Direct process integration of cell disruption and fluidised bed adsorption for the recovery of intracellular proteins†

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Abstract: An integrated process for the primary capture of an intracellular enzyme, where cell disruption is directly coupled with fluidised bed adsorption of the product, was proposed as a generic approach to benefit the yield and molecular integrity of labile protein products. The purification of glyceraldehyde 3-phophate dehydrogenase (G3PDH) from waste brewers' yeast was selected for the demonstration of this principle. Cell disruption by bead milling was combined with direct adsorption of the enzyme on a Cibacron Blue derivative of a kieselguhr–agarose composite adsorbent in a fluidised bed contactor operated immediately downstream of the cell disrupter. The short process time and immediate sequestration of product from the hostile disruptate environment facilitated the recovery of partially purified preparations of this labile enzyme from yeast aged at −20°C for 9 months. The recovered specific activity of 7.6 IU mg^{-1} bettered that expected from the extended time-scale of sequential batch operations of milling, centrifugation or microfiltration, and fixed bed chromatography. The potential role for this novel approach to process integration is discussed in the context of the adsorbent and contactor optimisation necessary to establish efficient, continuous primary recovery of labile intracellular proteins.

Keywords: cell disruption; bead mill; fluidised bed; protein recovery; process integration; intracellular proteins

1 INTRODUCTION

There is much current interest in the achievement of process integration in respect of the upstream operations of fermentation and the downstream recovery processes conventionally associated with the manufacture of bioproducts. There are three common approaches. In the first, new technologies are implemented wherein two or more process objectives, previously attained by a number of discrete operations, are achieved in a single operation. Thus, the technique of fluidised bed adsorption of proteins from whole broths or cell disruptates in a single operation circumvents the need for discrete operations of solid–liquid separation, feedstock dewatering and product fractionation.[1–3] The second approach is more conservative and seeks merely to efficiently interface two sequential operations. Thus, scheduling ammonium sulfate precipitation of proteins directly upstream of hydrophobic chromatography is commonly recommended to avoid the desalting procedures required if ion-exchange chromatography was to be selected.[4,5] A third approach attempts to combine two operations into one in order to achieve specific objectives not efficiently met by discrete processes. Thus, the operation of a fluidised bed contactor as an integral, external loop to a productive fermenter facilitates extractive biotransformations wherein substrate is converted microbially into product which is instantly recovered. Such direct product sequestration has been shown to improve both the yield and molecular quality of an extracelluar protease produced by *Yarrowia lipolytica*.[6]

The third approach to process integration is the subject of the current paper. Cell disruption is a mandatory first step for the recovery of intracellular protein products and is commonly performed mechanically using process homogenisers or bead mills.[7,8] The particle size range of cell debris so generated can

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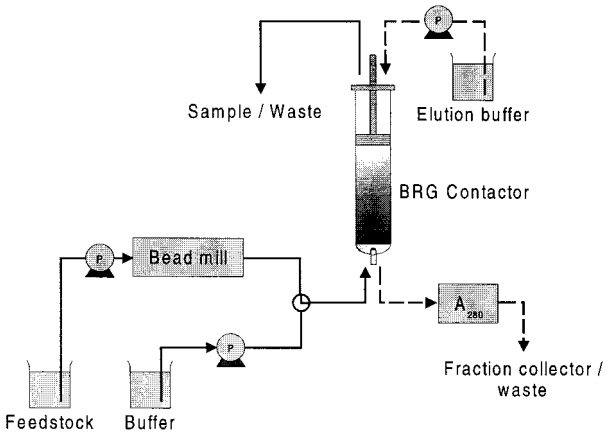

