Methods for disruption of microbial cells for potential use in the dairy industry—a review

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Abstract

Lactic acid bacteria are being increasingly exploited for use in production of microbial bioproducts such as enzymes for food industry applications. The different techniques available for release of intracellular products include mechanical, physical, chemical, enzymatic and combined methods. The effectiveness of the various methods differs for different microbial species. Publicly accessible information is available mainly for yeasts or several well studied bacterial host cells such as *Escherichia coli* or *Bacillus subtilis*, while reports on disruption of bacterial cells applicable to uses in the dairy industry are scarce.

In general, mechanical methods are non-specific, but their efficiency is higher and application broader in comparison to any of the other methods. Disruption characteristics such as pressure or number of passes for each device vary according to microbial strain, age of culture, temperature of cultivation, and cultivation medium. High-pressure homogenizer, Microfluidizer, and Bead Mill appear to be devices best suited for use in the dairy industry due to their wide range of applicability and accessible scale-up. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Microbial cells as sources of valuable enzymes, proteins and other bioproducts have been exploited very intensively in the last 30 years. Biotechnology, supported by development of understanding of molecular biology, genetic engineering and efficient separation techniques, has been a fast growing industry. Microorganisms, both native and genetically modified, produce two basic types of biological compounds; extracellular, which are excreted into a growth medium; and intracellular, which are retained inside the cell's cytoplasm. A variety of host microorganisms have been studied. The following characteristics identify usefulness of the host microorganisms: simple genetic material, presence of plasmids, availability of strongly inducible promoters, high growth rates on simple media, excretion

of proteins into growth medium, low protease levels and for production of food related compounds, a "food grade status" of the microorganism. The most often used organisms are *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* (Middelberg, 1995). Table 1 illustrates some advantages and disadvantages of these organisms as guidelines for technology applications in general.

Several other microbial strains have been used for production of microbial enzymes; some of these and their applications are (Chisti & Moo-Young, 1986): *Aspergillus niger* and *Kluyveromyces fragilis* (for production of catalase, EC 1.11.1.6.); *Saccharomyces lactis* and *Kluyveromyces lactis* (β-galactosidase, EC 3.2.1.23); *Bacillus coagulans* and *Streptomyces* sp. (glucose isomerase, EC 5.3.1.5.); or *Penicilium notatum* (glucose oxidase EC 1.1.3.4).

A large proportion of potentially useful microbial products is retained within the cells of their producers. The isolation of intracellular material requires either the cell to be genetically engineered (so that intracellular products can be excreted into the growth medium) or the cells must be disintegrated by physical, chemical or enzymatic means to release their cytoplasmic content

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Table 1
Microorganisms frequently used for production of biological compounds (adapted from Middelberg, 1995)

Microorganism	Advantages	Disadvantages
E. coli	Extensively studied	Not GRAS[a]
	Strongly inducible promoters available	Low excretion levels of proteins into growth medium
	High growth rates on simple media	
	Low protease levels	
S. cerevisiae	GRAS microorganism capable of simple glycosylation	Low excretion levels of proteins into growth medium
	Genetic system well understood	
	High growth rates	
B. subtilis	GRAS microorganism	Excretes high levels of proteases
	Easy excretion of proteins	Plasmid instability
		Limited range of vectors and promoters available

[a] Generally regarded as safe.

(Chisti & Moo-Young, 1986). Permeabilization of cell walls to make the enzymes in the cytoplasm easily accessible for a substrate is also possible (Somkuti, Dominiecki, & Steinberg, 1996, 1998).

This review examines the different cell rupture techniques having a potential for possible use in the dairy industry; compares their applicability for different producing microorganisms, emphasizing the applications of mechanical techniques for use with bacteria; and further narrows the focus to the uses with lactic acid bacteria for production of enzymes with a potential use in the dairy industry.

2. Microbial cell wall

The nature of the bacterium or yeast cell wall is greatly dependent on the type of organism, as well as on external growth conditions including temperature of cultivation, presence of certain chemical compounds in the environment, the composition of the growth medium, growth phase, or specific growth rate (Sutherland, 1975; Engler & Robinson, 1981; Engler, 1985; Collis, O'Neill, & Middelberg, 1995; Middelberg, 1995). Knowledge of the cell wall structure is important in selecting a disruption method and rationalizing the parameters of disruption to suit the character of the feed cells (Middelberg, 1995).

2.1. Bacterial cell walls

The composition of a bacterial cell surface is different for Gram-negative or Gram-positive bacteria (Sutherland, 1975). In Gram-positive bacteria, including strains of lactic acid bacteria (*Lactobacillus*, *Streptococcus*, *Lactococcus*, or *Leuconostoc* sp.), as well as those of the *Bacillus* sp. and many others, a thick layer of mucopeptide (peptidoglycan network) confers a considerable rigidity to the cell wall. This polymeric compound is composed of *N*-acetyl-D-glucosamine and *N*-acetyl-muramic acid in β-$(1 \rightarrow 4)$ linkage as alternating units in polysaccharide (glycan) chains (Sutherland, 1975). The units on adjacent glycan chains are cross-linked by peptide bridges. The lactyl groups of the *N*-acetylmuramic acid residues provide the points at which peptides are linked to the glycan. At least portions of them may be substituted by tetrapeptide units (Engler, 1985), containing D-glutamic acid, D-alanine, and other amino acids (Sutherland, 1975). The degree of cross-linking varies considerably among different organisms. In *E. coli*, about 50% of the tetrapeptide units are not cross-linked and the others are linked only as dimers. In *Lactobacillus acidophilus*, about 90% of the tetrapeptide units are cross-linked and about 30% of the units are cross-linked as trimers (Engler, 1985). In general, the walls of Gram-positive organisms are composed predominantly of a mucopeptide, teichoic acids (polyribitol phosphate; polyhexosamine phosphates; and similar polymers) and other polysaccharides. Proteins are either essentially absent or comprise only a small portion of the cell walls.

