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Freeze/thawing and sonication of *Escherichia coli* TB1 cells for cytochrome b_5 recovery

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Abstract

The influence of sonication power, suspension volume and cell concentration on the kinectics of cytochrome b_5 and intracellular protein release by sonication of *Escherichia coli* TB1 cells was studied. The influence of freezing and thawing of the cell suspension was also evaluated. Freezing and thawing increased the recovery yield of cytochrome b_5. The sonication efficiency increased with the increase of sonication power and with the decrease of the suspension volume and cell concentration.

Introduction

In recent years owing to the introduction of genetic engeneering and the development of biotechnology, the number of economically attractive intracellular products with applications in food and pharmaceutical industries, such as chemicals, proteins, enzymes and antibiotics, has significantly increased.

The isolation of intracellular materials requires that the producing cell either be genetically modified to excrete the intracellular product into the extracellular environment, or be disintegrated by physical, chemical or enzymatic methods to release its contents into the surrounding medium. As making the cell fully permeable is still limited (Christi et al., 1986), microbial cell disruption for intracellular product isolation is becoming of increasing importance. Cell disruption, as the first stage in the isolation of intracellular materials, is a crucial step in downstream processing because it limits the performance of subsequent recovery and purification steps, and, on the other hand, the achievement of high disruption yields will allow more flexibility in the subsequent treatment of the product (Keshavarz et al., 1987).

In this work, we have studied the release of cytochrome b_5, a recombinant protein, from *Escherichia coli* by sonication, a well recognized effective procedure at laboratory scale.

Cytochrome b_5 of the endoplasmic reticulum of mammalian liver is a small heme protein, with a well-known three-dimensional structure, that plays an important role in a variety of electron transfer reactions related to fatty acid desaturation, hepatic cytochrome P-450 reduction and regeneration of ferrous hemoglobin in erythrocytes. This protein is composed of two domains: a soluble heme-containing globular core and a smaller carboxy-terminal tail anchored in the microsomal membrane. The synthetic gene of the soluble domain of cytochrome b_5 was cloned into a plasmid and expressed into *Escherichia coli*. The transformed cells containing the cytochrome b_5 sequences are red coloured due to the intracellular production of this protein in amounts up to 8% w/v of the total cellular protein (von Bodman et al., 1986; Karim et al., 1993).

Materials and methods

Cell growth and storage

Escherichia coli TB1 cells, genetically modified with the plasmid pUC13 (von Bodman et al., 1986), which contains the cytochrome b_5 (MW = 13.6 kDa) expression gene and confers ampicillin resistance to the cells, were grown in a 2 L *Erlenmeyer* flask (with a lateral port for a pH electrode), with a working volume of 1.1 L, using a fermentation medium composed of 1 % peptone (BDH), 0.5% yeast extract (Difco), 1% glucose (Merck), 0.5% sodium chloride (Merck) and supplemented with 100 μg/mL ampicillin (Boehringer Mannheim). Fermentations were carried out for 32 h at 37 °C, 200 rpm, and the pH was automatically adjusted to 7.0 with the addition of 2N NaOH or 2N HCl.

After cell growth, the *Escherichia coli* cells were harvested by centrifugation at 4 °C and 9000 rpm for 10 min, washed twice and ressuspended in 20 mM phosphate buffer pH 7.0 up to the experimental volumes. The cells were then stored frozen at -20 °C and thawed just prior to the experiments.

Cell disruption

Cell suspensions were sonicated at 20 kHz, using a Labsonic 2000 sonicator (B. Braun) with a 3/4" standard titanium probe. All experiments were performed in a batch system using a cup (with a cooling jacket) with a total volume of 280 ml (with a diameter and a height of 6 and 10 cm, respectively). The probe was immersed 1.5 cm below the liquid surface. During sonication, a large amount of heat is transfer to the liquid. To prevent the temperature rising above 15 °C the suspension was cooled by running a 10% cold solution of ethylene glycol in the cooling jacket and by sonicating for 1 min followed by another minute of waiting before proceeding with the next sonication step (Braun, 1984).

