CHAPTER 3

MICROALGAE AND CELL DISRUPTION

3.1   Algae

3.1.1   A Definition for the Algae

In brief algae are aquatic organisms (with frequent exceptions) with capacity of photosynthesis, oxygenic autotrophy. They are (except for the seaweeds) typically smaller and less structurally complex than land plants. This rather inelegant definition allows including the cyanobacteria (blue-green algae, chloroxybacteria), which, although prokaryotic, is similar to some members of other algal groups (eukaryotic) in terms of overall structure and ecosystem function. Cyanobacteira are distinguished from other bacteria mainly by their possession of the biochemical apparatus necessary for oxygen production (some other bacteria are photosynthetic but do not generate molecular oxygen). Algae consist of several groups which have acquired plastids through endosymbiosis. Although it is possible to clearly distinguish algae from plants, animals, and fungi, they cannot as a whole be seperated from the rest of the protists.

A great deal of variation exists in the morphology of the algal thallus (the algal body), while the most commonly encountered forms could be described briefly as follows;

- Unicells and colonies

    Many algae exist as solitary cells (unicells) while others may be made up of several to many individual cells held together loosely or in a highly

organized fashion. Some unicellar algae are nonmotile, while other possesses one or more of the various means of locomotion (for examples, *Chlorella* and *Chlorococcum*). Those locomotory structures are known as flagella. A colony is an assemblage of individual cells in which there may be either a variable number of cells or a predictable number and arrangement of cells that remain constant throughout the life of the individual (for examples, *Chroococcus*). A colony of the latter type is reffered to as a coenobium. Depending on the organism, cells in coenobia may be either flagellated or nonmotile.

- Filaments

A common growth form among the algae is the filament, where daughter cells remain attached to each other following cell division forming a chain of cells. Filaments may be unbranched or branched and may be uniseriate (a single series of cells) or multiseriate (pluriseriate), where a few to many individual filaments fused together to form a larger, more complex structure. Linear colonies, from by some diatoms, for examples, can be distinguished from true filaments by the fact that cells of the former each possess their own individual walls, whereas adjacent cells of true filaments share their wall.

3.1.2 Cell Wall

In order to design equipment for breaking microalgal cells, it is important to understand the cell wall structure. Here, information of cell wall of some typical microalgal cells is briefly introduced.

- Blue-green algae

There seem to be three types of investment surrounding blue-green algal cell wall (Figure 3-1): (1) a sheath that is immediately adjacent to the cell wall and visible without straining; (2) a slimy, mucilaginous "shroud" that surrounds the organism (with or without a sheath); this has indefinite, not sharply definded, limits; (3) similar mucilaginous shrouds with well-defined limits. The available evidence indicates

that they are composed of pectic acids and mucopolysaccharides. Upon analysis, the latter from various organisms have yielded glucose, hexauronic acids, D-xylose, ribose, galactose rhamnose, and arabinose. The outer investments of blue-green algae, which are somewhat analogous to the capsules of bacteria, are sometimes visible without special treatment, but they become strikingly clear when the organisms are mounted in dilute India ink. Electron microscopy reveals that the outer investmints are fibrillar. The fibrils are embedded in an amorphous matrix. The sheaths of some oscillatoriacean algae vary in thickness and consistency as the environmental conditions change.

