DISINTEGRATION OF CELLS AND PROTEIN RECOVERY

L. EDEBO and K.-E. MAGNUSSON

Department of Medical Microbiology, Linköping University, Sweden

ABSTRACT

In order to prepare intracellular substances from microorganisms the barrier of the cell envelope has to be disrupted. Since this barrier is usually very resistant, the methods available for the preparation of substances with unstable activity are restricted due to the inactivation occasioned by some disintegration processes. Some methods, usually applying physico-mechanical shearing stress, are inherently well suited to disintegrate cells to release unstable substances. In other cases, when more inert substances like e.g. nutrients are prepared from microbes, denaturing treatments like cell envelope disruption by heating followed by enzymatic dissolution of the protoplasm may be advantageous.

Some recent results with disintegration by freeze-pressing are described. Procedures giving selective release of certain cell constituents and ways to influence the yield qualitatively are also reported.

The main obstacle to the disintegration of microorganisms lies in their tough cell walls. In the true bacteria (Eubacteriales), the mechanically-resistant part of the wall is the murein which can be considered as a giant bag-shaped macromolecule. Consequently, covalent bonds have to be broken in order to disrupt the bacteria. In other microbes analogous conditions often exist.

Table 1. Methods for disintegration of microorganisms.

Chemical	Alkaline or detergent breakdown
	Solvent extraction
	Enzymatic digestion
	Freeze-thawing
Physical	Heat-treatment rupture
	Osmotic disruption
	Decompression
	Crystal transformation
	Sonic vibration
Mechanical	Agitation with abrasive particles
	Grinding
	Pressure extrusion

The disruption of the cell wall can be accomplished in several ways and a great number of methods have been described. Based on the mechanism of disintegration, division into chemical, physical and mechanical methods can be made. Each division contains groups of methods with similar action (*Table 1*). In order to prepare native proteins and other substances on a fairly large scale the choice of methods is limited. I will focus this review on such methods and the problems involved with them. Several reviews dealing with disintegration of microbes in general have been published recently[1,2].

REVIEW OF METHODS

Enzymatic digestion

The search for cell-wall-degrading enzymes has been extensive, and a wide variety of active enzymes have been found e.g. from the snail gut, from actinomycetes (*Micromonospora*[3]), and from gram-negative bacilli (Flavobacterium[4,5]). The high yields recovered of the Flavobacterium enzyme[5] seem promising for large-scale use. Extensive reference lists on enzyme digestion have been published recently[4,6]. Since then lytic activity against several yeast and *Chlorella* species has been demonstrated from a thermophilic actinomycete, *Micropolyspora sp.*[7].

Agitation with abrasive particles

Agitation of suspensions of microorganisms mixed with small glass beads has in many cases been used for cell disruption. Usually, cell concentrations around 10–100 mg ml^{-1}, alkali-free glass beads with diameter 0.1–0.5 mm at approximately the same weight proportion as the microbial suspension, and frequencies around 50–100 Hz have been employed. The efficiency of disintegration is greater when the treatment vessel is not filled up. After the treatment, the beads are usually separated from the cell homogenate by filtration and may be used again, but some silicate is released into the medium. For small-scale laboratory use several apparatuses are available. A continuous-flow shaker with plastic beads seems to be particularly efficient[8]. For bacteria, the velocity of disintegration is generally more rapid with small bead sizes –diameters 0.1 mm and less– whereas the disintegration of yeast and algae seems to be less dependent on bead size. A high concentration of glass beads improves the disintegration[9]. The disintegration is thought to result from shear forces[8].

An equipment for continuous operation on a larger scale has been developed in Czechoslovakia[10,11]. The suspension was agitated by rotating disks fastened to a horizontal axis. *Saccharomyces cerevisiae* was efficiently disintegrated in a couple of minutes. Applying the same method to the microalgae *Scenedesmus obliquus*[9] increased the pepsin digestibility of the algae much more than hydrogen peroxide or cellulolytic enzyme (meicelase) treatment which were run in parallel tests. In further experiments[12] soluble nitrogen recovered in the supernatant fluid after agitation and centrifugation was highest at pH-values above 11 for the microorganisms tested viz. fresh *Saccharomyces cerevisiae*, lyophilized *Methanomonas sp.* and spray-dried *Scenedesmus obliquus*. The amounts of nitrogen precipitated at pH 4 from these supernatants were maximal, when the pH of extraction was approxi-

mately 11–12 indicating hydrolysis at higher pH-values. The precipitate contained 60–70 per cent of the cell nitrogen.