Figure 1. Experimental configuration for integrated wet-milling and fluidised bed adsorption. Solid lines indicated flow direction during sample application (fluidised bed single pass mode). Dashed lines indicate flow during elution (fixed bed mode). The bed was initially expanded to 80% in buffer A. Bed expansion was maintained at 130% throughout loading by manual adjustment of pump settings

pose severe demands upon the resolution and throughput demanded of centrifuges or microfilters used for subsequent feedstock clarification. In addition, the act of cell disruption can initiate a heightened level of endogenous protease activity which may degrade or modify labile protein products at source. Direct product sequestration at cell disruption could therefore enhance product yield and quality in a manner analogous with the extractive biotransformation outlined above.[6] Such product capture could be achieved in a fluidised bed adsorber reliant on the output flow of a cell disrupter for its bed expansion and adsorption performance. As a demonstration of principle, we report preliminary data concerning the linked operation of a bead mill and a fluidised bed contactor to disrupt yeast cells and directly capture the cytoplasmic enzyme glyceraldehyde 3-phosphate dehydrogenase (G3PDH) upon a pseudo-affinity adsorbent (see Fig. 1). The primary purification of this enzyme is exploited here as the focus for a discussion of the generic applicability of such an approach to the processing of labile intracellular products.

2 MATERIALS AND METHODS

The bead mill was a Dyno Mill KDL-I (Willi A Bachofen AG, Switzerland) consisting of a glass chamber cooled by re-circulating iced water (<4°C) from a reservoir at a flow rate of 320 $dm^3 h^{-1}$. The chamber was loaded with glass beads (0.2–0.5 mm) to 83% settled volume occupancy. The agitator speed was 3200 rpm corresponding to a peripheral speed of the agitating discs of 10.5 $m s^{-1}$. Waste brewers' yeast was thawed overnight below 4°C in buffer A (10 mmol dm^{-3} Tris/HCl, pH 7.5 containing 1 mmol dm^{-3} EDTA) at various biomass concentrations of 15–50% frozen wet weight per volume (ww/v). The temperature of the suspension was maintained below 4°C by storage in an ice-bath. Scouting experiments were conducted to establish efficacy of wet-milling alone in respect of protein release and temperature rises, exploiting a range of biomass concentrations (15–50% ww/v) and feedstock flow rates (4–25 $dm^{-3} h^{-1}$, see Table 1). In integrated experiments, cell suspension (15% ww/v) was fed to the mill by a peristaltic pump at a flow rate of 4.05 $dm^3 h^{-1}$ and the temperature of the disruptate was maintained at the outlet of the chamber between 21 and 22°C. In order to achieve a steady-state condition with regard to effluent protein and G3PDH concentrations, the first five chamber volumes of effluent were discarded before switching the disruptate to the contactor. The variation in sample properties taken after processing two to six chamber volumes of feedstock was found to be less than 5% at feed rates of 5–15 $dm^3 h^{-1}$ (data not shown).

The contactor comprised a glass column (500 mm length, 45 mm id) with a hemispherical inlet. The latter was separated from the bed by a stainless steel mesh (98 μm) acting to retain the matrix particles. The inlet was packed with glass beads (3.5–4.5 mm) to evenly distribute the inlet flow over the cross-sectional area of the column. The top of the column was fitted with an adjustable adapter. Cibacron Blue 3GA was immobilised as a pseudo-affinity ligand upon Macrosorb K6AX as described by Gilchrist[9] and the adsorbent was packed to give a settled bed height (SBH = H_0) of 155 mm. Following condition-

Feedstock	*Feed rate* ($dm^3 h^{-1}$)	*Outlet temperature* (°C)	*Total protein released* ($mg cm^{-3}$)	*G3PDH released* ($IU cm^{-3}$)
50% biomass	5	23.8	19.4	402
	15	17.1	19.4	437
	25	13.6	18.7	427
40% biomass	25	12.6	13.8 (17.4)	298 (376)
30% biomass	25	11.9	8.6 (15.2)	144 (256)
15% biomass	4.05	21.5	[3.0]	[5.9]

The experiments were carried out in the Dyno Mill KDL-1 using glass beads (diameter 0.2–0.5 mm) at a bead occupancy of 83% of the chamber volume and an agitator speed of 3200 rpm. The yeast was suspended in buffer A (10 mmol dm^{-3} Tris/HCl, pH 7.5, and 1 mmol dm^{-3} EDTA). The inlet feedstock was maintained at a temperature of 2°C. Values in parentheses are normalised to a 50% (ww/v) fresh yeast feedstock to facilitate comparison. Values in brackets were obtained using yeast aged for 9 months at −20°C.