The cell wall of blue-green algae, which lies between the plasmalemma and mucilaginous sheath, is a complex, usually multilayered structure (Figure 3-1). The outer layer is a lipopolysaccharide similar to that of Gram-negative bacteria. This surrounds a mucopolymer layer of peptidoglycans which is dissolved by lysozyme, which also digests the cell wall of Gram-negative bacteria. Upon fractionation, this mucopolymer has been shown to be composed of muramic acid, glucosamine, alanine, and glutamic acid and α- and ε-diaminopimelic acids. In the filamentous blue-green algae very delicate plasmodesmata or protoplasmic connections can affect protoplasmic continuity across the walls.
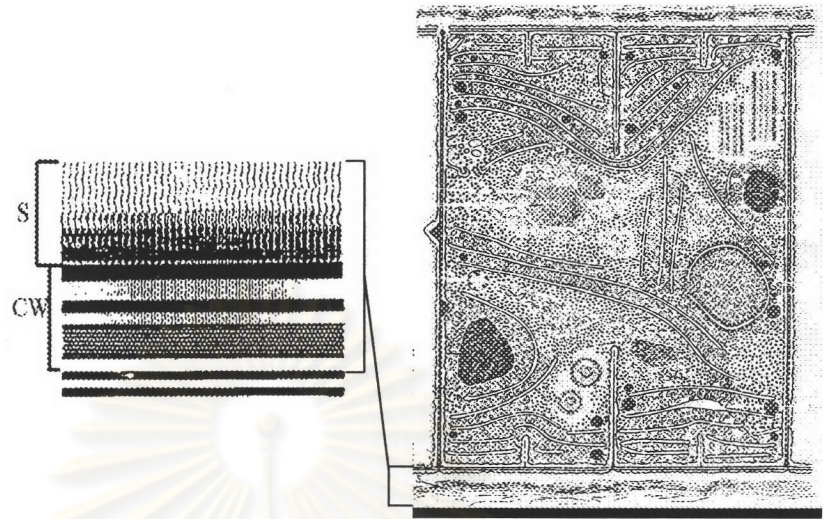
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Figure 3-1 Diagram of the organization of the cellular envelopes of certain blue-green algae, as seen in transection, base on electron microscopy. CW, cell wall: S, Sheath.

(Redraw from Hoek, 1995, 38)

- Chlorophyta

The vast majority of green algae have a distinct, fairly rigid wall around their protoplast. The cell wall consists of two or three layers of which the inner may be either completely cellulosic, i.e., consisting of pure glucose residues, or a mixture of cellulosic and noncellulosic chains. Outside the inner cellulosic layer is a layer of pectose or mucilaginous substance. The latter is water-soluble and hence is continuously regenerated in aquatic species. The gelatinous wall layer may be impregnated with lime in some members of the Siphonales.

Since long, the chlorophytan cell walls have been supposed to be mainly cellulosic, but several recent researches have tended to disprove this belief.
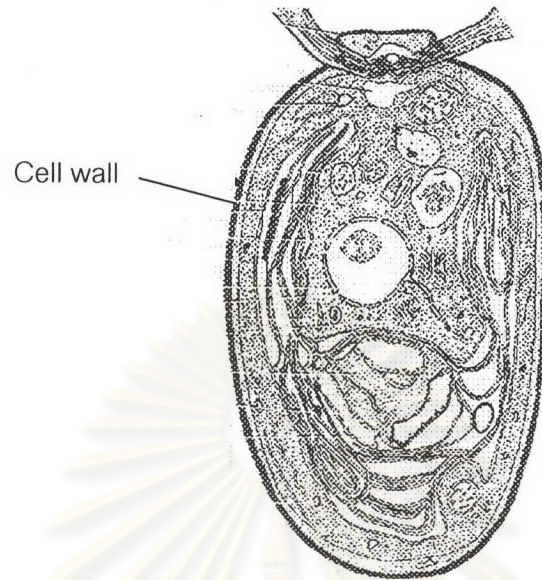
Cell wall

Figure 3-2 Diagram of the organization of *Chlamydomonas eugametos*

(Redraw from Hoek,1995, 220)