Pressure extrusion in liquid suspension

Pressing of liquid suspensions of bacteria through a narrow orifice at pressures 400–3500 kp cm^{-2} disrupts many microorganisms. Recently an industrial homogenizer, the Manton–Gaulin homogenizer, was adopted for disintegration of microorganisms by Lilly, Dunnill and coworkers at University College, London[13–15]. It was observed that a knife-edge valve-seat improved the disintegration over a flat valve-seat. This might be due to a steeper pressure gradient which gives a more rapid flow through the shorter constriction. Increase of the temperature of the yeast suspension to be pressed from 5°C to 30°C increased the disruption rate 1.5 times. At moderate cell concentrations and pressures the protein release was independent of the cell concentration. The rate of release of protein could be described by the relationship (the original equation is slightly rearranged to agree better with the general equation for disintegration):

$$\log \frac{R_m - R}{R_m} = -KNP^{2.9} \qquad (1)$$

where R = amount of soluble protein released, R_m = maximum amount of soluble protein that can be released, K = dimensional temperature-dependent rate constant, N = number of passages through valve, and P = operating pressure.

At high operating pressures, say above 800 atm, and high cell concentrations ($C_y = 0.75$) the disruption rate was reduced and no longer first-order. This effect as well as the effect of temperature might be due to the reduction of flow with increased viscosity.

The rate of release of seven different enzymes was studied in relation to the release of protein. At temperatures over 35°C, significant losses of enzyme activity were observed. At lower temperatures, no loss in activity on prolonged treatment was observed except in the cases of fumarase and invertase. In all other instances the various operating pressures, temperatures and initial yeast concentrations used did not affect the rate of release of the individual enzyme relative to protein release. The rate of release of the individual enzymes was dependent on their location in the cell: the release of acid phosphatase and invertase, which are supposed to be located predominantly outside the cell membrane, was faster than the overall protein release. The dehydrogenases, which belong to the cell sap, were released slightly faster or at the same rate as the overall protein. Alkaline phosphatase and fumarase, which probably mainly come from the plasma membranes and the mitochondria, respectively, were released more slowly. In principle the same type of slow release of bacterial cell membrane enzymes was shown at sonic disintegration of *Azotobacter*[16].

Pressure extrusion in freeze-pressing

Freeze-pressing as adopted in the X-press[17, 18] exploits the phase-changes in ice under pressure[19]. The X-press consists of two identical cylindrical axial chambers separated by a disk with a central round hole about 2 mm

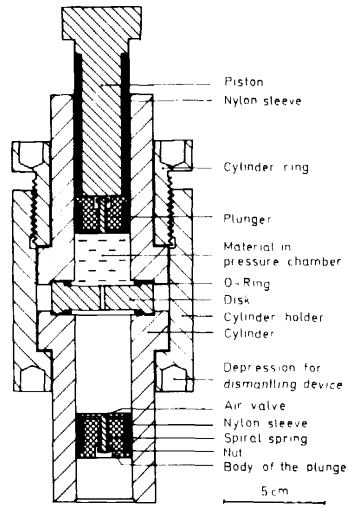

Figure 1. Section through the X-press.

wide (*Figure 1*). The material to be disintegrated is forced from the frozen state through this hole. The pressure required for this is dependent on the temperature in a way similar to that in which changes in the crystal structure of water vary with pressure (*Figure 2*)[19]. By moving the piston, on which pressure is applied, from one chamber to the other, the material could be pressed through the hole several times without uncoupling the press. Repeated pressings increased the disintegration both with regard to the percentage of cells disrupted and the degree of disintegration of the cellular constituents[20, 21].