The effective energy of the sound waves generated by the transducer was estimated by subtracting the power output in the air from that in the cell suspension (James et al., 1972), and from an adiabatic temperature increase measured in the experimental cup with a known volume of water (Thacker, 1973; Augenstein et al., 1974; Bar, 1988). However, as these two methods are not accurate measurements of that energy, and lead to quite different results, it was decided to use the output power as the ultrasound power (Davis, 1959).

The influence of freezing and thawing the cellular suspension was also evaluated, as well as the influence of output power, suspension volume and cell concentration on cell disruption by sonication. The disruption was evaluated by the release of cytochrome b_5 and the total soluble intracellular protein. Experimental conditions for the sonication process are indicated in Table 1.

Optical microscope observations

The photographs were taken on a optical microscope (Zeiss Axioplan), and observations were made at a magnification of 1000×.

Particle size determinations

Particle size analysis of *Escherichia coli* cells and cell debris were carried out by light scattering (Brookhaven Instruments Corporation), and by direct measurement from the photographs (between 30 and 50 particles measured for each sample) taken from the optical microcope.

Viscosity determinations

Viscosity determinations were made at 30 °C in a rotation viscometer (Brookfield LVTDV - II CP), at a shear rate of 450.0 s^{-1} (60 rpm), using the spindle #40 and a cup containing sample volumes of 0.5 mL.

Analytical methods

Cell concentration (g Dry Cell Weight/L) was determined by drying a known cell suspension volume for 24 h at 80 °C. After sonication, cell debris was removed by centrifugation at 4 °C and 12 000 rpm for 45 min, and the supernatant assayed for cytochrome b_5 and total protein. Quantification of cytochrome b_5 was achieved by absorbance measurements at 410 nm, and using the Lambert-Beer law with a known extinction coefficient ($\epsilon = 130\ mM^{-1}\ cm^{-1}$) (von Bodman et al., 1986). The total soluble protein was obtained from the clear supernatant by the Bradford's method (Bradford, 1976).

Table 1. Experimental conditions for the sonication process

Parameter studied	Output power (W)	Sonication time (min)	Suspension volume (mL)	Cell concentration (g DCW/L)
Output power	100–250	20	100	27
Suspension volume	100	10–30	50–200	28
Cell concentration	150	20–30	100	5.7–58

Results and discussion

1. Effect of freezing and thawing on the cell disruption

Freezing/thawing is a physical method of cell breakage, with known applications in the recovery of intracellular bioproducts since the twenties (Hugo, 1954). It has the advantage of simplicity, reproducibility, and the cells are not subjected to harsh mechanical treatment or high temperatures, and, when needed, can be performed on a sealed vessel. However, many microorganisms are resistant to rupture and many enzymes are inactivated by repeated freezing and thawing. When used as a method for releasing periplasmic substances, it has advantages over conventional techniques such as of osmotic shock or chemical treatment (the absence of use of lysozyme, chloroform, toluene, sucrose or EDTA) (Paoletti, 1987).

Freezing/thawing depends on many factors, including composition of fermentation medium, growth phase and rate, rate of freezing (Calcott, 1975), and the presence of cryoprotective compounds in the freezing medium (e.g. ethanol, under conditions of rapid freezing (Lewis, 1993)). It is easily performed in medium or large scale, however it is not extensively used due to it is time-consuming. The main application of this method has been as an adjunct to other cell-breaking methods. In this work the cells were frozen only to preserve the cell suspensions until the sonication test was done. Cells were frozen for one week, at -20 °C and were thawed, at room temperature, just prior to sonication experiments.

It was observed that the freezing/thawing treatment released a considerable amount of cytochrome b_5 and of intracellular protein to the medium. These amounts, which were independent of the volume processed in the range used (50 to 200 mL), were, respectively, 2.99 ± 0.23 and 14,7 ± 1.3 mg/gDCW, or 21.1 ± 2.7% and 4.02 ± 0.32% of total amounts of cytochrome b_5 and total soluble intracellular protein released by freezing/thawing plus sonication, for the suspension with a cell concentration of 25 gDCW/L.