3.1.3 Nonmotile Forms Lacking Vegetative Cell Division

This group of algae contains some of the most abundantly distributed microalgae in the world. It consists of unicells and colonies that are not motile and always lack of vegetative cell devision. These organisms release the products of division as individuals, or young colonies, from within the parent cell wall. Along with the previously studied flagellated types, they are among the easiest of algae to obtain in axenic culture. Member of this group such as *Chlorella*, *Scenedesmus*, and *Chlorococcum* (Figure 3.3) have been cultured in the laboratory for many years.
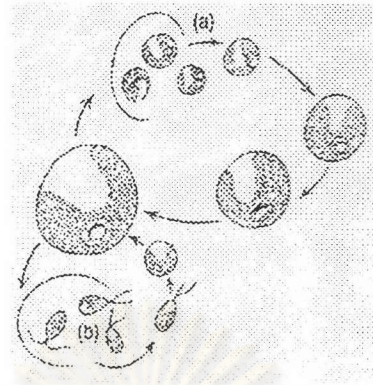
Figure 3.3. *Chlorococcum* sp. When it reproduced by aplanospores (cycle a), it resembles chlorella. But it may form planospores (cycle b).

3.2   Cell Disruption Methods

A variety of disruption methods can be employed to break the cellular walls of microalgae for liberation of the cell contents.
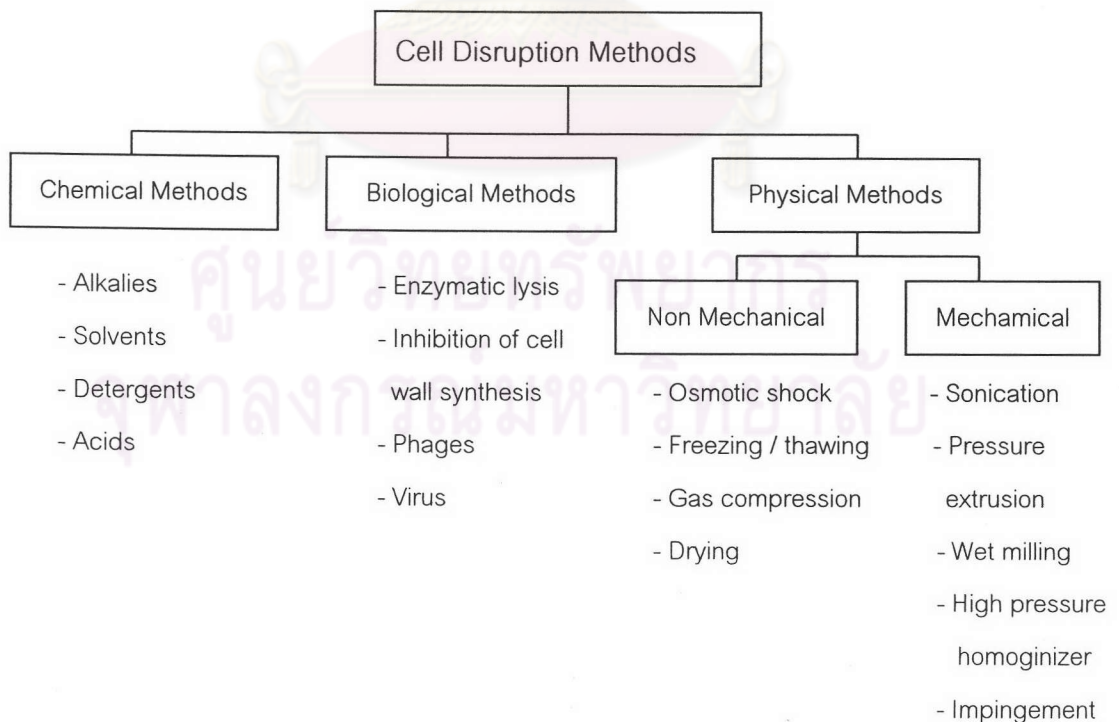

Figure 3-4. Cell disruption methods.

Typical methods shown in Figure 3-4 could liberate microbial cells' content more or less effectively and also can be classified into chemical, biological, and physical methods, depending on the basic disintegration mechanism. A knowledge of cell wall structure and composition is of course especially important for developing more efficient methods of cell disruption and is already elaborated previously. However, since the mechanical disruption is one of the most comprehensively employed methods, hereafter an couple of representatives of the mechanical cell disruption will be elaborated.