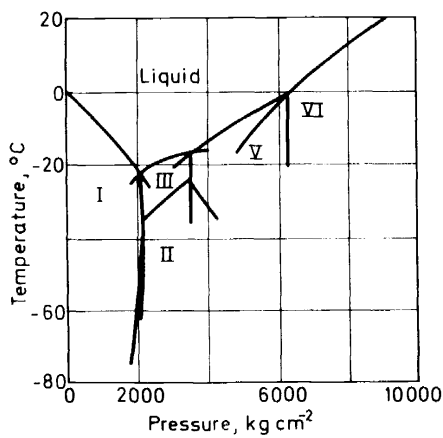

Figure 2. A part of the phase diagram of water in the liquid-solid region. (Reproduced from P. W. Bridgman, *Proc. Amer. Acad. Arts Sci.* **47**, 439 (1912)).

The kinetics of the freeze-pressing was tested with a number of different bacteriophages. The phage suspensions were pressed at $-$ 25°C and 2000 kp cm^{-2} (Ref. 22). The inactivation obeyed the general equation

$$\log S = -KN \qquad (2)$$

where S = the fraction of surviving plaque-forming units, N = the number of pressings, and K = a constant characteristic of a given phage.

The T-even phages T2L, T2H r13 h (two different T2 mutants), T4D and T6 had K-values between 6.6 and 7.6 which means that generally one phage in 10 millions remained intact after one pressing. For T5, P2 and lambda the K-values were 4 to 5.2 and for ϕX-174, T1 and T3 0.6 to 1.8.

With the ordinary X-press the pressure could be regulated up to about 2000 kp cm^{-2} by selecting the corresponding temperature. Pure water is liquid at $-$ 20°C and 2000 kp cm^{-2} but is solid ice below $-$ 23°C whatever the pressure. However, material was pressed through the orifice of the X-press even at lower temperatures. This was originally thought to be a result of ice I–ice III changes. However, when pressure was applied on cell suspensions at $-$ 30°C, the conductivity of the suspension increased[23] which indicated that a change into the liquid state occurred. Several experiments on freezing serum and other biological products suggest that in the presence of sugars and proteins, salts do not form eutectic mixtures but instead form glasses which slowly harden as the temperature falls[24]. In what way different kinds of biological material affect the structure of water under pressure is little known. This is a problem of great importance particularly for the operation at higher pressures, since pressures above 2100 kp cm^{-2} rather promote the different crystal forms than the liquid state of water.

Pressures above 2100 kp cm^{-2} were attained by providing the X-press with a spring-loaded piston (diameter = 2 mm) fitting the orifice of the disk (*Figure 3*). At pressures of 4 to 4.8 tons cm^{-2}, 90 per cent disintegration of *S. cerevisiae* was obtained with a suspension containing 13.5 per cent dry

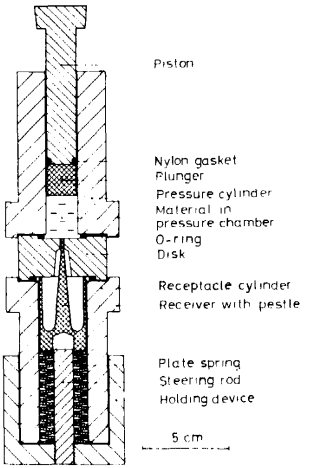

Figure 3. Section through the high-pressure X-press.

weight. At 27 per cent dry weight (ordinary baker's yeast) 4 to 5 tons cm^{-2} produced 50 per cent, and 5 to 6 tons cm^{-2} 75 per cent disintegration (*Table 2*).

In an investigation to elucidate the influence of different conditions for freeze-pressing with the X-press on the efficiency of disintegration, we faced the problem of distinguishing between cell-wall disruption and release of

Table 2. Disintegration of *Saccharomyces cerevisiae* in the high-pressure X-press.

Temperature (°C)	Number of plate springs	Yeast 27% dry wt		Yeast 13.5% dry wt	
		Pressure (Tons cm^{-2})	Disintegration (%)	Pressure (Tons cm^{-2})	Disintegration (%)
−8	20	2.4–3.2	15		
−8	20 + 1	2.0–2.4	10		
−12	20	2.4–3.6	15		
−17	20	2.4–4.0	40	4.0, drops	35
−17	20 + 3	4.4–6.4	50		
−22	20	4	50	4.0–4.8	90
−22	20 + 1	4.8–5.2	50	4.0–4.8	90
−22	20 + 2	5.6–6.0	75	4.0–4.8	90
−22	20 + 3	4.8–6.0	75	4.8	90

intracellular material. Counting of intact cells in a microscope is inexact and laborious. Viable counts and turbidity measurements are hard to translate into degree of disintegration. Determination of the release of enzymes or other proteins is often intimately connected to the actual scope of the problem, i.e. to extract cell-bound components, but it measures not only disintegration but also dissolution.