In Gram-negative bacteria (e.g. *E. coli*), the cell wall is thinner than that of the Gram-positive species, but its structure is more complex, both in physical configuration and number of macromolecular components (Sutherland, 1975). It is formed of two distinct layers. Lipopolysaccharides, lipoproteins and phospholipids compose the outer membrane, while the thin inner layer consists of mucopeptides without teichoic acids.

The major resistance to disruption of bacterial cells appears to be the peptidoglycan network. The strength of this network depends both on the frequency with which peptide units occur on the glycan chains and the frequency with which peptide units are cross-linked (Engler, 1985). During the transition from exponential to stationary phase, considerable changes in peptidoglycan structure occur. The degree of crosslinkage increases significantly. The frequency of trimers is higher in slow growing or stationary-phase bacteria than during the exponential growth, implying a denser or thicker peptidoglycan layer (Pisabaro, de Pedro, & Vazquez, 1985). Stationary-phase bacteria have a thicker and more highly cross-linked peptidoglycan layer (Middelberg, 1995).

2.2. Yeast cell wall

Because of their larger size and different cell wall structure, disruption of yeasts is generally easier than bacteria. The basic structural components of the yeast cell wall have been identified as glucans, mannans and proteins. The overall structure is thicker than in Gram-positive bacteria. Glucans are moderately branched molecules composed of glucose residues, primarily in β-(1→3) and β-(1→6) linkages. Mannans are characterized by a backbone of mannose residues in α-(1→6) linkage having short oligosaccharide side chains. Many of the proteins found in yeast cell walls are enzymes rather than structural components (Engler, 1985).

Glucan fibrils constitute the innermost part of the cell wall and give the cell its shape. A layer of glycoprotein covers these fibrils, beyond which there is a mannan mesh covalently linked by 1,6-phosphodiester bonds.

Although characteristics of yeasts, with respect to disruption technologies, have been extensively studied, this review will be mainly involved with bacteria due to their greater relevance to the direct dairy industry application.

3. Methods of cell disruption

Complete destruction of the wall and the release of all intracellular components requires destruction of the strength-providing components of the wall, i.e. peptidoglycan in Gram-negative bacteria and glucan in yeast (Middelberg, 1995). Non-specific destruction is usually achieved by mechanical means, while non-mechanical (physical, chemical, and enzymatic) methods are more specific and gentler. Fig. 1 summarizes the most common methods used for disruption of microbial cells.

3.1. Mechanical methods

The common principle of these methods is that the cells are subjected to high stress produced by high pressure, abrasion during rapid agitation with glass beads, or ultrasound (Engler, 1985). Mechanisms of disruption are cavitation, shear, impingement or their combination (Sauer, Robinson, & Glick, 1989). Intensive cooling of the cell suspension subjected to the treatment is necessary to remove the heat generated by dissipation of the mechanical energy (Engler, 1985).

Some high-pressure methods are applicable for use on laboratory scale only; these include the Hughes press or the French press, in which a frozen suspension of cells is forced through a small opening by high pressures (Engler, 1985). In contrast the bead mill, the high-pressure homogenizer or the Microfluidizer methods are scalable for industrial use.

3.1.1. Bead mill

The bead mill, originally used in the paint industry, has been successfully adapted for cell disruption both in the laboratory and in industry. It provides a simple and effective means for disrupting different types of micro-organisms. Different designs are available. The basic scheme is a jacketed grinding chamber (vertical or horizontal) with a rotating shaft running through its center. The shaft is fitted with agitator(s) of varied design that impart the kinetic energy to small beads in the chamber, forcing them to collide with each other (Chisti & Moo-Young, 1986; Middelberg, 1995). The choice of bead diameter and bead loading is of a major importance for the efficiency in relationship to the location of the desired enzyme in the cell (Shütte, Kroner, Hustedt, & Kula, 1983). The degree of disruption increases with bead loading due to increased bead-to-bead interaction; however, heating and power consumption also increase, because of this interaction. A bead loading of 80–85% is generally considered optimal (Kula & Shütte, 1987) since the heating and power consumption for bead loads >90% often outweigh the minimal increase in disruption efficiency.

Generally, the enzymes found in soluble form in the cytoplasm are released with higher efficiency by using smaller beads; for enzymes bound to cytoplasmic membrane or in periplasmic space, bigger glass beads can be used, as complete disintegration of the cells is not
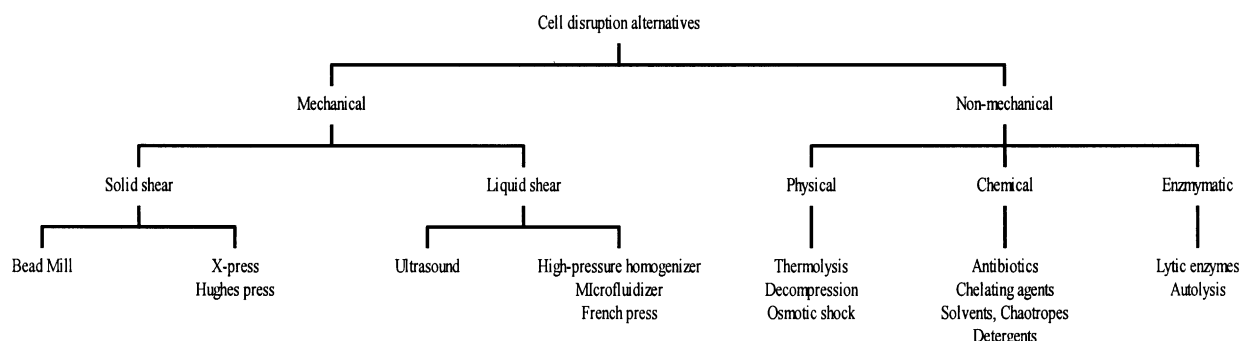

Fig. 1. Methods of microbial cell disruption (adapted from Chisti and Moo-Young, 1986; Middelberg, 1995).

necessary (Shütte et al., 1983). More rapid disruption is generally achieved with smaller beads. Beads 0.10–0.15 mm in diameter are considered optimal for the disruption of bacteria while beads 0.25–0.75 mm in diameter are used for the disruption of yeasts. Industrial machines must use beads larger than 0.4–0.6 mm in diameter because of the mechanism for separating the beads from the suspension (Kula & Shütte, 1987). Increasing the impeller tip speed increases the disruption effect at the expense of increased power usage and heat generation. Smaller cells, in general, require higher tip speeds for disruption. A tip speed of 8 m s^{-1} is adequate for yeasts while a tip speed of at least 10 m s^{-1} is recommended for the disruption of bacteria (Kula & Shütte, 1987). Moderate to high cell concentrations (40–50% wet weight) in the broth introduced into the chamber are optimal for maximization of the disruption efficiency (Middelberg, 1995). A typical effect of bead milling on rod-shaped *Lactobacillus delbrueckii* ssp. *bulgaricus* grown in skim milk is shown in Fig. 2.