Physical Disruption Method

3.2.1. Bead mill

These kinds of machines are originally developed for the communication of pigment of printing industry, requiring dispersion of solids into micrometer-sized particales with rather narrow size distribution. In biotechnology these mills are used in an adapted form for cell disruption (Figure 3-5)
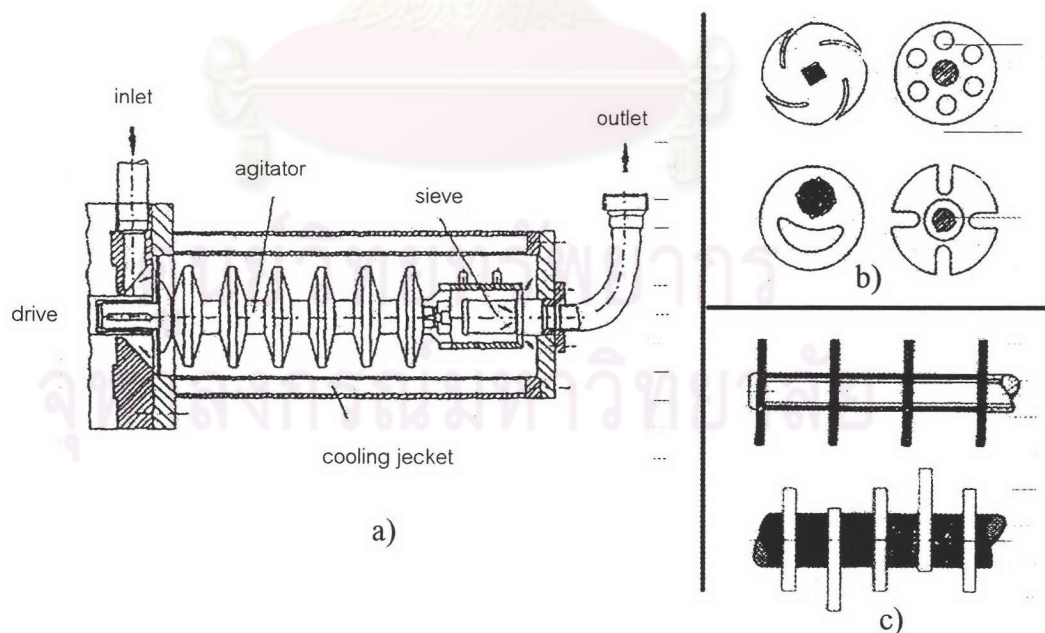

Figure 3-5. Characteristics of a bead mill (a) schematic view of a bead mill (b) centric and eccentric arrangement of agitator disk on the drive shaft. (c) section of disks used in milling.

Generally the cells will be pumped through the horizontally arranged grinding vessel, which contains a motor-driven central shaft supporting a collection of disks or other agitating elements. The chamber is filled with abrasives, usually glass beads smaller than 1 mm. Disruption is basically brought about by shear, generated by the differential velocity of streaming layers of glass beads at high agitator speed. Cell disruption in these devices can be affected by a number of operation parameters as summarized in Table 3-1

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Table 3-1 Process variables of a bead mill

Agitator speed

Feed rate of suspinsion

Size of grinding beads

Packing density of beads

Cell concentration

Temperature

Design of the stirrer

Geometry of the gringing chamber

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In batch operation of bead milling, the rate of cell disruption can be considered to be directly proportional to the amount of unreleased protein. Therefore, it is worth to consider the protein release rate equation

$$\frac{dR}{dt} = k\,(R_m - R) \tag{3-1}$$

where R is the weight of protein released per unit weight of microbial cell, R_m is the maximum measured protein release, k is the rate constant. Integration of equation (3-1) for t = 0 and t = t (batch time) yieldes:

$$\ln\left[\frac{R_m}{R_m - R}\right] = \ln D = kt \tag{3-2}$$

where D is the reciprocal of the fraction of unreleased protein.