With cell freezing, the water inside the cells forms ice crystals, and the total water volume can increase by about 14% (Perry,1984), with the ice crystals being very destructive to membranes and organelles (Scopes, 1982). The stress due to the increase of water volume inside the cells can split membranes and cell walls, and low molecular proteins can be released. This is proved by the high amount of cytochrome b_5 (a 13.6 kDa protein) released comparatively to the amount of total intracellular protein.

With the freezing/thawing, the cell length decreased (Fig. 1A and B) and the distribution of cell sizes changed (Fig. 2). The *Escherichia coli* average cell length was 1.4 ± 0.3 and 1.1 ± 0.2 μm before and after freezing/thawing, respectively. The main reason for this decrease seems to be the premature breakage of cells that are in the division process, since in Fig. 1B, when compared with Fig. 1A, there are less cells with narrow isthmus and no cell debris are visible.

The increase in cellular concentration seems to promote the increase of cytochrome b_5 and total protein release (Table 2). An increase of the ratio cytochrome b_5/total protein was also observed, which suggests that the splits increase mainly in number and not in enlargement.

2. Cell sonication

(A) Effect of output power

Cytocrome b_5 and total soluble protein released with time are shown in Fig. 3 (results shown are only for the lowest and highest output power used). The release rate increases significantly with the sonication power, and after 10 min of sonication reaches a plateau.

If cell disintegration follows a first order kinetics, the rate of protein release, in a batch system, is directly proportional to the amount of unreleased protein (Davis, 1959; Augenstein, et al., 1974; Christi et al.,

Table 2. Cell concentration effect on cytochrome b_5 and total protein released by freezing/thawing

Cell concentration (g DCW/L)	Released (mg/g DCW)		(Cyt. b_5/protein) (mg/mg)
	Cytochrome b_5	Total protein	
5.7	2.8	16	0.18
25	4.2	20	0.21
41	4.8	21	0.23
58	5.5	22	0.24

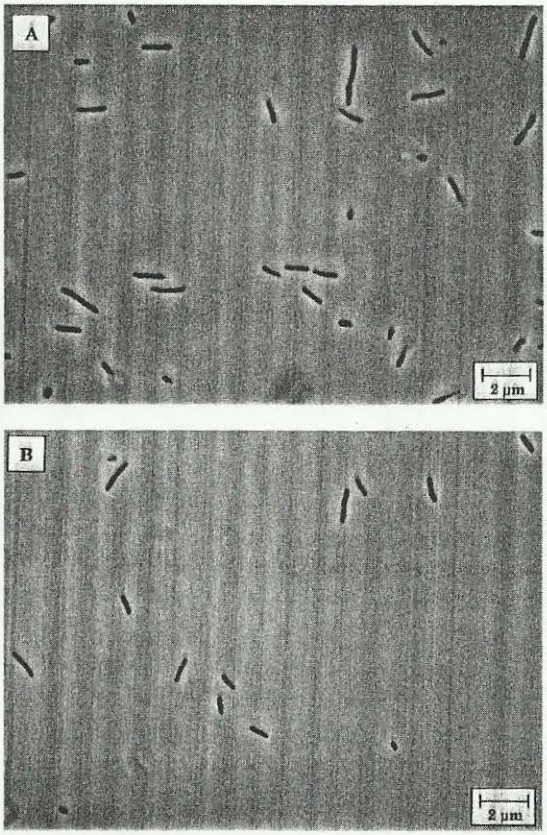


Fig. 1. Escherichia coli cells as observed through an optical microcope before freezing (A) and after freezing/thawing (B)

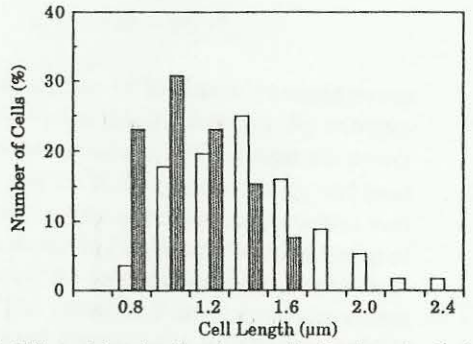


Fig. 2. Effect of freezing/thawing on distribution of cell sizes in *Escherichia coli* populations (before freezing – □, after freezing/thawing – ■).

