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The effect of chemical pretreatment combined with mechanical disruption on the extent of disruption and release of intracellular protein from *E. coli*

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Abstract

The combination of non-mechanical (chemical) and mechanical methods such as high pressure homogenisation (HPH) can increase the release of intracellular components and decrease the exposure to mechanical disruption required for the breakage to attain maximum intracellular release. Through these, the energy requirement of microbial cell disruption can be decreased along with the reduction in the micronisation of cell debris. Pretreatments to permeabilise or weaken the cell envelope were selected and the optimum conditions determined through a screening process. The permeabilisation of *Escherichia coli* with EDTA was successful in achieving maximum intracellular protein release at a lower pressure of 13.8 MPa on high pressure homogenisation, compared with 34.5 MPa in the absence of EDTA. Significant reduction in energy input required was observed with the use of this combination method. Pretreatment with guanidium hydrochloride (G-HCl) and Triton X-100 also resulted in increased intracellular release and decreased energy usage. Chemical pretreatment can be useful in enhancing mechanical disruption, however, careful selection of pretreatment conditions is required to avoid protein deactivation and chemical interference in the protein assay.

Keywords: Cell disruption; Downstream processing; Protein recovery; Chemical pretreatment; EDTA; High pressure homogenisation

1. Introduction

Mechanical methods are most widely used to achieve microbial cell disruption for intracellular product release on an industrial scale. However, several disadvantages result. When microorganisms are disrupted mechanically, their periplasmic and cytoplasmic components are released non-selectively, resulting in challenging separation and purification of the desired product subsequently. To increase the extent of disruption, the cells are passed repeatedly through the disruption equipment causing a considerable decrease in the particle size of the debris and hence resulting in a more difficult solid–liquid separation. Further, the mechanical process is energy intensive and very inefficient in terms of energy. Much of the energy input to the mechanical disruption process is dissipated as heat, negatively affecting heat labile intracellular products and increasing duty of heat removal processes required.

Non-mechanical cell disruption methods are less energy intensive and some have been demonstrated to achieve selective release of products. However their application is often limited to a small scale, owing to restrictions of process economics or efficiency. Consequently, they have found limited commercial application to date. Researchers have suggested that the combination of a mechanical method with a non-mechanical pretreatment has potential for improved protein and enzyme release in combination with a reduction in the energy requirement [1–4].

The use of guanidine hydrochloride (G-HCl) and Triton X-100 for bacterial permeabilisation has shown enhanced release of periplasmic proteins [5] owing to their pronounced synergistic effect. The permeabilisation involves a solubilisation of the inner membrane and an outer wall alteration occurring on a molecular level, not detectable by microscopy. The process is dominated by G-HCl which was reported to give a 20% protein release. Its use in combination with Triton X-100 is reported to enhance the protein release to 35% [6] and 50% [5]. Pretreatment of recombinant *E. coli* with 1.5 M G-HCl and 1.5% Triton X-100 resulted in 62% protein release on pretreatment, and 82% after one pass at 41 MPa through the homogeniser,

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compared to 93% release from untreated cells at 41 MPa and two passes [7].

In this study, a combined cell disruption approach has been investigated using *E. coli*. Chemical methods were chosen based on their ability to attack the specific resistant regions in the cell envelope. These methods were implemented for the permeabilisation or weakening of the cell wall and the cell membrane. The permeabilised cells were further exposed to high pressure homogenisation to enable cell disruption with advantages sought in terms of reduced operating pressures and hence improved energy efficiency and reduced micronisation of cell debris. The effectiveness of cell disruption was determined by both the extent and rate of release of total soluble protein and of specific enzymes from defined locations within the microorganism.

2. Release kinetics theory

Microbial disruption kinetics, described in terms of protein release kinetics for high pressure homogenisation, were studied by Hetherington et al. [8]. Protein release by high pressure homogenisation is first order with respect to the protein available for release and can be described by Eq. (1):

$$\ln\left(\frac{R_m}{R_m - R}\right) = kP^a N = k'N \quad (1)$$

where R_m is defined as the maximum protein available for release, R the amount of protein released after N passes through the homogeniser, P the operating pressure (kPa) and k is the release rate constant with units of $1/Pa^a$. The terms kP^a can be grouped together to represent the effective disruption rate constant, k', with units of 1/pass. This approach of Hetherington et al. [8] has been supported through numerous subsequent studies of yeast and bacterial cell disruption [9–13].