The bead mill is more efficient for rupturing yeast cells and especially molds in comparison to bacteria (Middelberg, 1995). The small dimensions of bacterial cells hamper the disintegration of bacteria, which are approximately 1/10th the size of a yeast cell (Shütte et al., 1983). Complete disintegration of a myceliar mold culture (e.g. *Aspergillus niger*) is possible only in bead mills, because mycelia can blind the high-pressure homogenizer valve (Chisti and Moo-Young, 1986).

The longer a cell remains in the chamber, the higher is the probability of its destruction. Therefore, increasing the milling time (batch operation) or mean residence time (continuous operation) will normally increase the level of disruption. However, for a continuous operation, increasing the mean residence time by decreasing the flow rate can sometimes lead to an overall reduction of the effectiveness as the degree of back-mixing increases. To increase the effectiveness and to narrow the residence time distribution, two or more mills in series can be used to increase the mean residence time instead of slowing the flow rate (Kula & Shütte, 1987).

The effect of the cell concentration on the release of protein appears to be minimal; Kula and Shütte (1987) recommended 40–50% cells (wet weight) to optimize the ratio of power consumption to cell disruption. Bury, Jelen, and Kaláb (2001) also observed no apparent effect of biomass concentration (ranging from 12% to 46%, wet weight) on β-galactosidase release, while Mogren, Lindblom, and Hedenskog (1974) indicated no influence of yeast concentration in the 4–20% on disruption rate. In contrast, Dunnill and Lilly (1975) found that the disruption rate was dependent on yeast concentration between 30% and 60% packed weight per volume and decreased with increasing cell concentration.

3.1.2. Sonication

Sonication is one of the most widely used laboratory disruption methods (Engler, 1985). Ultrasound, i.e. sound waves of frequency higher than 15–20 kHz, can cause both inactivation and, at higher acoustic power inputs, disruption of microbial cells in suspension.

The mechanism of the cell disruption is associated with the cavitation phenomena, i.e. shear stress developed by viscous dissipative eddies arising from shock waves produced by imploding cavitation bubbles. Shear forces creating eddies larger than the cells are more likely to move the cells rather than disrupt them, whereas eddies smaller than the cells are capable of generating disruptive shear stresses. Thus, larger cells will experience more disruptive eddies than smaller cells.
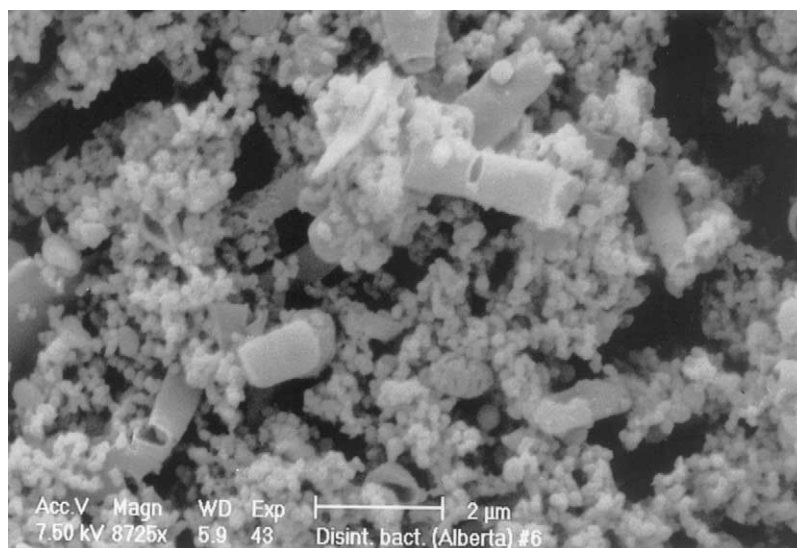

Fig. 2. Scanning electron microscopy of a *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 cell culture grown in skim milk after bead mill treatment (courtesy of Dr. M. Kalab, Agriculture and Agri-Food Canada, Ottawa, Ont.).

Increasing the power (intensity) will shift the size distribution towards smaller eddies, which in turn, will increase the number of disruptive eddies acting on the cells resulting in greater disruption (Doulah, 1977). James, Coakley, and Hughes (1972) showed that the protein release constant increased linearly with acoustic power when sonicating 200 mL of a 20% yeast suspension in the range of 67–187 W, as did Feliu, Cubarsi, and Villaverde (1998) who sonicated *E. coli* suspensions (5–30 mL) using 35–95 W of acoustic power. Feliu et al. (1998) noticed that the release of protein decreased as the volume of the sample being sonicated increased. In terms of eddies, increasing the sample volume would reduce the power dispersed per unit volume. This would favor the formation of larger eddies and reduce the number of eddies per unit volume, which would result in decreased disruption efficiency. Kuboi, Umakoshi, Takagi, and Komasawa (1995) showed that initial cell concentration of *E. coli* cells in suspension from 3.5 to 20 g L^{-1} had no effect on the disruption rate constant. The rate constant increased linearly with the power and decreased linearly with the working volume (2.5–10 mL tested).