Table 1. Disruption of brewers' yeast by bead milling

ing,[9] the bed was fluidised using buffer A at a flow rate of 122 $cm^3 min^{-1}$ (superficial velocity 460 $cm h^{-1}$) to give a stable expanded bed height, where $H/H_0 = 1.8$ (ie 80% bed expansion, see Fig 2). Samples were clarified by centrifugation (7600 *g*, 10 min). Eluted samples were desalted using gel filtration chromatography and immediately assayed for G3PDH activity and protein concentration as described by Gilchrist.[9]

3 RESULTS AND DISCUSSION

Results of preliminary disruption experiments are summarised in Table 1. With respect to total protein or G3PDH release the data indicated that total cell disruption was effectively achieved over a wide range of feed rates from 5 to 25 $dm^3 h^{-1}$. Lower feed rates resulted in extended residence times and risked increased temperature for unit volumes of processed feedstock. The outlet temperature at low feed rates, eg 5 $dm^3 h^{-1}$, was close to ambient temperature and thus heat denaturation of the labile product was possible. The efficiency of cell disruption apparently increased with biomass concentration of the feedstock as evidenced by product release at 30 and 40% (ww/v) biomass concentration when normalised to that achieved at 50% biomass (see Table 1). These findings indicate that bead milling can effectively be carried out at high biomass loads, eg 50% (frozen wet weight/volume), and high feed rates, eg 25 $dm^3 h^{-1}$ in order to maximise throughput. Such physical characteristics define the specification limit for an integrated fluidised bed contactor.

However, in this demonstration of principle, the operating parameters of the bead mill had to be matched with the dimensions and the expansion characteristics of the 45 mm diameter fluidised bed contactor available for preliminary experiments. Figure 2 depicts the bed expansion of underivatised Macrosorb K6AX when fluidised in buffer A or in the presence of various concentrations of disrupted biomass. The elevated viscosity associated with increased biomass strongly increased the expansion of the bed for a given flow rate. Experimental conditions, ie 15% biomass concentration, feed rate 4 $dm^3 h^{-1}$, were established for the demonstration of process integration with the 45 mm contactor.

During adsorption, disruptate was applied to the bed until apparent saturation of the adsorbent capacity for G3PDH had been achieved (see Fig 3). The bed was initially expanded ($H/H_0 = 1.8$) using buffer A as determined in preliminary experiments (see Fig 2). However, during loading, the bed expanded further ($H/H_0 = 2.3$) in the face of the elevated viscosity of chilled feedstock. Immediate breakthrough of enzyme activity was evident which might be associated with the channelling clearly visible in the lower segment of the expanded bed on switching the column inlet from buffer to disruptate. This indicates that mixing of Macrosorb K6AX, a highly heterogeneous particle,[6,9] is greater than for other matrices,[3] which promotes poor adsorption behaviour. An idealised breakthrough curve is included in Fig 3 to indicate the extent of improvement of fluidised bed adsorption required in the process.