With the use of a pretreatment, Eq. (1) can be modified to incorporate the release of protein during permeabilisation. Baldwin and Robinson [14] modified the equation to describe protein release on mechanical disruption following enzymatic pretreatment of the cell suspension to the form:

$$\ln\left(\frac{R_m - R_0}{R_m - R}\right) = kP^a N = k'N \quad (2)$$

where R_0 is the amount of protein released during the pretreatment.

In the case of the procedures used for this study, the pretreated cells were analysed for the release of proteins. The chemical and protein-containing supernatant was then removed by centrifugation and the cells were washed to remove remaining chemicals. Therefore, when the cells were homogenised, the amount of protein available for release was $R_m - R_0$. The equation describing first order cell disruption in this case is a modified form of Eq. (2) and is given by Eq. (3):

$$\ln\left(\frac{R_m - R_0}{R_m - R_0 - R}\right) = kP^a N = k'N \quad (3)$$

3. Materials and methods

3.1. Microorganism

Escherichia coli (*E. coli* CSH 36) was sub-cultured in sterile tryptone yeast extract (TYE) growth media containing: 10 g/l tryptone, 5 g/l yeast extract and 5 g/l sodium chloride. A 50 ml inoculum was cultured for 12 h on an orbital shaker at 120 rpm and 37 °C. This was used to inoculate 200 ml in 2 l shake flasks, under the same conditions. The *E. coli* cells were harvested at 24 h and washed with a sodium phosphate buffer (0.025 M NaH_2PO_4, pH 7.0).

3.2. Experimental apparatus

A Rannie high pressure laboratory homogeniser (Model MINI-LAB, type 8.30 H), manufactured by APV, with a working pressure range of 0–1000 bar (0–100 MPa) was used. The capacity of the apparatus was 400 ml with a maximum flow rate of 10 l/h. The homogeniser consists of a dual piston design with a single stage homogenising system. The ceramic Rannie homogenising valve housing contains a Stellite ball valve with a flat edge valve unit and an orifice diameter of 3 mm. The cooling water is available at 5 l/h. Experiments were performed at operating pressures of 13.8–34.5 MPa. All experiments were performed using a 1% cell concentration (wet weight, w/v).

3.3. Permeabilisation procedure

A 2% cell suspension (wet weight, w/v) was used for permeabilisation. The final concentrations of chemicals used, time and temperature of incubation are given in Table 1. Following the treatment, the suspension was centrifuged at 10,000 rpm using the Avanti J-25 Beckman high speed centrifuge and the JA-10 Beckman rotor for 10 min at 20 °C to remove the chemicals. A sample of the supernatant was analysed to determine the amount of protein and enzyme released during the permeabilisation procedure. The cells were subsequently washed twice with a sodium phosphate buffer (0.025 M NaH_2PO_4, pH 7.0) to remove any remaining chem-

Table 1
Permeabilisation and HPH conditions for *E. coli*

Chemical for outer membrane permeabilisation	Concentration (M)	Chemical for inner membrane permeabilisation	Concentration	Pretreatment time (min)	Pretreatment temperature (°C)	HPH conditions
EDTA	0.040	–	–	10	37	13.8 MPa, 20 pass
G-HCl	0.1	Triton X-100	2%	60	4	13.8 MPa, 20 passes

Table 2
Protein and enzyme release from *E. coli* (1% wet weight) following permeabilisation with EDTA at 37 °C for 10 min with agitation at 120 rpm

EDTA concentration (M)	Total soluble protein (mg/g)	Periplasmic acid phosphatase (U/g)	Cytoplasmic β-galactosidase (U/g)
0.02	2.25	14.2	18.4
0.04	3.58	14.9	28.0
0.06	2.43	4.07	16.6
0.08	1.86	4.07	21.6
0.10	1.00	4.74	5.03

icals prior to their re-suspension and further breakage by homogenisation.

3.4. HPH protocol

A 1% suspension (wet weight, w/v) of permeabilised cells was prepared in sodium phosphate buffer solution (0.025 M NaH_2PO_4, pH 7.0) and homogenised at a pressure of 13.8 MPa. Aliquots of 1 ml were taken every pass for the first four passes and every 4th pass thereafter for analysis. The total soluble protein concentration and activities of the periplasmic enzyme acid phosphatase and cytoplasmic enzyme β-galactosidase in the sample supernatant were determined.