Most of the ultrasound energy absorbed into the cell suspensions ultimately appears as heat, and a good temperature control is necessary (Chisti & Moo-Young, 1986). Sonication may cause significant degradation of enzymes due to heat denaturation because of insufficient cooling in close proximity of the sonication probe (Chisti & Moo-Young, 1986; Engler, 1985). Although flow-through cells are available for larger volumes (James et al., 1972), sonication is still inefficient and largely ineffective for pilot scale or industrial use.

3.1.3. High-pressure homogenizer

Disruption in a high-pressure homogenizer is achieved by passing a cell suspension under high pressure through an adjustable, restricted orifice discharge valve. The major parameters determining efficiency are operating pressure and number of passes through the valve (Engler, 1985), suspension temperature (Hetherington, Follows, Dunnill, & Lilly, 1971), and homogenizer valve design (Keshavarz-Moore, Hoare, & Dunnill, 1990). The basic homogenizer design consists of a positive-displacement pump that forces a cell suspension through the center of a valve seat and across the seat face. Adjusting the force on the valve controls the pressure. The fluid flows radially across the valve and strikes an impact ring (Middelberg, 1995). Disruption results from non-specific tearing apart of the cell wall. Impingement is an important mechanism of disruption, particularly for yeasts (Engler & Robinson, 1981). The homogenizer is a vital unit for the dairy processing industry for milk fat globule disruption and size control. Milk is often homogenized in two stages (two valve assemblies in series) using pressures around 15 MPa for the first stage, followed by a lower pressure for the second stage, to break large fat globules to smaller ones (<1 μm), which prevents the separation of cream from the milk. These pressures are much too low to be effective for microbial cell disruption. Instead, pressures ranging from 55 to 200 MPa are often utilized for the disruption of cells.

The most widely used homogenizer type today is the Manton–Gaulin APV design (Middelberg, 1995). Temperature rise of about 2°C per 10 MPa in a homogenizer is common, due to the adiabatic compression (Chisti & Moo-Young, 1986). There is a strong influence of the operating pressure on the disruption process in the homogenizer. By operating the homogenizer at higher pressures, it is possible to decrease number of passes of the cell slurry through the homogenizer for a given degree of disruption (Chisti & Moo-Young, 1986; Bury et al., 2001). However, the deactivation of heat sensitive proteins may limit the operating pressure, which in turn may increase the number of passages required (Kula & Shütte, 1987). Three to five passages are usually required to release more than 90% of the protein but fewer passes are typically used for practical purposes, as the incremental amount of protein released by additional passages may not be economically justified. Disruption has been found to be independent of the cell concentration in the feed over a wide range (Engler, 1985; Bury et al., 2001).

One pass of a cell suspension through a Manton–Gaulin homogenizer typically ruptures the cell or breaks it into several distinct parts. After further passes the cell debris may be degraded to smaller fragments resulting in less effective downstream separation with increasing number of passes (Baldwin & Robinson, 1990). The effect of multiple passes through the homogenizer is demonstrated in Figs. 3(b–d), which show SEM pictures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 bacteria after 1–3 passes through a high-pressure homogenizer, in comparison to untreated sample (Fig. 3a).

Siddiqi, Titchener-Hooker, and Ayazi Shamlou (1997) showed that for the yeast, cell debris particle size distribution and the extent of soluble protein release are independent of the scale of operation and of differences in the design of the valve head. However, this is contrary to the generally accepted view regarding the importance of the valve design. Laboratory high-pressure homogenizers are suitable for disrupting cell suspensions with volumes as low as 100 mL. High-pressure homogenizers are routinely used in pharmaceutical and biotechnology industries to disrupt bacteria and yeast on an industrial scale. The disadvantage of the valve homogenizers when applied to extraction of heat-sensitive materials is the need for external cooling which can only be applied after the disruption—with the concomitant temperature increase—has taken place.
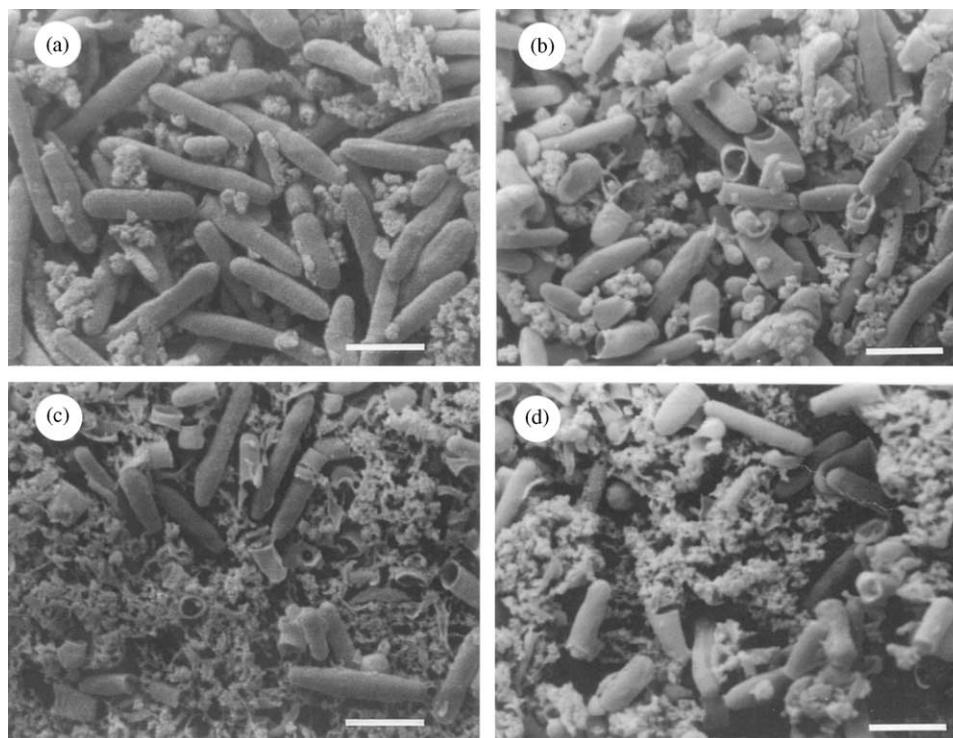
Fig. 3. Scanning electron microscopy of disrupted cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842. (a) Culture prior to disruption; (b)–(d) culture after one, two or three passages through a Rannie high-pressure homogeinizer operated at 135 MPa. Bar: 2 μm (Bury, 2000).