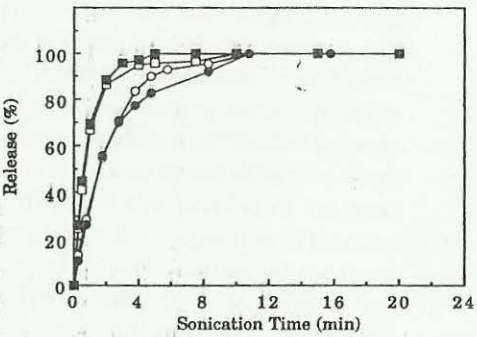


Fig. 3. Time dependence of cytochrome b_5 ((○), (□)) and total soluble protein ((●, (■)) released at 100 ((○)) and 250 W ((□), (■)).

1986):

$$dR/dt = k_r(R_m - R)$$

where k_r is the protein release constant, t is the sonication time, R is the protein released and R_m is the maximum protein releasable by sonication. After integration it can be obtained:

$$R = R_m[1 - \exp(-k_r t)]$$

Escherichia coli cell disintegration follows a first order kinetics in the first 2 or 3 mins of sonication (Figs. 4 and 5). For higher sonication times, the disintegration rate decreases and the kinetics order is lower than unity. This may be explained by cavitation unloading, that markedly affects the efficiency of sonication. This is due to the increasing of the surface tension caused by the release of proteins and nucleic acids (e.g. surface-active products), and to the intense foaming that can result and contributes to the denaturation of proteins at the gas-liquid interface decreasing the amount of soluble protein (Garcia, 1993).

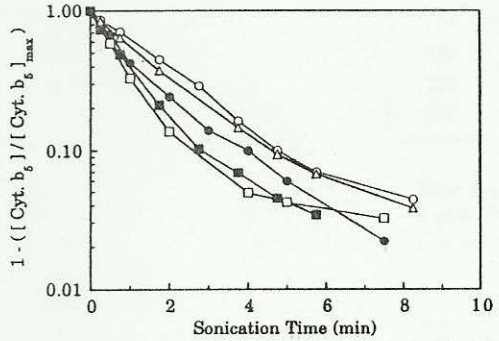


Fig. 4. Effect of output power (100 (○), 125 (△), 150 (●), 200 (■) and 250 W (□)) on kinetics of cytochrome b_5 release.

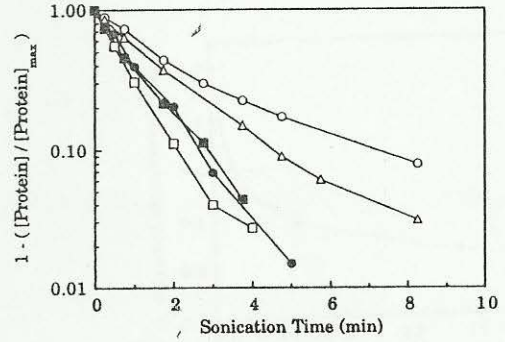


Fig. 5. Effect of output power (100 (○), 125 (△), 150 (●), 200 (■) and 250 W (□)) on the kinetics of total intracellular protein release.

In Table 3 are indicated the kinetic constants and the maximum cytochrome b_5 and protein released for different output powers. The kinetic constants increase almost linearly with the output power (see also Fig. 6), in the range tested. A doubling of output power roughly leads to a two fold increase of k_r. As the kinetic constants for the release of cytochrome b_5 and for total soluble intracellular protein are identical, it can be confirmed that cytochrome b_5 is a cytoplasmic protein (von Bodman et al., 1986; Follows et al., 1971).