ESTIMATION OF CELL DISINTEGRATION

The desire for a distinction between disruption and dissolution was particularly pertinent, when the effect of different suspending media on the disruption was tested. For such a distinction, a direct examination of each cell of a sample subjected to the disintegration treatment seemed to be preferable. Information about individual cells is provided by the Coulter Counter. Since the electrical signals produced by most bacteria are close to the background noise, larger baker's yeast cells (*Saccharomyces cerevisiae*) where chosen as models to study the disintegration. The Coulter Counter was equipped with a 100 μm aperture, and calibrated with polystyrene latex spheres. Provided that the cross-sectional area of the particle is much smaller than that of the aperture, then the expression

$$\Delta E = \{Ev(1 - \rho_0/\rho)\}/V \qquad (3)$$

is valid where V and v are the volume of the aperture and the particle, respectively, ρ_0 the resistivity of the suspending solution and ρ the resistivity of the particle. For a particle of very low conductivity in a salt solution, such

as a polystyrene-latex sphere in 0.15M NaCl, $\rho_0 \ll \rho$ and to a good approximation

$$\Delta E \approx Ev/V \tag{4}$$

i.e. the pulse is proportional to the volume of the particle.

Untreated yeast cells were suspended in 0.15M NaCl solution and counted in the Coulter Counter with different threshold levels for the electrical pulse. The number of particles in each volume interval was calculated by subtracting the count at the upper threshold level from that at the lower one. The volume distribution with the apparatus calibrated with polystyrene-latex spheres is shown in *Figure 4*. The peak of the curve was at 37 μm^3. However, when the volumes of the cells of the same suspension were measured microscopically the peak was at 56 μm^3.

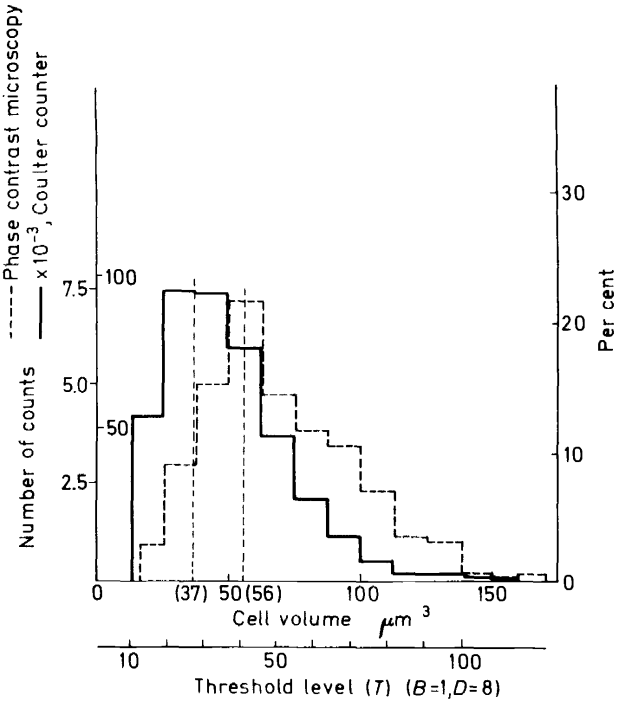

Figure 4. Counting and sizing of yeast cells with the Coulter Counter and phase contrast microscopy. The cells were suspended in 0.15M NaCl and counted at $B = 1$, $D = 8$ and different threshold levels (T).

Considering a yeast cell with volume V_c and the functional resistivity ρ_c, the assumption that $\rho_0 \ll \rho_c$ is not valid. Under such circumstances it can be calculated[26] that ΔE acquires a maximum when $\rho_0 = \rho_c/2$.