3.1.4. Microfluidizer homogenizer

The Microfluidizer® operates on a principle different from that of the high-pressure valve homogenizer. In this laboratory or industrial-size equipment, two streams of a cell suspension are impacted at high velocity against a stationary surface and the energy input is dissipated almost instantaneously at the point of impact leading to disruption of cells. The operation pressure is a function of the flow rate, the disruption unit, and the backpressure unit when used (Middelberg, 1995; Agerkvist & Enfors, 1990).

The residence time of the cell suspension in the Microfluidizer® disruption chamber, which is the hottest part of the device, is only 25–40 ms. Efficient cooling in place is easily achievable for the laboratory models by immersion of the disruption chamber in an ice bath (Sauer et al., 1989; Geciova, personal experience). The fraction of disrupted cells increases with increasing pressure and number of passes. The efficiency of the disruption is also dependent on the initial cell concentration and on the specific growth rate of the cells during the upstream fermentation stage. Similar to other mechanical methods, Microfluidizer® can disrupt cell suspensions of a relatively low concentration, i.e., as low as about 5 g of dry matter per liter, a level which is readily achievable in a fermentor with no need of further concentration (Sauer et al., 1989).

The disruption mechanism utilized in the Microfluidizer® leads to larger particle sizes after disruption as compared to the Manton–Gaulin APV homogenizer, resulting in more effective separation during the following centrifugation, if included (Baldwin & Robinson, 1990). Increasing the number of passes degrades the cell debris further, which decreases the separation efficiency of the centrifugation. In addition, the viscosity of the fluid containing the cells can decrease somewhat after multiple passes, as the DNA long chain is broken up.

3.2. Non-mechanical methods

3.2.1. Physical disruption

A limited number of methods exist with potential process-scale application. One of these is decompression, based on introducing a pressurized subcritical or supercritical gas into the cells causing disruption after release of applied pressure by expansion. Another possibility is the osmotic shock, where a cell suspension is diluted after equilibration in high osmotic pressure. A third alternative, thermolysis involves heat treatment of the producing microorganism cells. Yet another laboratory alternative is a method referred to as "nebulization" (formation of droplets by flow of gas over a surface of a liquid causing formation of tiny droplets connected with surface by a capillary-like formations,

where tearing apart of the cells occurs; Lewis, 1993). These methods are gentle and result in large cell debris, which is an advantage for separation of soluble proteins, enzymes or other bioproducts. The disadvantages of these methods are in their limited general applicability and in most cases, low efficiency (Middelberg, 1995).

3.2.2. Chemical permeabilization

The outer wall of a microorganism can be permeabilized by a large variety of chemical compounds, which differ in selectivity and efficiency towards different microbial species. Chemical permeabilization could be accomplished by antibiotics, chelating agents, chaotropes, detergents, solvents, or by hydroxides and hypochlorites (Table 2).

Chemical permeabilization with solvents or detergents does not result in the release of intracellular enzymes (Somkuti et al., 1998). In general, for food applications, chemical treatments introduce another complicating factor, the "contamination" of the cell suspension by the active compound (often non-food grade), resulting in higher degree of complexity of the downstream process operations.

3.2.3. Enzymatic disruption

Enzymatic lysis has the advantage of being specific and gentle. Autolysis and use of foreign lytic enzymes are two alternative approaches with possible uses in industry (Chisti & Moo-Young, 1986; Middelberg, 1995). The technique of autolysis was employed to prepare autolysed yeast and yeast hydrolyzates, but an

Table 2
Methods of chemical permeabilization (adapted from Middelberg, 1995)

Permeabilizing compound type	Examples of specific compounds	Effect	Note
Antibiotics	β-Lactam antibiotics (penicillin)	Gram-negative bacteria (lysis of peptidoglycan)	Effectiveness depends on state of culture, high cost
	Cationic polypeptide (polymyxin)	Binding to and distorting the cytoplasmic membrane	Effectiveness depends on state of culture; high cost; used in combination with lysozyme (Dean & Ward, 1992)
Chelating agents	EDTA	Gram-negative bacteria; binding Mg^{2+} and Ca^{2+} resulting in release of lipopolysaccharide molecule	Strain specific effect; influenced by buffers; no effect on inner membrane; used in combination with lysozyme (Dean & Ward, 1992)
Chaotropes	Urea	Weakening solute–solute interaction—solubilization of membrane proteins	Strain dependent minimal concentration required
	Guanidine		
	Ethanol	Disrupts the hydrogen bond—inhibition of the assembly of cross-linked peptidoglycan	Used in combination with toluene. Used for permeabilization of *Streptococcus thermophilus* culture (Somkuti et al., 1996, 1998)
Detergents	Triton X series (100, 114); non-ionic	Formation of mixed micelles containing lipid from the membrane. Used mainly for Gram-negative bacteria, for yeast less effective; extensively studied with *Streptococcus thermophilus* (Somkuti & Steinberg, 1994)	Used in combination with EDTA, chaotropes; possible denaturation of proteins (by SDS), problematical removal (Triton X-100); mainly for recovery of specific membrane proteins
	Sodium dodecyl sulphate (SDS); anionic		
	Brij series (58); non-ionic		
	Sodium lauryl sarcosinate (Sarcosyl)—anionic		
Solvents	Toluene	Dissolving hydrophobic components in Gram-negative bacteria (inner membrane phospholipids); enhanced by EDTA	Effect dependent on concentration; efficiency of release is low and highly dependent on the organism and conditions; toxic; expensive
	Chloroform	Extraction of periplasmic proteins from Gram-negative bacteria	
	Acetone	Combination with SDS detergent; extraction of cytoplasmic proteins from Gram-positive bacteria	
Hydroxide and hypochlorite	Sodium hydroxide and Sodium hypochlorite	Saponification of lipids in the cell wall	Cheap and effective; extremely harsh; product of interest has to be resistant to degradation at high pH

acceptable rate of autolysis occurred in specific conditions only (Reed & Nagodawithana, 1991). To trigger autolysis of growing *E. coli* cultures, antibiotics that interfere with peptidoglycan synthesis were tested. By the degradation of peptidoglycan structure, the porosity of the cell wall is increased and may cause an eventual lysis (Middelberg, 1995). However, the use of antibiotics for most food applications can be problematic.