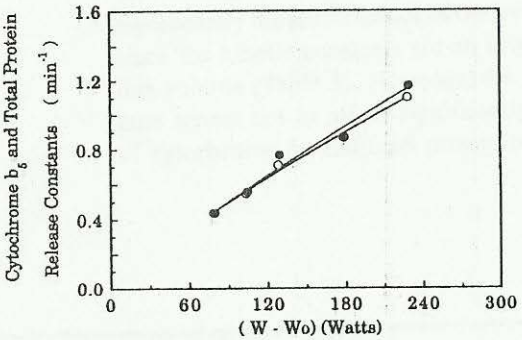


Fig. 6. Cytochrome b_5 (○) and total protein (●) release constants as function of output power.

The dependence of the protein release constant on output power was established (Doulah, 1977) as:

$$k_r = \alpha(W - W_o)^{\beta}$$

where α and β are constants, W is the ultrasound power and W_o is the cavitation threshold power. By extrapolating the experimental values, W intercepts the power axis (W_o) at 22 and 21 W for cytochrome b_5 and total protein, respectively. The experimental k_r values versus ($W-W_o$) are shown in Fig. 6, and from the fitting of the equation above, the values of α and β were calculated (Table 4). The values of β are in good agreement with those obtained theoretically by Doulah, (1977) (that was 0.895), and experimentally with yeasts by James et al. (1972) (that was 0.9).

For the experiment carried out at 100 W, the evolution of the particle size with sonication time was measured by light scattering. With the exception of the initial sample (0 min of sonication), two distinct populations were found. However, it was not possible to estimate the highest population parameters because its dimensions were too close to the detection. limit of the equipment used and also because of the presence of dusty particles in the suspension. Therefore only the smallest population was considered, the mean distribution value being determined. In order to find out whether the unbroken cells and dusty particles left in the suspension could interfere with the particle size determination of much smaller cell debris, the mean particle size was measured directly in the photographs taken on the optical microscope for the initial sample (0 min of sonication) and for the sample corresponding to 5 min of sonication (in this sample it was not considered the measurements higher than 1 μm). The results, indicated in Fig. 7, show a reasonable reproducibility (error around 10%). This figure shows that particle size pronouncedly decreased in the first minutes of sonication (the apparent particle size was 0.5 after 2 min of sonication), followed by a slower decrease. The evolution of mean particle size can also be seen in Fig. 7.

After 20 min of sonication several particles were still observed in the suspension with a length close to 1 μm, which could be whole or partially desintegrated *Escherichia coli* cells. In Fig. 8B a small particle is visible (according to *Escherichia coli* particle size distribution (see Fig. 2)) with a length of 0.93 μm. This was also observed with yeast cells (Thacker, 1973), with *Escherichia coli* cells and several other microorganisms (Wase et al., 1985) and was theoretically

Table 3. Effect of output power on the kinetics of cytochrome b_5 and total intracellular protein release constants

Output power (W)	Cytochrome b_5		Total protein	
	Maximum released (mg/g DCW)	k_r (min^{-1})	Maximum released (g/g DCW)	k_r (min^{-1})
100	11	0.44	0.35	0.44
125	11	0.55	0.38	0.56
150	11	0.77	0.38	0.77
200	10	0.87	0.36	0.87
250	11	1.1	0.38	1.2

Table 4. Coefficients α and β from the fitting of the equation $k_r = \alpha(W-W_o)^{\beta}$

	α ($Watt^{-\beta}\ min^{-1}$)	β (-)	Correlation coefficient
Cytochrome b_5	0.011	0.85	0.997
Total protein	0.0093	0.89	0.985