For continuous disruption, first-order kinetics could be assumed, and D can be related to the mature of mixing in the mill expressed in terms of a continous stirred tank reactor (CSTR) in-series model, thus:

$$D = \frac{R_m}{R_m - R} = \left[1 + \left(kx \frac{\tau}{j} \right) \right] \tag{3-3}$$

where τ is the mean residence time in the mill (total volume of the mill, V, divided by the total throughput q) and j is the number of CSTRs. The values of j could be obtained experimentally from residence time distribution studies.

The rate constant, k, is known to be a function of several operating parameters, such as, agitation speed, feed rate of suspension, bead size, bead loading, temperature, equipment design, and cell concentration. Influence of those on the performance of cell disruption will be discussed below.

a) Influence of agitator speed

Generally, increasing the agitator speed will induce higher shear force generation, which leads to the large of cell lysis. At the same time, the erosion of glass beads, temperature and the power consumption will also become increased. So, it is necessary to analyze the efficiency of the cell disruption as a function of the agitator speed. The working range of agitator speed usually lay between 5 and 15 m/s, and more commonly between 8 and 10 m/s. Below these values, lysis will become slow and inefficient but on the other hand, at higher values heat generation will become and then lead to the degradation of released product excessive.

b) Influence of feed rate of suspension

The feed flow rate of cell suspension concerns the residence time distribution within the mill. Generally, cell disruption is not a simple function of residince time. It is known that the cell disruption in the mill is only slightly influenced by the feed flow rate.

Typically, the rate constant of the process is first order and constantly decreases with flow rate.

c) Influence of equipment design

The major variables in the design of bead mill lie in geometry of mill, the agitator disk design, the arrangement of the disk on the drive shaft, and the means of separation the homogenate from the media. Various designs of bead mills have been used for microbial cell disruption. The mill can be arranged in either a vertical or horizontal configuration. Generally, it is comprised of a cylindrical chamber equipped with a motor-driven central shaft support in a collection of off-centered discs or the other agitating element. The chamber is filled to the desired level with glass beads, which provide the grinding action. In vertical units, the charge of grinding beads is retained in the chamber by a sieve plate covering the bottom inlet, while in horizontal units the fluid entry is above the level of the beads in the chamber and no retention mechanism is required.

d) Influence of bead size

Lead-free glass or porcelain beads are the medium of choice for most biotechnology applications because of their inertness, abrasion resistance and low cost. Smaller beads, for example 0.2-0.5 mm, are used efficiently in smaller mills and are often selected for bacterial cell disruption. Smaller beads can generate more impacts with reduced energy requirement. They, however, require very narrow slots or screen for seperating the grinding media from the homogenate at the exit of the mill. Slot diameters are restricted to approximately one-third-bead diameter. Also, smaller beads (less than 0.8 mm.) may float, thereby reduce effectiveness of cell disruption.

For breaking larger cells and in larger mills, beads with sizes of 0.5-1.5 mm, are preferred. Above 1.5 mm, effectiveness of disruption could drop rapidly. The larger beads allow larger separation dimensions hence higher flows. Schutte and Kula (1990) reported that larger beads (> 1 mm.) are preferred for periplasmic enzymes or proteins. In that case, it does not require complete disintegration of cell, whereas smaller beads are preferred for cytoplasmic enzymes of proteins. Large beads also

suffer less reduction in diameter from abrasion. Useful lifetimes for beads are 100-200 h. for beads with size smaller than 0.5 mm. However it can be extended to 500-700 h. for beads with size smaller in the range of 0.5-1.0 mm.