The enzymatic disruption of yeasts by foreign lytic enzymes has been extensively studied (Middelberg, 1995). Disruption requires the use of protease and glucanase to attack, at first, the mannoprotein complex of the cell wall and then the glucan backbone (Kitamura, 1982). To date the most efficient system for yeast cell wall lysis appears to be the commercial product Zymolase-20T (Seikagaku America, Inc., Rockville, MD) produced from *Oerskovia xanthineolytica* (Kitamura, 1982; Middelberg, 1995).

Lysozyme is often used for lysis of peptidoglycan layers as it catalyses hydrolysis of β-1,4-glycosidic bonds. The enzyme is commercially available at reasonable cost, produced from egg-white preparations. Gram-negative bacteria are less susceptible than the Gram-positive ones as the outer lipopolysaccharide layer of Gram-negative bacteria shields the peptidoglycan from the enzyme. However, the combination of lysozyme with EDTA for Gram-negative bacteria allows the disruption of the lipopolysaccharide layer and subsequent attack on the peptidoglycan structure (Salasbury, 1989).

4. Effectiveness of mechanical cell rupture techniques for individual microbial species

4.1. Traditionally used strains of host microorganisms

Most of the published research has been focused on the disruption characteristics of genetically well-characterized host microorganisms such as *E. coli* (Gram-negative bacteria), *B. subtilis* (sporeforming Gram-positive bacteria) and yeasts, mainly bakers' or brewers' yeast *S. cervisiae* or *Candida utilis*.

The cell disruption techniques with the best industrial potential are the mechanical methods. The characteristics monitored during and after mechanical disruption include the efficiency of disruption (measured by amounts of released proteins, activity of released enzymes, or numbers of surviving cells) as well as physical properties, very important for downstream processing. Different characteristics of the disintegrated cells caused by different mechanical devices (e.g. size of cell debris, particle size distribution, viscosity) influence the efficiency of separation techniques used for isolation of a desired product (Baldwin & Robinson, 1990).

Engler and Robinson (1981) found out that cell wall strength is related to the specific growth rate of the bacterial cells. Cells of a particular bacteria grown at high specific growth rates were easier to disrupt than cells grown at a lower growth rate. In their study there appeared to be no relationship between the type of organism (bacteria or yeast) and their susceptibility to disruption. Changing growth conditions can alter disruption characteristics of a given organism. Pressure required for disruption is related to the shape of the microorganism, the rods being more easily disrupted than the cocci (Kelemen & Sharpe, 1979). *E. coli,* a Gram-negative rod, is disrupted more easily in comparison to the *B. subtilis*, which is similar in size, but is Gram-positive (Kelemen & Sharpe, 1979). Residence time of the cells in a batch fermentor is usually greater than the average residence time in a continuous fermentor. Thus, cells in batch culture have a longer time to repair any cell wall defects, which results in stronger cell walls (Sauer et al., 1989).

E. coli, a host organism for many different bioproducts, is often genetically modified to produce foreign proteins. As a genetically modified organism, *E. coli* should be inactivated before removal from the fermentor (GMAC, 1994). According to Collis et al. (1995), thermal deactivation by raising temperature to 60–70°C had a significant effect on subsequent downstream processes (homogenization, centrifugation). Thermally deactivated cells were considerably smaller than cells from the exponential growth phase; their disruption degree was considerably lower and cell-debris was larger. For release of enzyme β-galactosidase from recombined *E. coli*, Ariga, Yoshinaga, and Sano (1995) developed an efficient method combining thermal treatment (60°C) and presence of glycine (2%) as an effective inducer for release of periplasmic proteins.

Dean and Ward (1992) studied the recovery of intracellular products from *E. coli* by the use of EDTA or polymyxin with lysozyme. They found that the use of EDTA, resulting in removal of the lipopolysaccharide content with exposure of the hydrophobic phospholipid layer, rendered the cells susceptible to lysozyme. They showed that stationary cells were most susceptible to lysis by EDTA/lysozyme whereas polymyxin/lysozyme had an optimal effect on log-phase cells.

Agerkvist and Enfors (1990) compared three mechanical devices (high-pressure homogenizer, Manton–Gaulin APV; Microfluidizer®; and Bead Mill, Dyno Mill KDL) for cell rupture of *E. coli* and the influence of these methods on protein release, the particle size distribution and the growth medium viscosity. All three methods gave approximately the same protein and enzyme release, but considerably different physical properties of the medium containing disintegrated cells. Increasing the degree of disruption in the bead mill affected the separation degree only slightly, while in the high-pressure homogenizers the effect was much stronger.

Kleinig, Mansell, Nguyen, Badalyan, and Middelberg (1995) concluded that the optimal feed concentration of *E. coli* cell suspension for high-pressure homogenizer (APV-Gaulin) should be as high as possible, the limitation being practical handling difficulties due to high viscosity.

Woodrow and Quirk (1982) studied conditions for breakage of *Enterobacter cloacae* or *E. coli* in their study with a bead mill. The optimal conditions found involved homogenization of a 1:2.5 (w:w) cell suspension in a buffer solution with 0.25 mm diameter of glass beads at an agitation speed of $15\,\mathrm{m\,s^{-1}}$.

Su and Feng (1999) used horizontally stirred bead mill for extraction of intracellular proteins by combination of cell disruption and aqueous two-phase extraction in a single processing step. Their target microorganisms were recombinant *E. coli* and *S. cerevisiae*. In this study, better results were achieved for extractive disruption than for mechanical disruption alone.