3.5. Analytical methods

The effect of the pretreatment and the extent of disruption were quantified in terms of the total soluble protein and enzymes released. The release of total soluble protein was measured by the Bradford method [15] at a wavelength of 595 nm. The activity of acid phosphatase was measured by the hydrolysis of 4-nitrophenyl phosphate to 4-nitrophenol determined at a wavelength of 410 nm [16]. The activity of β-galactosidase was determined by the hydrolysis of ONPG (*ortho*-nitrophenyl-β-galactoside) quantified spectrophotometrically at a wavelength of 420 nm [17].

4. Results

4.1. EDTA pretreatment

A range of EDTA concentrations between 0.020 and 0.1 M was studied to determine the optimum conditions for permeabilisation or weakening of the cell wall. Release of total soluble protein and marker enzymes was measured. These showed a maximum release of both cytoplasmic and periplasmic components at an EDTA concentration of 0.040 M (Table 2). The release of acid phosphatase showed a small increase when EDTA concentration was increased from 0.020 to 0.040 M, but further increase in EDTA concentration was accompanied by a much reduced extracellular activity. Hence, 0.040 M EDTA was chosen for the combined cell disruption study. The pretreatment yielded a release of 2% of the total soluble protein R_m and approximately 2% of both the cytoplasmic and periplasmic components. Thus 98% of the protein remained available for release on mechanical disruption ($R_{m,HPH} - R_0$).

4.2. Guanidium hydrochloride (G-HCl) and Triton X-100 pretreatment

G-HCl is known to inhibit the cross linking of peptidoglycan and cell wall synthesis. It also solubilises protein from membrane fragments and alters hydrophobic interactions. Triton

Table 3
Protein and enzyme release from *E. coli* (1% wet weight) following permeabilisation with G-HCl and Triton X-100 (2%) at 4 °C for 1 and 2 h with intermittent shaking

G-HCl concentration (M)	Total soluble protein (mg/g)	Periplasmic acid phosphatase (U/g)	Cytoplasmic β-galactosidase (U/g)
1 h incubation			
0.10	0.43	395	35.9
0.50	1.53	268	22.3
1.00	2.25	89.0	0.26
1.50		116	1.03
2.50	12.3	48.8	1.94
G-HCl concentration (M)	Protein (mg/g)	Acid phosphatase (U/g)	β-Galactosidase (U/g)
2 h incubation			
0.10	0.68	304	35.0
0.50	1.14	322	21.6
1.00	1.88	88.1	0.13
1.50		167	1.03
2.50	12.2	49.5	1.48

Table 4
Interference of chemicals on proteins measured as protein concentration and % denatured in relation to release achieved from untreated bacteria at the same pressure

	Bacterial homogenate (R_i)	0.040 M EDTA pretreatment		0.1 M G-HCl + 2% Triton X-100 pretreatment	
		HPH	% Denatured	HPH	% Denatured
Protein (mg/g)	126	122	3.02	116	7.55
Acid phosphatase (U/g)	427	369	13.5	357	16.5
β-Galactosidase (U/g)	1203	712	40.8	923	23.3

X-100 was used to permeabilise the cell membrane. It is reported that the main cause of permeabilisation in bacterial cells in the presence of G-HCl and Triton X-100 is due to G-HCl [5–7]. Triton X-100 assists through membrane destabilisation. However, the detailed mechanism of action of G-HCl in the presence of Triton X-100 is not reported elsewhere. The G-HCl concentration was varied in the range 0.1–2.5 M. The Triton X-100 concentration was only used at the optimum reported concentration of 2% [5–7]. Treatment times of 1 and 2 h were used. The protein concentration and enzyme activities per unit biomass released on these treatments are reported in Table 3.