Since the maximum count was obtained at a resistivity 130 ohm cm

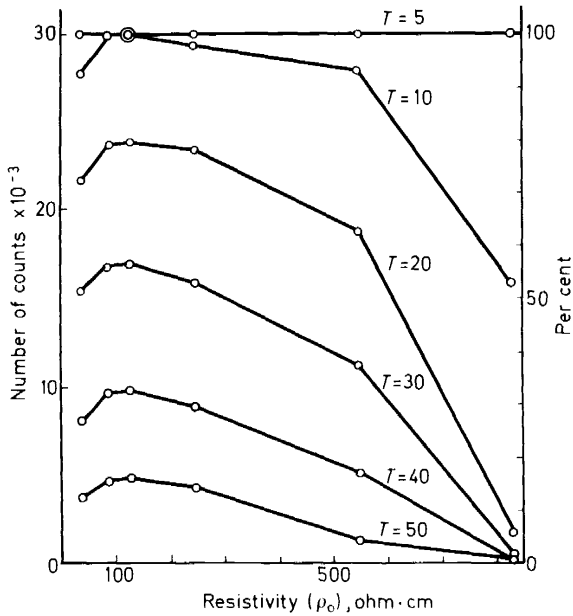

Figure 5. Counting of yeast cells at different resistivities of the suspending medium (ρ_0) and different threshold levels (T). $B = 1$, $D = 8$.

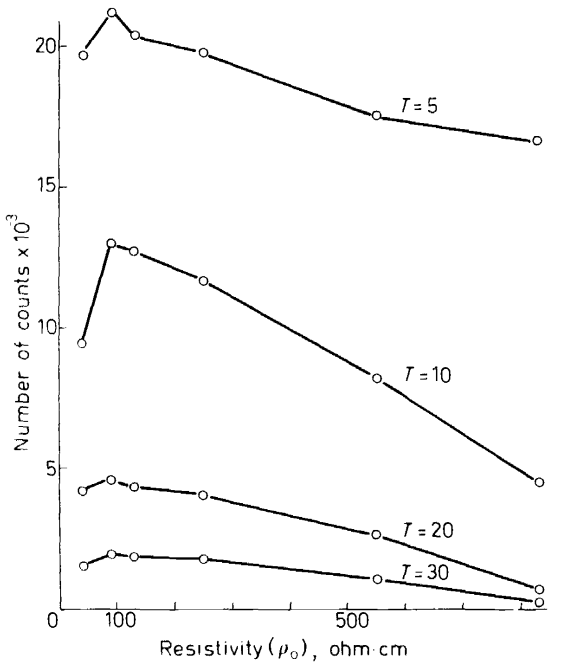

Figure 6. Counting of a yeast cell suspension freeze-pressed once at different resistivities of the surrounding medium (ρ_0) and different threshold levels (T). $B = 1$, $D = 8$.

(*Figure 5*), the functional resistivity of the intact cells was approximately 260 ohm cm. This gives a correction factor of 1.53 which changes the volume peak in the Coulter Counter to 57 μm^3 which is close to that of the microscopical peak, 56 μm^3.

When similar experiments were performed with pressed cells the peak was changed to a lower resistivity, 90 ohm cm (*Figure 6*). This indicates that membrane damage has occurred without disruption of the cell envelope. This corresponds to the loss of halo in the microscope as described earlier[1, 27]. A similar change of the peak was also observed after heat treatment or after addition of a quaternary ammonium compound (cetyl trimethyl ammonium bromide) which is known to damage the cell membrane.

For the quantitative estimation of the effect of suspension medium, cell concentration, temperature and flow geometry on the disintegration, the cells were counted in the Coulter Counter suspended in 0.10M NaCl with the threshold level set at $T = 10$ (B = 1, D = 8).

For testing the effect of the suspending medium the cells were suspended in deionized water (3 $\mu mho\ cm^{-1}$), and in 0.10M concentrations of sodium, potassium or calcium chloride. Gelatin in concentrations of 5, 10 and 20 per cent was also used with water or 0.10M NaCl as solvent. Pressing was performed at − 15, − 25 and − 35°C with the aid of a motor-driven press.

Higher pressures were needed at lower temperatures. In general, higher pressures promoted disintegration but the correlation was not close. Bangs occurred in the absence of gelatin.

In the absence of gelatin, salt improved the disintegration at − 25 and − 36°C at the same time as it reduced the pressure required. There is a relation between the disintegration and the eutectic point of the salt. KCl, which has the highest eutectic point (− 11.1°C), showed the greatest disintegration at − 15°C and the smallest disintegration at − 35°C of the salt solutions tested. $CaCl_2$, which has the lowest eutectic point (− 54.9°C) showed the lowest disintegration at − 15°C and the greatest at − 35°C.