At the termination of the adsorption stage residual particulate material was washed from the expanded bed with buffer A (50 bed volumes) until a clear effluent was achieved. Enzyme elution was achieved in a four-stage process (see Fig 4 and Table 2). Initially, 0.2 mol dm^{-3} NaCl in buffer A eluted weakly-bound contaminants together with traces of enzyme product which were discarded. Following re-equilibration of the bed in buffer A, the enzyme was desorbed in 0.25 mol dm^{-3} KSCN in buffer A. A final treatment using 3 mol dm^{-3} KSCN in buffer A stripped residual material bound to the matrix as a prelude to the re-equilibration and the re-use of the adsorbent. The mass balance of G3PDH purification is documented in Table 2. Specific activities were

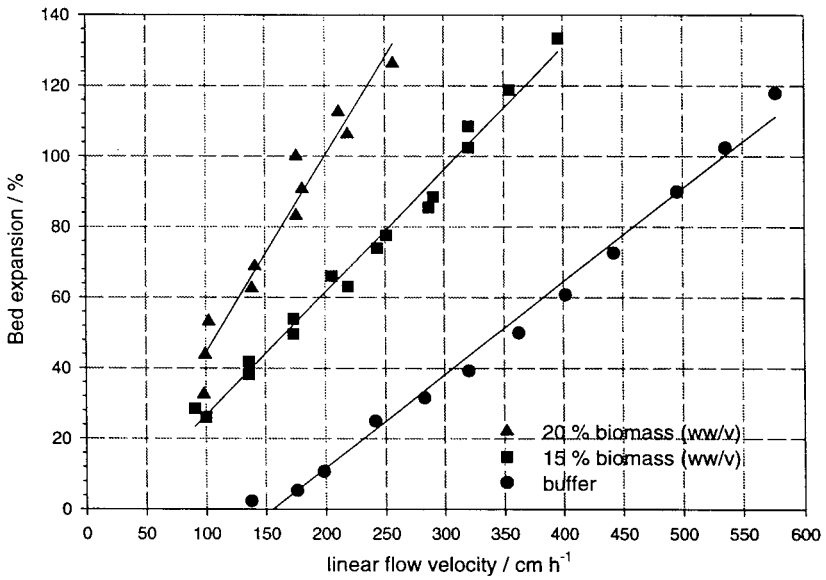

Figure 2. Bed expansion characteristics of underivatised Macrosorb K6AX. The cell disruptate was generated in the dyno mill KDL-I from brewers' yeast suspended in 10 mmol dm^{-3} Tris/HCl buffer at pH 7.5 containing 1 mmol dm^{-3} EDTA. Biomass content of feedstock is expressed as percentage frozen weight of original cells per unit volume (ww/v). Experiments were carried out using a settled bed height of 150 mm in a contactor having a diameter of 45 mm.