As the G-HCl concentration is increased from 0.1 to 1.0 M, the soluble protein release increased gradually. A sharp increase in the release was found at 2.5 M G-HCl (Table 3), resulting in a release of 8% of the total soluble protein available for release ($R_{m,HPH}$). Hettwer and Wang [6] observed that a peak in the protein release was achieved with 0.12 M G-HCl and 2% Triton X-100, correlating to 45% of the maximum attainable protein release. Contrary to the protein release, the acid phosphatase activity in the supernatant decreased with increasing G-HCl concentration from 0.1 to 2.5 M. The extracellular β-galactosidase activity also showed a steady decline when the concentration of G-HCl was increased from 0.1 to 1.0 M with little or no activity at higher G-HCl concentrations. Protein release was similar following the treatment time of 1 or 2 h, hence the shorter treatment time was selected. Since the maximum extracellular activity of acid phosphatase and β-galactosidase observed at a G-HCl concentration of 0.1 M suggested inhibition or denaturation of these enzymes at increased G-HCl concentrations and weakening of the bacterial cells is sought in the pretreatment rather than actual release of the protein, 0.1 M G-HCl was chosen for use as a pretreatment prior to mechanical cell disruption.

4.3. Interference of chemicals

The amount of enzyme denatured by the pretreatment chemicals was assessed by treating the *E. coli* homogenate prepared at 13.8 MPa with the chemical at the concentration, temperature and duration used in the pretreatment. The sample was analysed for residual soluble protein and marker enzyme activities. This allowed direct attack of the chemical on the released proteins to evaluate denaturation.

The potential for denaturation by the chemicals used in chemical permeabilisation of microorganism has generally not been considered, however, direct interference with the protein assay has been described [18,19]. A concentration of 0.1 M EDTA has been reported to cause a change of 0.004 in the optical density units at 595 nm with the assay [15]. This was acknowledged by adding the chemical at the selected concentration to the blank for samples containing the permeabilising solution. Underestimation of protein may result from reduced dye binding in the Bradford assay in the presence of G-HCl as a result of competition with the dye. These competitive effects were overcome through their inclusion in the standard calibration [20] by introduction of the chemicals into the blank at the appropriate concentration. After the washing procedure, it was expected that all chemicals had been removed and therefore no inclusion in the blank was necessary.

The results, presented in Table 4, revealed that EDTA showed little interference with soluble protein and acid phosphatase. However, a 41% reduction in β-galactosidase activity occurred. The treatment of *E. coli* homogenate with G-HCl and Triton X-100 revealed a small amount of denaturation of soluble protein, and slightly larger amounts of deactivation of acid phosphatase and β-galactosidase (17 and 23%, respectively).

4.4. HPH combination with pretreatment

4.4.1. Extent of disruption

Table 5 presents the results of intracellular protein release with 0.040 M EDTA pretreatment used in combination with high pressure homogenisation at 13.8 MPa. The extent of release measured with the combination is compared to R_{max}, the maximum available for release in the absence of pretreatment. R_{max} was determined by measuring soluble protein and enzyme release from untreated bacteria over a range of pressures between 13.8 and 69.0 MPa. R_{max} was taken as the highest asymptote of release for soluble protein and each enzyme measured. The release observed with pretreated bacteria exceeded the release of proteins from untreated bacteria at the same operating pressure, giving 1.1-, 2.4- and 1.9-fold the release on HPH under the same conditions in the absence of pretreatment for soluble protein, acid phosphatase and β-galactosidase respectivley. Further it exceeded the maximum release achieved on mechanical disruption by HPH alone ($R_{m,HPH}$).

Pretreatment with 0.1 M G-HCl and 2% Triton X-100 for 1 h combined with homogenisation at 13.8 MPa increased the extent of release of soluble protein and acid phosphatase when compared with untreated bacteria homogenised at the same operating pressure (Table 5). The pretreatment resulted in a maximum release being achieved at an operating pressure of 13.8 MPa. The amount of β-galactosidase released was 1.4 times greater than the release from untreated bacteria at 13.8 MPa (17.7 U/g in comparison to 12.0 U/g) and 89% of the maximum available

Table 5
Protein release following pretreatment and homogenisation of *E. coli* at 13.8 MPa

Pretreatment	HPH conditions	Total soluble protein		Acid phosphatase		β-Galactosidase	
		mg/g	%	(U/g) × 10^2	%	(U/g) × 10^2	%
Pretreatment: 0.040 M EDTA	None	0.85	0.5	0.14	1.6	0.05	0.2
	13.8 MPa, 20 passes	176		10.5		22.8	
	Total (chem. and HPH)	176	112	10.6	121	22.9	113
Pretreatment: 0.1 M G-HCl, 2% Triton X-100	None	31.6	20.1	0.41	4.6	0.19	0.9
	13.8 MPa, 20 passes	138		9.15		17.7	
	Total (chem. and HPH)	170	108	9.56	108	17.9	89
No pretreatment	13.8 MPa, 20 passes	156	99.4	4.37	49.6	12.0	60
	34.5 MPa, 20 passes	157	99.5	8.88	100	20.2	100
	R_{max}	157		8.82		20.2	

Extent of release is compared following 20 passes through the homogeniser. Maxima may be achieved on fewer passes (Fig. 1).