With gelatin in the suspending medium salt reduced the disintegration efficiency and pressure. With 10 per cent gelatin bangs were seldom noted and with 20 per cent gelatin, never. In earlier experiments—at efficient disintegration with the manually operated laboratory press—bangs generally were heard. In the later experiments, which were performed with the motor-driven press which produced a more rapid streaming of material, the bangs did not seem to be essential for efficient disintegration.

The effect of the orifice geometry on the disintegration efficiency was studied at different temperatures and cell concentrations (*Table 3*). An increase in the width of the orifice decreased the amount disintegrated. An increase in the length of the hole had no clear-cut effect on the disintegration of diluted cell material. However, with the longest hole used, diameter 2.5 mm, length 100 mm, no flow was obtained with the most diluted material. This is probably due to plugging of the hole with frozen material. At − 35°C two series were run under the same experimental conditions (*Table 4*). When bangs were heard, the disintegration was conspicuously reduced in comparison to flow without bangs. With outflow orifices of 1 mm diameter, and no bangs, approximately 90 per cent disintegration was achieved in one pres-

Table 3. Disintegration of different concentrations of *S. cerevisiae* at −25 °C as a function of the diameter (D_1 mm) and length (L_1 mm) of the orifice of the X-press disk.

Orifice		5.4 mg yeast per ml				180 mg yeast per ml				270 mg yeast per ml			
		Pressure (tons cm^{-2})		Bangs at flow	Intact cells (%)	Pressure (tons cm^{-2})		Bangs at flow	Intact cells (%)	Pressure (tons cm^{-2})		Bangs at flow	Intact cells (%)
Diameter	Length	initial	after 2 s			initial	after 2 s			initial	after 2 s		
1	2	2.4	1.7	+	37	2.1	1.9	(+)	37	1.9	1.6	(−)	25
	10	2.3	1.8	+	37	2.4	1.6	(+)	38	2.4	1.2	(−)	23
2.5	2	2.4	1.7	+	50	2.0	1.8	+	43	1.5	1.4	(−)	29
	10	2.5	1.8	+	43	2.1	1.9	+	44	2.0	1.4	(−)	24
	40	2.4	2.0	+	50	2.6	1.6	+	40	2.4	1.5	(−)	29
	100	> 3		no flow		2.3	1.2	+	38	1.4	1.2	(+)	41
5	2	1.9	1.2	+	57	1.9	1.9	+	52	1.6	1.3	(−)	29
	40	1.8	1.1	+	60	1.9	0.9	+	48	1.5	1.2	(−)	34
10	40	1.8	1.1	+	75	1.6	1.3	+	49	0.8	0.8	(−)	51

Table 4. Disintegration of *S. cerevisiae* (270 mg dry wt per ml) at −35°C and −45°C as a function of the diameter (D_1 mm) and length (L_1 mm) of the orifice of the X-press disk. Two identical experimental series were run.

Orifice		−35°C								−45°C							
		Series 1				Series 2				Series 1				Series 2			
Diameter	Length	Pressure (tons cm^{-2}) initial	Pressure (tons cm^{-2}) after 2s	Bangs at flow	Intact cells (%)	Pressure (tons cm^{-2}) initial	Pressure (tons cm^{-2}) after 2s	Bangs at flow	Intact cells (%)	Pressure (tons cm^{-2}) initial	Pressure (tons cm^{-2}) after 2s	Bangs at flow	Intact cells (%)	Pressure (tons cm^{-2}) initial	Pressure (tons cm^{-2}) after 2s	Bangs at flow	Intact cells (%)
1	2	3.0	1.3	−	11	2.6	2.1	(+)	26	3.5	1.8	−	12	4.2	1.6	−	19
	10	3.6	1.4	−	13	2.9	1.6	−	14	3.5	1.6	−	13	4.3	1.7	−	20
2.5	2	2.5	1.2	−	26	2.2	2.2	+	44	2.9	2.1	+	46	3.3	2.0	+	48
	10	2.9	1.2	−	22	2.1	1.5	−	18	3.1	1.4	−	17	3.3	1.4	−	16
5	2	2.4	1.5	+	40					2.6	2.5	+	54	2.9	2.1	+	52

sing. The bangs occurred only with the 2 mm long holes. Holes 10 mm long might therefore be preferred to smooth the flow.