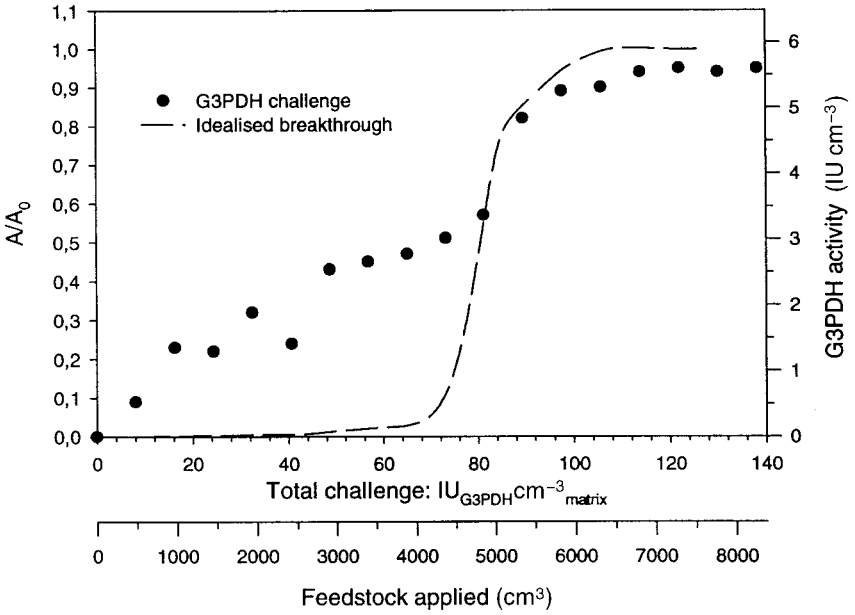

Figure 3. Expanded bed adsorption of G3PDH from wet-milled yeast onto Macrosorb K6AX-Cibacron Blue 3GA: The feedstock contained 15% (w/v) brewers' yeast (wet weight) and was fed to the bead mill at a rate of 4.05 $dm^{-3}\,h^{-1}$ which corresponded to a superficial velocity of 255 $cm\,h^{-1}$ within the contactor operated at a settled bed height of 155 mm (settled bed volume: 247 cm^3). Disruptate from the mill was directly applied to the fluidised bed contactor. Samples were taken from the outlet of the column and assayed for G3PDH activity. Additionally, results are expressed as the ratio of sample activity to feedstock activity (A/A_0). As a comparison, an idealised breakthrough curve is depicted for optimal fluidised bed adsorption.

higher than those previously recorded from wet-milling and fluidised bed adsorption operated as discrete processes over longer time scales.[11] This was encouraging given the low starting activity of yeast stored at −20°C for 9 months (<15% of fresh material, see Table 1).

The 0.25 mol dm^{-3} KSCN treatment resulted in a discrete peak of G3PDH activity coincidental with a broader peak of protein material. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) indicated the presence of the subunit of the tetrameric G3PDH in all samples taken throughout this protein peak (data not shown) but later fractions (3100–3400 cm^3) were inactive. This might reflect different affinities of active and inactive forms of G3PDH for Macrosorb K6AX Cibacron Blue 3GA

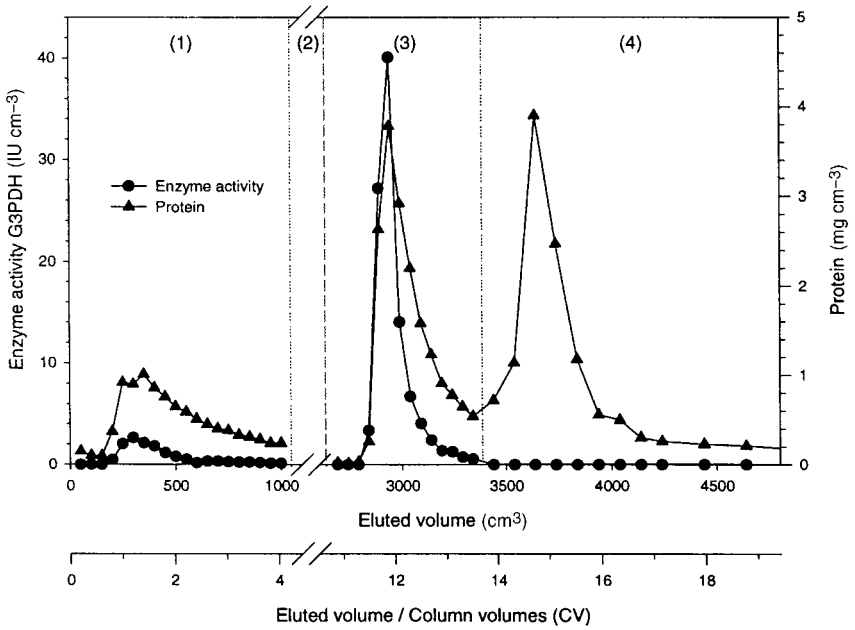

Figure 4. Elution of bound solutes from Macrosorb K6AX-Cibacron Blue 3GA in packed bed mode: The following elution procedures were used. (1) Four column volumes of 0.2 mol dm^{-3} NaCl in buffer A (10 mmol dm^{-3} Tris/HCl, pH 7.5 containing 1 mol dm^{-3} EDTA) at a superficial velocity of 75 $cm\,h^{-1}$ (2) Re-equilibration of the adsorbent by seven column volumes of buffer A at a superficial velocity of 75 $cm\,h^{-1}$. (3) Three column volumes of 0.25 mol dm^{-3} KSCN in buffer A at a superficial velocity of 25 $cm\,h^{-1}$. (4) Five column volumes of 3 mol dm^{-3} KSCN in buffer A at a superficial velocity of 75 $cm\,h^{-1}$.