(R_{max} of 20.0 U/g). The coefficients of variance of the total soluble protein, acid phosphatase and β-galactosidase measured were 8.8, 6.4 and 5.4%, respectively.

4.4.2. Rate of disruption

Maximum release of intracellular protein from untreated bacteria was obtained in four passes through the homogeniser at 34.5 MPa (Fig. 1), and on 16 passes at 13.8 MPa. Maximum release from untreated cells was not achieved for acid phosphatase and β-galactosidase at 13.8 MPa, even though the release stabilised such that an increase in the number of passes did not further increase release (Fig. 1). At a pressure of 13.8 MPa, the release of the total soluble protein reached 156 mg/g bacteria i.e. R_{max}, while 50% of the acid phosphatase and 60% of the β-galactosidase was released.

The release of total soluble protein on homogenisation at 13.8 MPa subsequent to EDTA pretreatment achieved R_{max} after four passes mimicking the release profile of untreated bacteria at 34.5 MPa (Fig. 1). The amounts of total soluble protein, acid phosphatase and β-galactosidase released on EDTA treatment followed by HPH at 13.8 MPa were 176 mg/g, 1050 U/g and 2278 U/g, respectively, achieving the maximum release of intracellular material determined by homogenisation at 34.5 MPa with untreated bacteria.

Increased release of acid phosphatase and β-galactosidase at 13.8 MPa was found with the G-HCl and Triton X-100 treatment in comparison to untreated bacteria homogenised at the same pressure (Fig. 1). Following this pretreatment, a soluble protein release of 85% of R_{max} was achieved following 12 passes through the HPH at 13.8 MPa. Maximum release of acid phos-

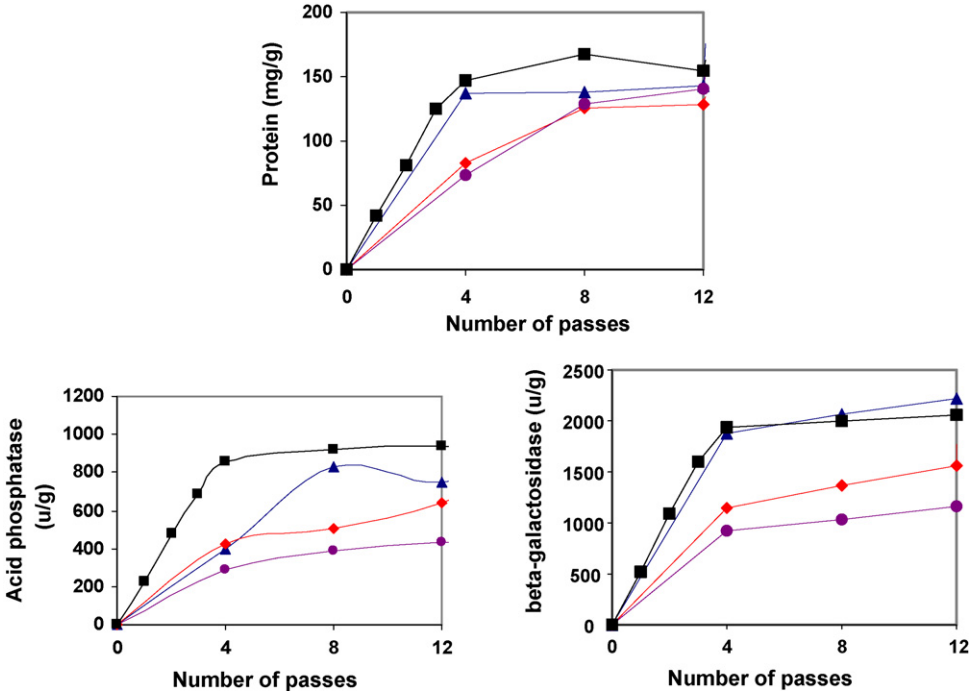

Fig. 1. Release of total soluble protein, acid phosphatase and β-galactosidase as a function of the number of passes. (▲) Pretreatment with EDTA, HPH at 13.8 MPa; (●) pretreatment with G-HCl and Triton X-100, HPH at 13.8 MPa; (◆) untreated bacteria and HPH at 13.8 MPa; (■) untreated bacteria and HPH at 34.5 MPa.