S. cerevisiae, commercially available common bakers' yeast, is often used as a GRAS host microorganism to produce valuable bioproducts, and as a model microorganism to study the cell rupture techniques. Baldwin and Robinson (1990) proposed a combined method of disruption for this yeast. Enzymatic pretreatment by Zymolase-20T significantly enhanced the ability of the Microfluidizer® to achieve high degree of disruption comparable to that achieved by industry scale Manton–Gaulin high-pressure homogenizer.

As shown in Table 3, efficiency of mechanical disruption is highly dependent on the strain, cultivation conditions, and type of mechanical devices. With the optimal conditions for disruption, it is possible to achieve about 82–99% disruption of bacterial cells depending on the method. However, obtaining 100% efficiency of disruption as presented in the paper by Woodrow and Quirk (1982) is highly questionable. With the initial cell concentration of approximately $10^9\,\mathrm{cfu\,mL^{-1}}$ and efficiency of disruption 99.9%, the cell suspension still contains $10^6\,\mathrm{cfu\,mL^{-1}}$ of intact cells. From a technological point of view, 99.9% disruption efficiency (estimated as viable counts before and after the treatment) is obviously more than adequate; however, from the microbiological point of view, the level of surviving microorganisms is still very high; this is why these methods cannot replace the heat sterilization. Nevertheless, the above-mentioned results (as well as our own experience) indicate that it is not necessary to achieve 100% cell disruption to release sufficient levels of the desired intracellular protein or enzyme.

4.2. Dairy starter cultures

Literature reports on cell disruption of dairy starter cultures are scarce. Starter cultures used in the dairy industry include strains of *Lactobacillus* sp. (*delbrueckii* subsp. *bulgaricus*, *casei* subsp. *casei*); *Streptococcus thermophilus*; several strains of *Lactococcus* sp. (*lactic*, *cremoris*); *Leuconostoc* sp.; and some strains of *Pediococcus* sp. Recently studied intracellular enzymes with current or promising utilization in dairy industry include β-galactosidases (lactase, β-D-galactoside galactohydrolase, EC 3.2.1.23) for use in dairy products suitable for lactose malabsorbing consumers, and aminopeptidases (Choi, Laleye, Amantea, & Simard, 1997) for acceleration of cheese ripening. The microorganisms available for production of these enzymes are GRAS. There is no need for expensive purification of the enzyme solution. The whole culture after disruption can be utilized in manufacture of some dairy products (Choi et al., 1997; Somkuti et al., 1996, 1998). All of the traditionally used dairy cultures are Gram-positive microorganisms, usually grown in a nutritionally rich, complex food grade medium milk. In comparison to the growth conditions and cell wall structure of the often-tested strains of *E. coli* it is not surprising that the cell rupture of the dairy cultures is more difficult.

In a study by Choi et al. (1997), release of an aminopeptidase from *Lactobacillus casei* subsp. *casei* was investigated. Effectiveness of cell disruption by the Microfluidizer® was a function of both operating pressures and number of passes. The operating pressure had a greater effect on the disruption than did a number of passes.

Disruption of *Lactobacillus delbrueckii* subsp. *bulgaricus* (ATCC 11842) was studied by Bury et al. (2001). Sonication, bead milling and high-pressure homogenizer (Manton–Gaulin APV and Rannie Lab 2000) were compared. With respect to the released enzyme β-galactosidase, bead milling and high-pressure homogenizer were comparable, while laboratory scale sonication was the least effective. The desired degree of disintegration in the homogenizer was achieved in fewer passes by using higher pressure, in agreement with reports by others (Chisti & Moo-Young, 1986; Kula & Shütte, 1987). After one pass at 200 MPa in a Rannie Lab 2000 homogenizer, the enzymatic activity was comparable to that obtained after two passes at 135 MPa (Bury et al., 2001).

Somkuti et al. (1996, 1998) studied chemical permeabilization and enhancement of β-galactosidase activity in *Streptococcus thermophilus*. Among the tested chemical compounds (SDS, TritonX-100, bile salts and ethanol), only ethanol could be considered for further use. As a food grade chemical, its traces left after permeabilization of *Streptococcus thermophilus* biomass could be perhaps acceptable.

Toba, Hayasaka, Taguchi, and Adachi (1990), Sakakibara, Wang, Ikeda, and Suzuki (1994), and Wang, Sakakibara, Kondoh, and Suzuki (1996) studied sonication as a method of accelerating dairy fermentation

Table 3
Comparison of disruption efficiency of mechanical methods applied to specific bacteria

Organism	Method of disruption (equipment/parameters)	Cultivation medium/ treatment before disruption	Efficiency of cell disruption (%)	Literature source
Lactobacillus delbrueckii subsp. *bulgaricus* (ATCC 11842)	Sonication (Braun–Sonic 2000/75 W)	MRS *Lactobacilli* broth/ 18–24 h of cultivation	89[a]	Kreft et al. (2001)
			85.3[a]	Kreft and Jelen (2000)
Lactobacillus delbrueckii subsp. *bulgaricus* 2515	MSK cell homogenizer (glass beads, ⌀ 0.1 mm, 90 s)	MRS broth, 16 h	> 99.9[a]	Shah and Lankaputhra (1997)
Streptococcus thermophilus 2010			> 99.9[a]	
Lactobacillus delbrueckii subsp. *bulgaricus*	Sonic 300 dismembrator (16 khz, 4 min)	12% reconstituted non-fat dry milk, 18 h	> 99.9[a]	Shah and Jelen (1991)
E. coli JM 101	High-pressure homogenizer (APV–Gaulin 15 MR-8TBA/ single pass; 70 MPa)	Synthetic medium:		Collis et al. (1995)
		/Late exponential phase /Stationary phase Thermal deactivation: 9 min 13 min	98.6±0.1[b] 90.1±0.9[b] 62.3±2.1[b] 36.5±3.0[b]	
E. coli JM 101	High-pressure homogenizer (APV–Gaulin 15 MR-8TBA/ single pass; 64 MPa)	Synthetic medium	About 85[b]	Kleinig et al. (1995)
Lactobacillus casei subsp. *casei*	Microfluidizer® (M-110 Y/ 158.5 MPa; 15 passes)	MRS *Lactobacilli* broth/24 h	About 94[a]	Choi et al. (1997)
B. subtilis (ATCC 6051)	High-pressure homogenizer (Manton–Gaulin APV/ 88 MPa, 1pass)	Synthetic medium/ continuous fermentation	82[d]	Engler and Robinson (1981)
E. coli (ATCC 15224)	Bead Mil (Dyno Mill KDL/ ⌀ 0.25–0.50 mm glass beads, 4 min)	Glycerol-mineral salts, until late exponential phase	79[c]	Agerkvist and Enfors (1990)
	High-pressure homogenizer (Manton–Gaulin APV 15M-8TA/ 60 MPa, 3 passes)		82[c]	
	Microfluidizer® (M110/ 60 MPa, 3 passes)		88[c]	