Stage	*Volume* (cm^3)	*Protein* (*mg*)	*Enzyme activity* (*IU*)	*Specific activity* ($IU\,mg^{-1}$)	*Purification factor*
Disruptate	7763	23 290	45 802	2.0	1
Flow through	7763	20 823	30 808	1.5	—
Elution	750	669	5081	7.6	3.9

Table 2. Mass balance of G3PDH recovery

Brewers' yeast, stored at −20°C for 9 months, was disrupted by bead milling and directly applied to the fluidised bed contactor containing Macrosorb K6AX Cibacron Blue 3GA.

generated during the disruption process. Alternatively, the enzyme may be partially inactivated during the elution process due to the time of exposure to the chaotrope KSCN. It has been reported that high ionic strength promotes sub-unit dissociation of the enzyme and that only the intact tetramer retains catalytic activity.[10] In order to assess the influence of KSCN on the activity of G3PDH, samples were taken from eluted fractions (2900–3000 cm^3, Fig 4) and stored for up to 36 h at 4°C undiluted (ie in 250 mmol dm^{-3} KSCN in buffer A) as well as diluted ten-fold (ie in 25 mmol dm^{-3} KSCN in buffer A). This indicated that 30–50% enzyme inactivation occurred due to prolonged exposure to KSCN and was dependent on the chaotrope concentration (data not shown). The labile nature of G3PDH was confirmed by the loss of activity seen on prolonged storage at −20°C (see Table 1). The time of exposure to high concentrations of KSCN could be shortened by online application of the eluate to a gel filtration column for desalting[9] or direct elution of the G3PDH peak into a buffer reservoir in order to dilute the KSCN. Both options would considerably increase the volume of the eluate. Preliminary screening of alternative specific eluents for dehydrogenases such as NADP or NADPH yielded low elution efficiencies.[11]

4 CONCLUSIONS

Early breakthrough was attributed to the poor fluidisation properties of Macrosorb K6AX which promoted visible bed channelling. The fluidisation characteristics could be improved by an increased bed height and by the use of a matrix having a higher density and a more regular particle geometry. Such measures reduce the mixing of adsorbent particles and improve overall adsorption performance as illustrated in the idealised breakthrough curve in Fig 3.[12–14] Composites of silica–dextran, pyrex–agarose, titanic–cellulose and zircon–agar all possess such characteristics,[6,9,14] but were not available in forms suited to the chemical derivatisation required for this study.[9]

Throughput of feedstock in this experiment was limited by the cross-sectional area of the contactor but could be increased by use of denser matrix requiring higher fluid velocities for equivalent bed expansion. Using a matrix of a density of 1.6 g cm^{-3}, Beveridge[14] has recorded that a superficial flow velocity of 1250 cm h^{-1} was required to generate 100% bed expansion in an equivalent contactor (45 mm diameter). Assuming that a disruptate originating from 15% initial cell biomass extends the degree of bed expansion by a factor of 2.5 at a superficial flow velocity of 300 cm h^{-1} (see Fig 2), then such a high density matrix could be operated at superficial flow velocities of 700 cm h^{-1} in the present contactor, representing a three-fold increase of throughput (ie milling at 12 dm^3 h^{-1}).

The approach to process integration as reported here for labile enzymes clearly proves that a bioproduct can rapidly be removed from the potentially harmful environment of the cell disruptate by performing cell disruption and primary capture almost simultaneously. This methodology deserves further study and will be particularly important for labile proteins (such as the G3PDH studied herein) in order to improve both the yield and molecular integrity of the target products.

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