Table 6
Release rate constants (k') and regression coefficients R^2 calculated protein release by HPH prior to and post pretreatment

Pretreatment	HPH conditions	Protein		Acid phosphatase		β-Galactosidase	
		$k' \times 10^{-3}$	R^2	$k' \times 10^{-3}$	R^2	$k' \times 10^{-3}$	R^2
0.040 M EDTA	13.8 MPa, 20 passes	521	1.00	401	0.89	705	1.00
0.1 M G-HCl, 2% Triton X-100	13.8 MPa, 20 passes	268	1.00	111	1.96	129	0.97
No pretreatment	13.8 MPa, 20 passes	244	0.96	73.1	0.96	90.7	0.86
	34.5 MPa, 20 passes	516	0.94	506	0.96	867	0.97

phatase was achieved on 16 passes through the homogeniser at a pressure of 13.8 MPa. After 16 passes, the amount of β-galactosidase released was 1.4 times greater than the release from untreated bacteria at 13.8 MPa and 89% of the maximum available (R_{max}). Hence, the pretreatments used resulted in increased protein release on reduced exposure to homogenisation compared to that for untreated bacteria.

4.4.3. Release rate kinetics

The release rate kinetics on homogenisation of pretreated cells was modelled according to Eq. (3) to determine the release rate of soluble protein as well as individual enzymes. The correlation coefficients obtained illustrate that this modified model satisfactorily described the release following pretreatment (Table 6). The release rate of total soluble protein on HPH at 13.8 MPa following EDTA pretreatment was approximately two fold that of untreated bacteria at 13.8 MPa (calculated according to Eq. (1)), and equal to the release rate of untreated bacteria at 34.5 MPa. The release rates of acid phosphatase and β-galactosidase were five- to seven-fold greater at 401×10^{-3} and 705×10^{-3}, respectively, with the pretreatment in comparison to the release rates from untreated bacteria at 13.8 MPa of 73.1×10^{-3} and 91.0×10^{-3} and were approximately 80% of the release rate from untreated bacteria at 34.5 MPa.

The release rates of all proteins following G-HCl and Triton X-100 pretreatment and homogenisation were higher than untreated bacteria on homogenisation at 13.8 MPa; however, they remained lower than the release rates of untreated bacteria at 34.5 MPa. The order of release showed that soluble protein was released fastest at 268×10^{-3}, followed by 129×10^{-3} for β-galactosidase and lastly acid phosphatase. The trends observed with this method do not follow those observed by Follows et al. [21], but are due to the weakening or permeabilisation of the cell envelope during pretreatment with the chemicals. Once permeabilisation has occurred, there is no selective release of periplasmic or cytoplasmic enzymes from pretreated cells on further homogenisation.

4.4.4. Energy efficiency

The energy input required for one pass through the homogeniser (E_t) can be calculated in terms of the operating pressure P, the volumetric flow rate Q and the time of operation, according to Eq. (4):

$$E_t = PQt \tag{4}$$

In order to estimate total energy dissipated for multiple passes, E_t is multiplied by the number of passes. By replacing the time of operation for one pass by V/Q, the total energy input is obtained. Dividing by the total volume processed V, the energy dissipation per unit volume E is given by Eq. (5):

$$E = PN \tag{5}$$

where E is measured in terms of MJ/m^3, N the number of passes used and P is the operating pressure (MPa). Therefore, the number of passes and pressure contribute directly to the total energy input.

The use of EDTA as a pretreatment method in combination with high pressure homogenisation has shown significant decrease in energy consumption. Table 7 shows an energy reduction of 60% with the use of the combination on the release of soluble protein. Maximum release of soluble protein was obtained in four passes at 13.8 MPa with the pretreatment compared to four passes at 34.5 MPa for untreated bacteria. Maximum acid phosphatase release was obtained with the pretreatment in eight passes at 13.8 MPa resulting in a 20% reduction in energy usage. The release of β-galactosidase reached a maximum in four passes at 13.8 MPa resulted in a 60% reduction in energy usage. Clearly, this method is advantageous for its increased release and decreased energy consumption, especially for cytoplasmic enzymes such as β-galactosidase (60% reduction in energy requirements).