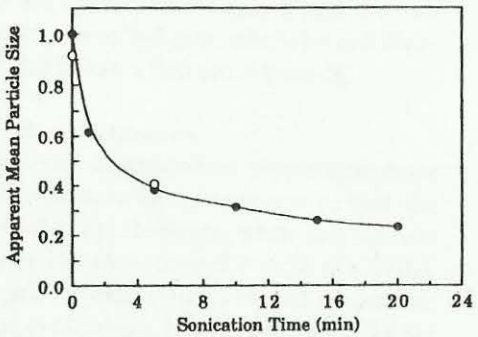
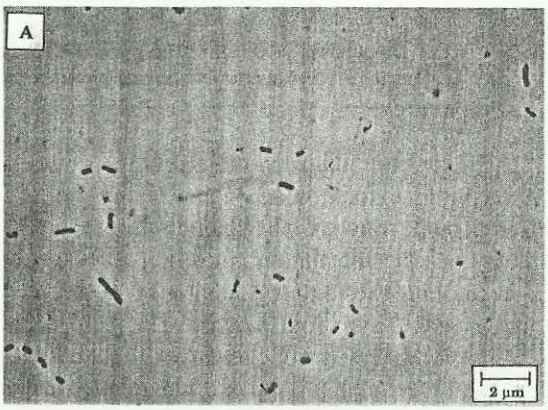
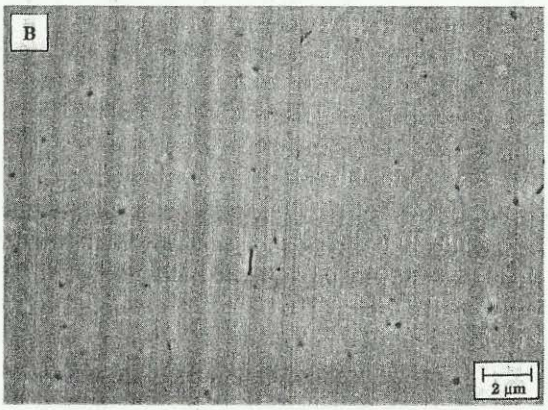


Fig. 7. Effect of sonication time on apparent particle size measured by light scattering (•) and directly on the photographs taken from the optical microscope (o).





explained by Doulah (1977, 1981). This author predicted a cell dimension below which the cells remain intact. This dimension (dimension of the largest stable cell) depends directly on the wall cell strength and inversely on the square root of the density and viscosity of the suspension and the energy dissipation rate (related with ultrasound power).

(B) Effect of the volume of suspension

The volume of the cell suspension to be treated affects significantly the performance of the sonication process, since the kinetic constants are an inverse function of this volume (Table 5). However, the increase in volume seems not to affect significantly the maximum of cytochrome b_5 and total protein released, and, as

seen before, the kinetic constants for the release of cytochrome b_5 and for total soluble intracellular protein are identical.

According to Davis (1959), k_r is proportional to ($\mathcal{C}$/V), where $\mathcal{C}$ is the number of cavitations per minute and V is the volume of cell suspension. If $\mathcal{C}$ is independent of the volume of the suspension and is only a function of the sonication power, k_rV should be constant for a given power. In this case, and for the range of volumes tested, it was found out that k_rV was independent of the suspension volume. This conclusion is particularly important to design a flow system, where the fraction of released protein, at equilibrium, should be dependent only on the flow rate (James et al., 1972). However, for this system, this is only true if the residence time is inferior to 2–3 min, otherwise cell disintegration will not follow a first order kinetics.

(C) Effect of cell concentration

The increase of cell concentration causes a decrease in the kinetic constants for cytochrome b_5 and the total protein (Table 6). However, when cell concentration increases 10 times (from 5.7 to 58 gDCW/L), k_r decreases only by half. After 5 min of sonication, the fraction of cytochrome b_5 released was 0.95 for the suspension with 5.7 g DCW/L, and 0.88 for the suspension with 58 g DGW/L, but it led to 90 and 940 mg of cytochrome b_5/L in solution, respectively.

The effect of cell concentration on the disintegration performance may be explained by the increase in the viscosity of the cell suspension (Fig. 9), which affects the rate of energy dissipation and inhibits the phenomenon of cavitation (Garcia, 1993). In the first seconds of sonication, an increase in the viscosity was observed due to the release of nucleic acids to the medium. Eventually the disruption of the nucleic acids caused by the liquid shear and the free radicals attack (Hughes and Nyborg, 1962), decrease significantly the viscosity.

The maximum cytochrome b_5 and total protein released decreased slightly with the increase in cellular concentration (Table 6). This is directly related to the intracellular amounts of cytochrome b_5 and total protein, since the previous freezing/thawing released a considerable amount of cytochrome b_5 and some intracellular protein to the medium, and these amounts increased with the cellular concentration. The total amount of cytochrome b_5 and total protein released by freezing/thawing plus sonication remained constant and equal to 16.1 ± 0.4 mg/g DCW and 0.331 ± 0.007 g/g DCW, respectively.