[a] Based on cell counts before and after disruption (cfu mL^{-1}).
[b] Based on data obtained from applied imaging disc centrifuge (integrated cell size distributions after and before homogenization).
[c] Based on protein released after disruption in comparison to maximal amount of protein released after 10 passes by microfluidizer.
[d] Based on Kjeldahl analysis of the supernatants from disrupted samples.

processes. After sonication of high β-galactosidase producing strains during yogurt fermentation, Toba et al. (1990) reported 71–74% depletion of the initial lactose content, whereas 39–51% lactose hydrolysis was obtained in non-sonicated milk. In comparison, Sakakibara et al. (1994) achieved about 55% lactose hydrolysis during sonication-enhanced fermentation compared with about 36% lactose hydrolysis during conventional fermentation. With the prolongation of the incubation period before sonication, the amount of intracellularly accumulated β-galactosidase released to the medium increased considerably (Toba et al., 1990; Wang et al., 1996).

4.3. Effect of cell rupture treatment on released enzymes

The intracellular β-galactosidase is one of the most thoroughly studied proteins used as indicators of

effectiveness of bacterial disruption. There are several reasons, including existence of sensitive methods of determination of its presence and the fact that the model microorganism *E. coli* is an abundant source of this intracellular enzyme (Agerkvist & Enfors, 1990). The β-galactosidase is one of the technologically most important enzymes produced by lactic acid bacteria with its potential application in the production of low-lactose milk (Somkuti et al., 1998).

When the mechanical methods of disruption are used, the release of proteins follows approximately first order kinetics as expected. The kinetics for the glass bead mill is a first order function of the mean residence time of the cell suspension in the grinding chamber; for the high-pressure homogenizers, it is a first order function of the number of passes through the disrupter (Agerkvist & Enfors, 1990). According to Sauer et al. (1989), for the Microfluidizer®, the disruption effectiveness does not show a simple first order dependency on the number of passes, being also dependent on the concentration of the cell suspension being disrupted, and on the type of cells (e.g. recombinant vs. native strains). Efficient cooling of the mechanical devices (the grinding chamber of a bead mill or the disruption chamber of the Microfluidizer® or the appropriate heat exchanger arrangements when using high pressure homogenizer) will prevent heat denaturation of the released proteins caused by heat dissipated during disruption. The temperature after treatment should not increase to more than 30°C (Hetherington et al., 1971), also for reasons of process economy. Increasing temperature during bead milling can reduce the total measured protein release (Limon-Lason, Hoare, Orsborn, Doyle, & Dunnill, 1979). Similarly, loss of enzyme activity can be caused by shear stress denaturation (Choi et al., 1997).

An enzyme is released during the disruption process continuously. In a bead mill chamber, with the use of small diameter beads (0.1 mm), the release of enzymes and other protein can be very rapid, often within 0.5 min (Woodrow & Quirk, 1982), but the bead milling is usually being run for several minutes (Woodrow & Quirk, 1982; Agerkvist & Enfors, 1990; Bury et al., 2001) probably without much measurable additional effect. Similarly, prolonging the homogenization treatment after the enzyme release was completed can cause some loss of enzyme activity, suggesting that homogenization can be so vigorous as to appreciably denature the enzyme (Woodrow & Quirk, 1982).

Choi et al. (1997) showed that the operating pressure had a greater impact on enzyme loss than the number of passes, when an aminopeptidase enzyme extract was subjected to Microfluidizer® treatment. Results indicated an enzyme activity loss of 10–20%. From their study it can be concluded that the release of the aminopeptidase can be completed at 75.8 MPa (10 passes) without serious enzyme loss. Agerkvist and Enfors (1990) showed that the release of β-galactosidase (both protein amount and enzyme activity) is slightly higher for the high-pressure homogenizers (Manton–Gaulin APV Homogenizer and Microfluidizer®) than for the bead mill (Dyno Mill KDL). Bury et al. (2001) found that a simultaneous release and deactivation of β-galactosidase could occur under certain process conditions as evidenced by the maximum observed using the bead mill.

5. Conclusions

Bead milling and high-pressure homogenizers (Manton–Gaulin APV or a Microfluidizer®) are accessible methods of cell disruption with good potential for industrial scale-up. For well known host microorganisms (*E. coli, B. subtilis, S. cerevisiae*) there are abundant literature sources dealing with equations and mathematical models describing influence of different parameters during disruption treatment. The literature data are relatively scarce for other microorganisms, especially bacteria with potential applications in the dairy industry. Generalizations from data obtained for other microorganisms can be used but more specific information is lacking. The progress in the last few years has been slow concerning advancement of new principles in microbial cell disruption technology. Most of the knowledge available publicly today has been generated from the early- to mid-1980s. In the last 15 years, the emphasis has been focussed on commercialization and new industrial applications of the known methods.

A new area of the disruption technology in the dairy industry is the use of dairy cultures as potential sources of various enzymes for accelerated cheese ripening, faster fermentation processes, lactose hydrolysis or various other novel applications. Direct food utilization without additional and costly purification procedure is possible, as these are GRAS organisms. However, publicly available research information about the specific aspects of disruption of the individual species and strains of conventional and new dairy starter organisms is scarce and more focused research should be encouraged.

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