Table 8 shows the protein release per energy input (MJ) following homogenisation of treated and untreated cells using four passes through the homogeniser as a comparison. This shows

Table 7
Energy efficiency calculated for maximum intracellular protein release with EDTA pretreatment combined with HPH

Compound released	No pretreatment		Pretreatment with 0.040 M EDTA		% Energy reduction
	HPH conditions	Energy for max. protein release (MJ/m^3)	HPH conditions	Energy for max. protein release (MJ/m^3)	
Total soluble protein	4 passes, 34.5 MPa	138	4 passes, 13.8 MPa	55.2	60
Acid phosphatase	4 passes, 34.5 MPa	138	8 passes, 13.8 MPa	110	20
β-Galactosidase	4 passes, 34.5 MPa	138	4 passes, 13.8 MPa	55.2	60

Table 8
Comparison of release per four passes per MJ of energy

Treatment	Protein release (mg/MJ)	Acid phosphatase (U/MJ) × 10^2	β-Galactosidase (U/MJ) × 10^2
Untreated bacteria, 13.8 MPa	22.9	0.90	2.88
Untreated bacteria, 34.5 MPa	18.4	1.08	2.42
EDTA + HPH at 13.8 MPa	42.8	2.60	6.46
G-HCl + HPH at 13.8 MPa	25.9	1.31	3.58

that for both pretreatments, total soluble protein and enzyme release per MJ of energy required is greater than that of untreated bacteria, further confirming the efficiency of including a pretreatment step prior to homogenisation of microbial cells for product release. The energy efficiency of HPH following EDTA pretreatment is approximately 1.7- to 2.0-fold greater than with G-HCL and Triton X-100 pretreatment and HPH. It is some 1.9- to 2.8-fold greater than on application of homogenisation only.

5. Discussion

EDTA has been successful in decreasing the resistance of Gram-negative bacteria to mechanical disruption. EDTA complexes with divalent cations resulting in the destabilisation of the outer membrane. The changes in the outer membrane also cause weaknesses in the inner membrane resulting in a high permeability of the cell after EDTA treatment [18,19]. De Smet et al. [18] reported a release of 17.7% protein following exposure to EDTA only.

The use of EDTA as a pretreatment method combined with HPH has proved to be successful with maximum release achieved with disruption at 13.8 MPa, whereas untreated bacteria required a pressure of 34.5 MPa to achieve the same release of protein. The enhanced intracellular release at a lower pressure with minimal denaturation of proteins on exposure to the chemical renders this method effective as a pretreatment method combined with HPH for increased release.

The G-HCl and Triton X-100 pretreatment method combined with HPH also resulted in an increased intracellular release compared to untreated bacteria at the same pressure, with maximum release of soluble protein and acid phosphatase achieved. This increase in the release at a lower pressure results in reduced mechanical energy requirements.

6. Conclusions

The use of pretreatment combined with mechanical disruption has proved successful in terms of yielding increased intracellular release or requiring reduced exposure to mechanical disruption. These result in decreased energy requirements and micronisation of cell debris. Optimisation of the permeabilisation process is necessary to avoid the denaturation of proteins. The removal of the chemicals is necessary to avoid denaturation of the proteins, since EDTA resulted in 3, 13.5 and 40% deactivation of the total soluble protein, acid phosphatase and β-galactosidase respectively. G-HCl and Triton X-100 have shown similar amounts of protein deactivation, with 7.55% for soluble protein, 16.5% for acid phosphatase and 23.3% for β-galactosidase. Therefore, insufficient removal of the chemicals by washing in the downstream processing will result in suboptimum yields of the desired product.

EDTA was successful in permeabilising bacterial cells, reducing its resistance to disruption and achieving maximum release of proteins on homogenisation at 13.8 MPa and four passes, whereas maximum release from untreated cells required a pressure of 34.5 MPa and four passes. The increased release at the reduced pressure resulted in a 60% decrease in energy requirements. The chemical was found to cause minimal interference with soluble protein and acid phosphatase but approximately 40% denaturation of β-galactosidase, further confirming the need for rigorous knowledge of interaction between pretreatment chemicals and proteins of interest.