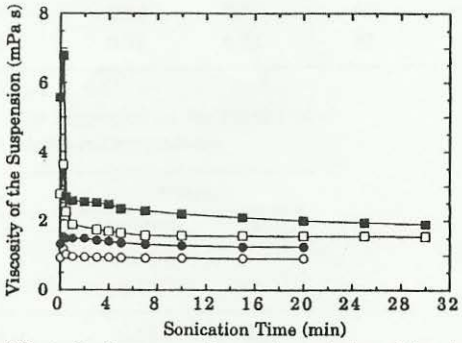


Fig. 9. Effect of cell concentration on the variation of the viscosity of the suspension with sonication time (5,7 – (o); 25 – (•; 41 – (□) and 58 g/L – (■)).

As previously mentioned, sonication is mainly a laboratory method for cell disintegration. It has, however, several experimental difficulties and limitations such as: high heat generation (a good temperature control is necessary), exposure of operators to high sound levels (the equipment needs to be shielding to reduce the ultrasound to a safe and comfortable level and the operator should use ear protection) and generation of free radicals (this problem, which can affect the integrity of the product but has no effect on cell breakage, can be alleviated by the addition of free radical scavengers such as glutathione or cysteine or by prepassing the cell suspension with hydrogen (Garcia, 1993)). Another limitation is the unavailability of equipment for large scale operation. A possibility for large-scale operation was suggested by Doulah (1981) using a continuous feed through a series of equal-sized tanks, each with a sonication probe. However, there are no published results of experiments using such a continuous system (Garcia, 1993).

In the system studied, for a power of 150 W, a suspension volume of 100 mL with 58 g DCW/L, and a global sonication time of 10 min (which corresponds to an efective sonication time of 5 min and a cellular desintegration of 90%), was possible to disintegrate 35 g DCW/h, which corresponds of about 12 L/h of fermentation broth with 3 g DCW/L. This flow rate could be increased using high sonication power and high cell concentration.

Table 5. Effect of the volume of suspension on the kinetics of cytochrome b_5 and total intracellular protein release constants

Suspension Volume (mL)	Cytochrome b_5 Maximum released (mg/g DCW)	k_r (min^{-1})	k_rV (mL min^{-1})	Total protein Maximum released (g/g DCW)	k_r (min^{-1})	k_rV (mL min^{-1})
50	9.7	0.93	47	0.34	0.95	47
100	10	0.44	44	0.34	0.44	45
200	11	0.23	46	0.33	0.23	47

Table 6. Effect of cell concentration in the suspension on the kinetics of cytochrome b_5 and on total intracellular protein release constants

Cell concentration (g DCW/L)	Cytochrome b_5 Maximum released (mg/g DCW)	k_r (min^{-1})	Protein Maximum released (mg/g DCW)	k_r (min^{-1})
5.7	13	1.0	0.32	0.97
25	12	0.82	0.32	0.78
41	12	0.61	0.31	0.62
58	11	0.52	0.30	0.51

Conclusions

Escherichia coli cell disintegration, for cytochrome b_5 release, by freezing/thawing plus sonication was carried out with high efficiency. The maximum amount of cytochrome b_5 released by the conjugation of these two methods was equal to 16 mg/g DCW (4.0% of total intracellular protein).

It was observed that the freezing and thawing treatment of the cell suspension plays an important role in the disintegration process, since, for the suspension with the highest cell concentration tested, 33 and 6.8% of cytochrome b_5 and total intracellular soluble protein, respectively, were released.

It was observed that the sonication efficiency (reduction on sonication time for the same fraction of release) increases with the increase of sonication power, and with the decrease of the suspension volume and cell concentration. However, there is an advantage in working with high cell concentration suspensions since the release constant k_r decreases only by half when cell concentration increases 10 times. It was also observed that, after ten minutes of sonication (20 min of operating time) the maximum amount of cytochrome b_5 and total protein released was achieved, these maxima being independent of the operating conditions and cell concentration (only in the first minutes of sonication are these parameters important).