e) Influence of beads loading

The bead loading is usually expressed as the percentage of the bead volume relative to the volume of the grinding chamber. The optimal beads loading depends on the size of the glass beads employed. Working with 0.5 mm. glass beads, the optimal loading is about 85% and for 1 mm. glass beads it is approximately 80%. At bead loading below 80%, the efficiency of the cell disruption will become inefficient, while at bead loading greater than 90%, the heat generation becomes a problem and the power consumption is markedly increased (Kula and Schutte, 1990).

f) Influence of concentration of cell suspension

The efficiency of disruption is not strongly influenced by cell concentration. Generally, the range of 4-20% by dry weight for bacteria and 17-18% for yeast is considered optimal (Mogren et al., 1974). The heat generation generally decreases with the decreasing cell concentration while the energy consumption per unit weight is oppositely increased.

g) Influence of temperature

The amount of heat generated in the chamber of a bead mill is influenced by the agitator speed and the bead loading. Temperature rising leads to a requirement of control by circulating cooling water or a refrigerant though the jacket to avoid protein denaturation and/or modification of physical properties of cell suspension such as viscosity (at higher temperatures, reduced viscosity will lead to greater back-mixing). However, it is known that temperature exerts a weak influence on the rate constant (Kula and Schutte, 1990).

3.2.2. High-Pressure Homogenizer

The high-pressure homogenizer is extensivesly used to disperse fat in milk in the dairy industry. In principle, a positive displacement piston pump with one or more plungers is also employed to deliver the cell suspension into a valve assembly (Fig 3-6). When the highly pressurized liquid enters the valve, at the present pressure a rapid change of velocity of up to 300 m/s can occur, accompanied by a rapid pressure drop down to the vapor pressure of the liquid. During discharge the suspension passes the valve and its seat and impinges on an impact ring. The pressure in the valve can be changed manually by a spring-load valve rod or by an automatic hydraulic regulator. The magnitude of the pressure drop is one of the parameters contributing to cell rupture, as well as the high shear forces occurring during the high acceleration of the liquid in the gap and impingement stress taking place at the valve.
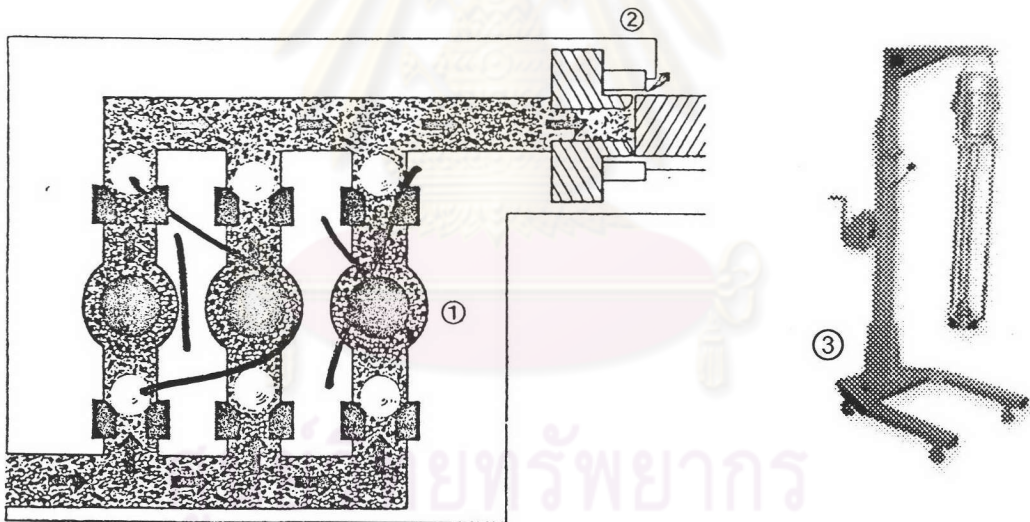
Figure 3-6 High-pressure homogenizer: (1) positive displacement piston pump; (2) homogenizing valve assembly; (3) photograph of homoginizer.