At -45°C no conspicuous increase in disintegration efficiency was observed whereas the pressures required were higher.

Parallel with these experiments to elucidate the parameters of freeze-pressing a moderate scaling-up of the process has been performed to get experience in the technical problems met then. The first prototype of a semi-continuous press now takes 5 to 10 kg of cell paste per hour. The scaling-up however, demands more from the mechanical properties of the materials used and also a closer control of the process with respect to temperature, pressure, material to be disintegrated and press. However, there should be no obstacle in principle to treating very large quantities.

PROTEIN RECOVERY

Few systematic studies have been performed on the extraction of cellular material after disintegration. However, since most of the cellular constituents are amphoteric electrolytes with their isoelectric points on the acid side, dissolution is favoured at higher pH-values. This was shown experimentally for *Escherichia coli* and *Bacillus megaterium* with a number of different disintegration methods[28]. Similarly, the effect on dissolution by salting-in and salting-out effects were recorded.

As mentioned earlier, Hedenskog *et al.*[12] working with agitation with abrasives, got increase in yields with increasing pH up to pH 11–12.

Bordetella pertussis cells disintegrated with the X-press were extracted 5 times with 0.1M phosphate + 0.15M citrate, 5 times with 1M NaCl + 0.015M citrate, and 3 times with distilled water[29]. The phosphate extracts contained most of the protein and RNA, the sodium chloride extracts contained DNA and some proteins, whereas very little organic material was dissolved with the distilled water.

Yeast grown on oil hydrocarbons, mycobacteria and *Chlorella* were dehydrated with acetone and ground in the medium of organic solvents in a cavitation mill[30]. By extracting with NaCl, nucleic acids were obtained. The remaining paste was extracted with dilute hot alkali to extract total protein.

DISCUSSION

The sensitivities of different cells in suspension to disintegration by different mechanical methods generally parallel each other roughly. We think that this similarity reflects the fact that the most decisive factor determining the resistance of the microorganism to disintegration is the mechanical strength of the cell wall, and that the most decisive factor for the efficiency of a disintegration method is mechanical shear. In general, quantitative rather than qualitative differences decide the outcome. For instance, when *E. coli* was disintegrated in the French press, Hughes press or by ultrasonics, the release of four Krebs-cycle enzymes was approximately equal[2]. One exception to this rule seems to be the particular resistance of fungal mycelium to sonic vibration. This might be caused by an impairment of cavitation

and eddy-formation by the filaments. Recently, when Zetelaki[31] compared different disintegration methods for release of glucose oxidase from *Aspergillus niger* mycelium, the X-press and a ball mill were particularly efficient. A few other examples of specific sensitivity have also been proposed[2]. The X-press has also been used for disintegration of tough solid tissue, e.g. skin.

Cell wall fractions prepared by pressure extrusion contain more of the cell membrane enzymes[2] than cell walls prepared by agitation. Sonic treatment seems to be particularly effective at 'solubilizing' the cell envelopes[32] which might make separation more difficult. Excessive pressures comminute the cell walls into small fragments[2]. Consequently, the disintegrating energy should be adjusted to the purpose.

For most disintegration purposes the ideal method should disrupt the cell envelope but leave the content. To achieve this the disintegrative force should either be working on whole cells only or be focused either in time or space. Otherwise, the contents of cells disrupted at an early stage of the process will be subjected to further disintegration. Osmotic disruption and decompression seem to fulfill the first criterion but are of low efficiency. Pressure extrusion probably only tears the cells passing through the orifice. To reduce the disintegration and inactivation of intracellular components one should strive at obtaining the disintegration in one passage, since then the cell content would be protected during most of the process. If pressing is used for disintegration, some way for stirring or agitation should be included in the extraction procedure.

For large-scale mechanical disintegration there are at present three main alternatives available: agitation with glass beads[12], the Manton–Gaulin homogenizer[15] and freeze-pressing[1].