Use of G-HCl and Triton X-100 as a pretreatment method for bacteria resulted in increased release of proteins compared to untreated cells at 13.8 MPa. This increased release resulted in a decrease in energy requirement of some 20% for release of periplasmic enzymes and 40% for release of cytoplasmic enzymes.

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References

[1] J.A. Asenjo, Separation processes in Biotechnology, Marcel Dekker Inc., New York, Basel, 1990.
[2] C. Baldwin, C.W. Robinson, Disruption of *Saccharomyces cerevisiae* using enzymatic lysis combined with HPH, Biotechnol. Techn. 4 (1990) 324–329.
[3] S.T.L. Harrison, The extraction and purification of PHB from *Alcaligenes eutrophus*, Ph.D. Dissertation, University of Cambridge, 1990.
[4] S.T.L. Harrison, J.S. Dennis, H.A. Chase, Combined chemical and mechanical processes for the disruption of bacteria, Bioseparation 2 (1991) 95–105.
[5] T.J. Naglak, H.Y. Wang, Recovery of foreign protein from the periplasm of *Escherichia coli* by chemical permeabilisation, Enzyme Microb. Technol. 12 (1990) 603–611.
[6] D. Hettwer, H. Wang, Protein release from *Escherichia coli* cells permeabilized with guanidine-HCl and Triton X-100, Biotechnol. Bioeng. 33 (1989) 886–895.
[7] S.M. Bailey, P.H. Blum, M.M. Meagher, Improved homogenization of recombinant *Escherichia coli* following pretreatment with guanidine hydrochloride, Biotechnol. Progr. 11 (1995) 533–539.

[8] P.J. Hetherington, M. Follows, P. Dunhill, M.D. Lilly, Release of protein from Baker's yeast (*Saccharomyces cerevisiae*) by disruption in an industrial homogenizer, Trans. Inst. Chem. Eng. 49 (1971) 142–148.
[9] P.P. Gray, P. Dunhill, M.D. Lilly, The continuous-flow isolation of enzymes, Ferment. Technol. Today (1972) 347–351.
[10] J. Limon-Lason, M. Hoare, C.B. Orsborn, D.J. Doyle, P. Dunhill, Reactor properties of a high-speed bead mill for microbial cell rupture, Biotechnol. Bioeng. 21 (1979) 745–774.
[11] C.R. Engler, C.W. Robinson, Effects of organism type and growth conditions on cell disruption by impingement, Biotechnol. Lett. 3 (1981) 83–88.
[12] E. Keshavarz-Moore, M. Hoare, P. Dunhill, Disruption of Baker's yeast in a high-pressure homogenizer: new evidence on mechanism, Enzyme Microb. Technol. 12 (1990) 764–770.
[13] S.T.L. Harrison, J.S. Dennis, H.A. Chase, The disruption of *Alcaligenes eutrophus* by high-pressure homogenisation: key factors involved in the process, Bioseparation 2 (1991) 155–166.
[14] C.V. Baldwin, C.W. Robinson, Enhanced disruption of *Candida utilis* using enzymatic pretreatment and high-pressure homogenization, Biotechnol. Bioeng. 43 (1993) 46–56.
[15] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[16] D. Galabova, B. Tuleva, D. Spasova, Permeabilization of *Yarrowia lipolytica* cells by Triton X-100, Enzyme Microb. Technol. 18 (1996) 18–22.
[17] M.V. Flores, C.E. Voget, R.J.J. Ertola, Permeabilisation of yeast cells (*Kluyveromyces lactic*) with organic solvents, Enzyme Microb. Technol. 16 (1994) 340–346.
[18] M.J. De Smet, J. Kingma, B. Witholt, The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*, Biochim. Biophys. Acta 506 (1978) 64–80.
[19] H. Felix, Permeabilized cells, Anal. Biochem. 120 (1982) 211–234.
[20] S.J. Compton, C.G. Jones, Mechanism of dye response and interference in the Bradford assay, Anal. Biochem. 151 (1985) 369–374.
[21] M. Follows, P.J. Hetherington, P. Dunhill, M.D. Lilly, Release of protein from Baker's yeast by disruption in an industrial homogeniser, Biotechnol. Bioeng. 13 (1971) 549–560.