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References

Augenstein DC, Thrasher K, Sinskey AJ & Wang DIC (1974) Optimization in the recovery of a labile intracellular enzyme. Biotechnol. Bioeng. 16: 1433–1447.

Braun B (1984) Operating Manual for Labsonic 2000. B Braun AG, Melsungen, Alemanha.

Bar R (1988) Ultrasound enhanced bioprocesses: Cholesterol oxidation by *Rhodococcus erythropolis*. Biotechnol. Bioeng. 32: 655–663.

von Bodman SB, Schuler MA, Jollie DR & Sligar SG (1986) Synthesis, bacterial expression, and mutagenesis of the gene coding for mammalian cytochrome b_5. Proc. Natl. Acad. Sci. 83: 9443–9447.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of Protein-Dye binding. Anal. Biochem. 72: 248–254.

Calcott PH & Macleod RA (1975) The survival of *Escherichia coli* from freeze-thaw damage: Permeability barrier damage and viability. Can. J. Microbiol. 21: 1724–1732.

Christi Y & Moo-Young M (1986) Disruption of microbial cells for intracellular products. Enzyme Microb. Technol. 8: 194–204.

Davis R (1959) Observations on the use of ultrasound waves for the disruption of microorganisms. Biochim. Biophys. Acta 33: 481–493.

Doulah MS (1977) Mechanism of disintegration of biological cells in ultrasonic cavitation. Biotechnol. Bioeng. 19: 649–660.

Doulah MS (1981) Cell disintegration by ultrasonic cavitation.. Process Biochem. 16: 26–27.

Follows M, Hetherington PJ, Dunnill P & Lilly MD (1971) Release of enzymes from bakers' yeast by disruption in an industrial homogenizer. Biotechnol. Bioeng. 13: 549–560.

Garcia FAP (1993) Cell wall disruption. In: Kennedy JF & Cabral JMS (eds.) Recovery Process for Biological Materials (pp. 47–66). J. Wiley, New York.

Hughes DE & Nyborg WL (1962) Cell disintegration by ultrasound. Science 138: 108–114.

Hugo WB (1954) The preparation of cell-free enzymes from microorganisms. Bacterial Revue 18: 87–105.

James CJ, Coakley CJJ & Hughes DE (1972) Kinetics of protein release from yeast sonicated in batch and flow system at 20 kHz. Biotechnol. Bioeng. 14: 33–42.

Karim A, Kaderbhai N, Evans A, Harding V & Kaderbhai MA (1993) Efficient bacterial export of a eukaryotic cytoplasmic cytochtome. Bio/Technology 11: 612–618.

Keshavarz E, Hoare M & Dunnill P (1987) Biochemical engeneering aspects of cell disruption. In: Verral MS & Hudson MJ (eds.) Separations for Biotechnology (pp. 62–79). Ellis Horwood, Chichester, U.K.

Lewis JG, Learmonth RP & Watson K (1993) Role of growth phase and ethanol in freeze-thaw stress resistance of *Saccharomyces cerevisae*. Appl. Environ. Microbiol. 59: 1065–1071.

Paoletti LC, Short KA, Blakemore N & Blakemore RP (1987) Freeze-thawing of *Aquaspirillum magnetotacticum* cells selectivety releases periplasmic proteins. Appl. Environ. Microbiol. 53: 2590–2592.

Perry R (1984) Perry's Chemical Engineers' Handbook. McGrow-Hill Book Company, New York.

Scopes RK (1982) Making an extract. In: Protein Purification: Principles and Pratice (pp. 21–37). Spring-Verlag, New York.

Thacker J (1973) An approach to the mechanism of killing of cells in suspension by ultrasound. Biochim. Biophys. Acta 304: 240–248.

Wase DAJ & Patel YR (1985) Effect of cell volume on the disintegration by ultrasonics. J. Chem. Tech. Biotechnol. 35B: 165–173.

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