As mentioned earlier, we consider focused activity an advantage. We also think that adding glass beads may lead to adsorption of some components and contamination of the product. These disadvantages may not be too great in some cases.

Comparing different pressing methods one should consider equation 1 where the disintegration is proportional to the third order of pressure. If great disintegrating activity is desired one should rather increase the pressure than press several times. Freezing balances out the heat production at higher pressures.

REFERENCES

[1] L. Edebo, In *Fermentation Advances*, D. Perlman, Ed. p. 249. Academic Press, New York (1969).
[2] D. E. Hughes, J. W. T. Wimpenny and D. Lloyd, In *Methods in Microbiology* Vol. 5B, J. R. Norris and D. W. Ribbons, Eds., p. 1. Academic Press (1971).
[3] J. Monreal, F. de Uruburu and J. R. Villaneuva, *J. Bact.*, **94**, 241 (1967).
[4] S. Yamamoto and S. Nagasaki, *J. Ferment. Technol.*, **50**, 117 (1972).
[5] S. Yamamoto and S. Nagasaki, *J. Ferment. Technol.*, **50**, 127 (1972).
[6] A. Wiseman, *Process Biochemistry*, **4**, 63 (1969).
[7] H. Okazaki and H. Iizuka, *J. Ferment. Technol.*, **50**, 228 (1972).
[8] J. W. Ross, *Appl. Microbiol.*, **11**, 33 (1963).
[9] G. Hedenskog, L. Enebo, J. Vendlová, B. Prokeš, *Biotechnol. Bioeng.*, **11**, 37 (1969).
[10] P. Novotný, *Nature*, **202**, 364 (1964).
[11] J. Reháček, K. Beran and V. Bičík, *Appl. Microbiol.*, **17**, 462 (1959).

[12] G. Hedenskog, H. Mogren and L. Enebo, *Biotechnol. Bioeng.*, **12**, 947 (1970).
[13] S. R. Tannenbaum and S. A. Miller, *Nature*, **214**, 1261 (1957).
[14] P. J. Hetherington, M. Follows, P. Dunnill and M. D. Lilly, *Trans. Inst. Chem. Engrs.*, **49**, 142 (1971).
[15] M. Follows, P. J. Hetherington, P. Dunnill and M. D. Lilly, *Biotechnol. Bioeng.*, **13**, 549 (1971).
[16] A. G. Marr and E. H. Cota-Robles, *J. Bact.*, **74**, 79 (1957).
[17] L. Edebo, *J. Biochem. Microbiol. Technol. Eng.*, **2**, 453 (1960).
[18] L. Edebo, *Biotechnol. Bioeng.*, **9**, 267 (1967).
[19] P. W. Bridgman, *J. Chem. Physics.*, **5**, 964 (1937).
[20] L. Edebo, *Acta path. microbiol. scand.*, **52**, 300 (1961).
[21] L. Edebo, *Acta path. microbiol. scand.*, **52**, 361 (1961).
[22] L. Edebo and T. Holme, *Abstr. Internat. Biophys. Congress, Stockholm*, p. 287 (1961).
[23] L. Edebo and R. I. N. Greaves, Unpublished.
[24] R. I. N. Greaves, *Fed. Proc. 24, suppl.*, **15**, 253 (1965).
[25] H. E. Kubitschek, In *Methods in Microbiology*, J. R. Norris and D. W. Ribbons, Eds., Vol. 1, p. 593. Academic Press (1969).
[26] K.-E. Magnusson and L. Edebo, to be published.
[27] B. von Hofsten and A. Tjeder, *J. Biochem. Microbiol. Technol. Eng.*, **3**, 175 (1961).
[28] L. Edebo, *Acta path. microbiol. scand.*, **52**, 384 (1961).
[29] H. Billaudelle, L. Edebo, E. Hammarsten, C.-G. Hedén, B. Malmgren and H. Palmstierna, *Acta path. microbiol. scand.*, **50**, 208 (1960).
[30] S. V. Rogozhin, A. M. Mamtsis and D. G. Valkovsky, *Prikl. Biochim. Mikrobiol.*, **6**, 638 (1970).
[31] K. Zetelaki, *Process Biochemistry*, **4** (12), 19 (1969).
[32] B. S. Roberson and J. H. Schwab, *Biochim. Biophys. Acta.*, **44**, 436 (1960).