In general, cell disruption in high-pressure homogenizer is described as first-order kinetics by the following equation;

$$\log\left[\frac{R_m}{R_m - R}\right] = kNp^a \tag{3-4}$$

where R_m = maximum protein release or enzyme activity; R = measured protein release or enzyme activity after N passes; k = rate constant; N = number of passes; p = operating pressure, and a = an exponent, which depends on the kind of microorganisms and the conditions under which the cells are grown.

In addition, it is known that the disruption rate constant for high-pressure homoginizer depends on the other parameters such as design of valve unit, concentration of cell suspension, and operating temperature. Detial of those parameters will be described below.

a) Influence of valve design

Design and selection of the valve can influence shear and impingement force on cell disruption. It has been reported that effective and versatile designs include a goulin SV valve, a goulin CR valve and a a goulin CD valve. Kula and Schutte (1990) has reported the influence of valve designs on yeast cell disruption (Figure 3-7). They reported that the knife-edge valve is favored for most cell disruption applications.

b) Influence of concentration of cell suspension

The cell concention, expressed as percent dry solids, has been found to exert a weak influence on performance of a homoginizer. Several authors have reported that the efficiency of disruption is not strongly influenced by cell concentration. Generally, the range of 4-20% by dry weight for bacteria and 17-18% for yeast, is considered optimal. The heat generation decreases with decreasing cell concentration while the energy consumption per unit weight is decreased.

c) Influence of temperature

The rate of cell disruption using a homogenizer increases with temperature. As a rule, the rate at $30^{\circ}C$ is approximately double of that at $5^{\circ}C$. However, in selecting the inlet temperature, one must consider both the temperature rising and the maximum allowable temperature of the product. Typical value of

temperature rising is 2.2-2.4^{o}C/100 bar. Using a typical operation pressure of 800 bar, temperature rising of 9.6^{o}C may be expected. The maximum allowable temperature for many proteins is considered to be 30-35°C. Regarding this data, it becomes nescessary to feed cell slurry to the homoginizer at 5-15°C. In order to recover more released proteins, the cooling unit is also necessary to prevent the denaturation of biological materials.
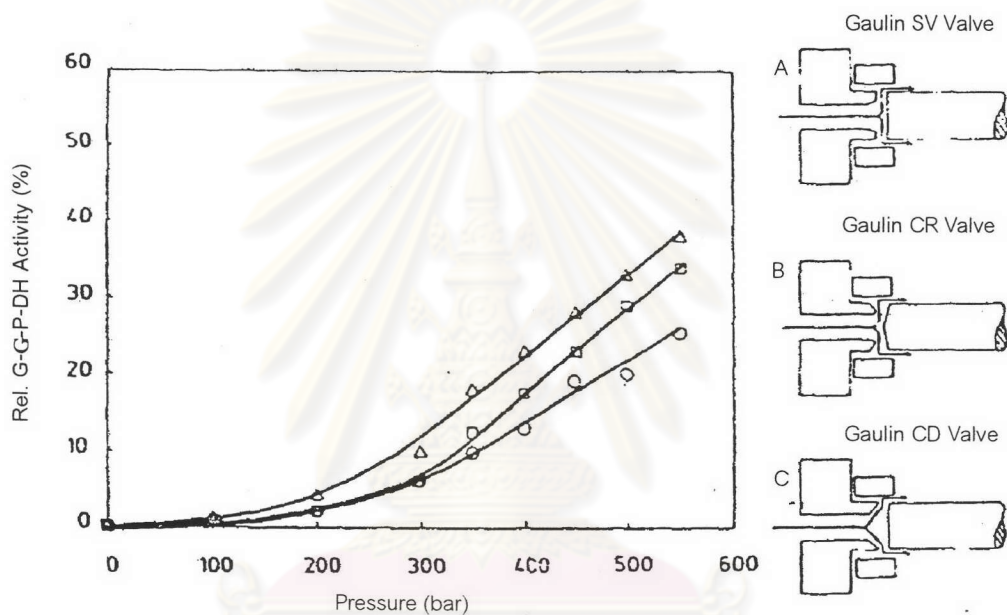

Figure 3-7. Disuption of *Saccharomyces cerevisiae* with a high-pressure homogenizer using different valve units. Symbol: (A) SV, standard valve: (B) CR, cell rupture valve: (C) CD, cell diruption valve. Enzyme; glucose-6-phosphate dehydrogenase.

