ratio of the enzymatic activity in the top and bottom phases. Values for systems 1 and 5 (TLL = 35% (w/w)) proved too low to plot accurately on the scale of Fig. 3. For all ATPS, V_r estimated from blank, non-biological systems) and the system pH were kept constant at 1.0 and 7.0 respectively.

ratio of the integrated process may be attributed to an unexplained, direct effect of the simultaneous extraction and disruption upon the final ATPS composition and its phase separation. In addition, the nature of the cell debris produced in the integrated process and its subsequent accumulation in the bottom phase would de facto increase the bottom phase volume and decrease the volume ratio.

Systems identified as 3, 4 and 5 in Table 3 exhibited a considerable increase in the volume ratio in integrated ATPS relative to the conventional process. Cell debris generated in the integrated exhibited a discrete top phase preference in contrast with the conventional process (exhibiting bottom phase and interface preferences). A converse conclusion to that drawn for systems 1,2 and 6 (see above) may apply here, and the change in the top phase volume inevitably increases the volume ratio. The different behaviour observed in these types of system cannot be currently explained, but may be associated with the nature of the debris and solids remaining after disruption in ATPS. In this context, previous research [4] has demonstrated that for PEG–sulphate systems, the addition of the phase-forming chemicals to a disrupter had a significant influence on the efficiency of cell breakage. This parameter was not evaluated in detail in the present study.

The findings reported here indicate that, although the integration of ATPS with cell disruption caused no effect on the partition behaviour of bulk protein and G3PDH (see Figs. 2 and 3), this was not the case for the partition of cell debris and residual solids. In terms of enzyme recovery, system 3 comprised 22.2% (w/w) PEG 1000, 19.0% (w/w) phosphate and a 50.0% (w/w) TLL, was the best apparent candidate for the primary recovery of G3PDH (see Section 3.1), However, the accumulation of both enzyme product and cell debris in the PEG-rich top phase compromises the application of this process for practical purposes of G3PDH recovery.

3.3. An integrated process for the recovery of protein and G3PDH from yeast

System 3 together with three additional PEG 1000 ATPS characterised by a common TLL of 50% (w/w)

Table 4
Influence of volume ratio on protein and G3PDH recovery in the integrated process

System	PEG1000 (% (w/w))	Phosphate (% (w/w))	V_r	Cell debris phase preference	Protein recovery Y_p (%)	G3PDH recovery Y_E (%)
I	7.0	33.0	0.26 (0.20)	B	40.0	63.0
II	12.0	28.0	0.45 (0.31)	B	41.0	73.0
III	22.2	19.0	1.0 (1.2)	T	79.8	66.5
IV	30.0	13.0	3.5 (7.1)	T	20.0	5.0

Systems (I–IV) were selected along a common same tie-line (TLL = 50% (w/w)) previously recorded for system 3 in Tables 1–3. The V_r, estimated from blank systems, was determined after phase separation in graduated tubes. Values of V_r in parentheses indicate volume ratios recorded for ATPS loaded with biomass as described in the legend to Table 3. T and B denote top and bottom phase preference for cell debris. The recovery of protein and G3PDH from the top phase (Y_P and Y_E) is expressed relative to values determined for the disrupted yeast feedstock. The ratio Y_E: Y_P indicates the relative purification achieved for G3PDH in systems I–IV.

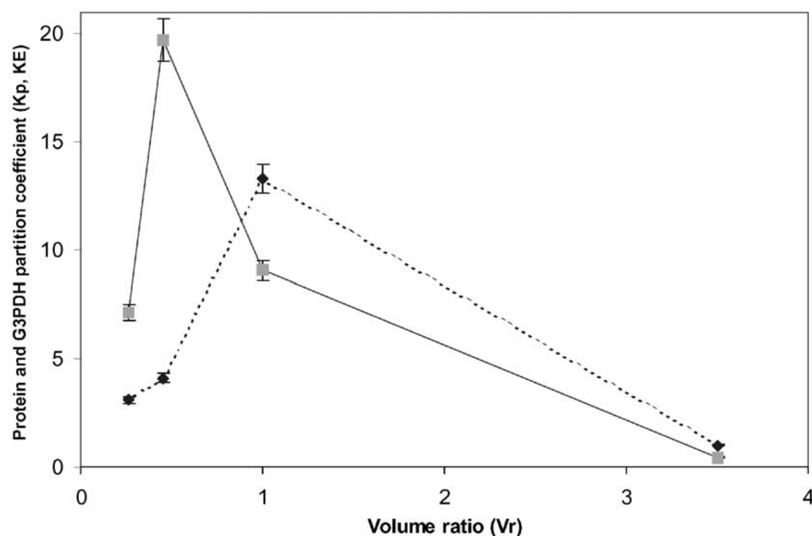

Fig. 4. Effect of system volume ratio on the protein and G3PDH partition coefficient in PEG 1000–phosphate ATPS cell disruption integrated process. The volume ratio of systems I–IV (refer to Table 4) were estimated from blank, non-biological systems (ATPS in the absence of biomass) as described in Section 2. Working volume ratios in loaded systems are indicated in Table 4. The protein (◆) and G3PDH (■) partition coefficients from the integrated process, respectively represent the ratio of the protein concentration and enzymatic activity in the top and bottom phases. For all ATPS, TLL and the system pH were kept constant at 50% (w/w) and 7.0, respectively.

(referred to as systems I–IV in Table 4) were further studied with respect to the variation of volume ratio (V_r) upon the partition behaviour of bulk protein and G3PDH (see Fig. 4). It is clear that system II having a V_r equal to 0.45 (clean system value) offers the best initial condition in this study for the preliminary recovery of G3PDH. Such a system (having a working $V_r = 0.1$; see Table 4) yields the highest enzyme partition coefficient (i.e. $K_E = 20$) together with low value for the partition coefficient of bulk protein (refer to Fig. 4). System II thus achieves the best fractionation of G3PDH enzyme and bulk contaminant protein as confirmed by inspection of the ratios of the enzyme and bulk protein recovered in the top phase (Y_E:Y_p in Table 4). In such an integrated system, where $V_r < 1.0$, the bottom phase preference of cell debris clearly benefits the degree of purification of the enzyme.

4. Conclusion

The process integration strategy presented here for the recovery of intracellular proteins demonstrates that simultaneous disruption and aqueous two-phase extraction can achieve the primary recovery of intracellular proteins from yeast. In particular, operating conditions have been established that facilitate the in situ, primary recovery of G3PDH directly and rapidly from disrupted yeast with a significant degree of purification in respect of the reduction of bulk protein and elimination of cell debris in a single operation. Further studies are required to intensify the biomass loading and increase the yield beyond the current 73% without compromise to the quality of fractionation and product purity. However, the preliminary data presented here demonstrate the potential of the integration of ATPS with cell disruption for the direct recovery of specific intracellular protein targets.

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