(Redraw from Kula and Schutte. 1990, 615)

Operation at higher pressures is desirable in order to achieve a higher efficiency for the single passage and to reduce the necessary number of passes for a high degree of disintegration. Besides pressure, further improvement is possible by optimizing the design of the valve unit. Cell disruption in a high-pressure homogenizer is also a first order process so that a degree of disruption of 60 –70% could only achieve in

one passage. It is indispensable to obtain complete breakage by employing two or three pass operations.

3.3   Measurement of Cell Disruption

Generally, the fraction of cells disrupted could be determined either by the estimation of the number of whole cell remaining after the disruption process (direct methods) or by a determination of the amount of biological materials released into the suspending medium (indirect methods). Various direct and indirect methods will be briefly reviewed and comparisons between the techniques will subsequently be discussed.
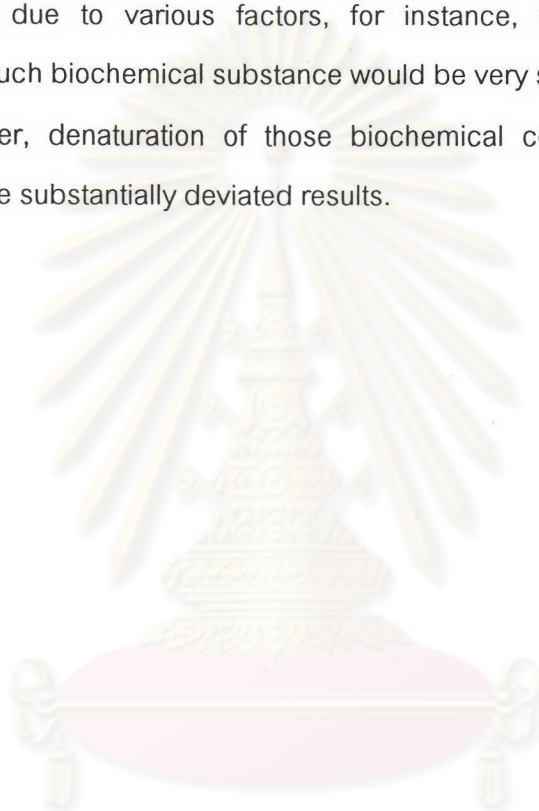
3.3.1   Direct Methods

Since disruption process typically give rise to a decrease in cell size, the direct methods for determining cell disruption generally involves with measurement of cell size before and after the disruption. The simplest measure of disruption may be determined by observational methods such as microscopy with staining to indentify a loss of wall intergrity. However, microscopy is too tedious and time consuming for a large number of samples. To overcome this, this study employed a Segwick raffter countering chamber for counting alive cell in the suspension.

The volume-fraction or number-fraction of physically destroyed cells can used to monitor the cell disruption. Shimizu et al (1998) used a Coulter couter to determine the number of whole yeast cell remaining after disruption process. Particle size analyzers have also been used to measure the cell size distribution, which could be adapted for determining cell disruption. Wong et al (1997) has monitored cell disruption with an Elzone particle size analyzer. A clear shift to smaller sizes is observed in the disruption of *Escherichia. coli.* disruption is defined as the ratio of the whole cell peak before and after disruption, which the cell peak is easily differentiated from cellular debris due to the high resolution of method.

3.3.2. Indirect Method

Indirect methods monitor the release of product from the cell. The most common approach is to measure total soluble chlorophyll release in supernatant of sample. The experiments reported by Heterington et al., 1971 and Schutte and Kula, 1990 demonstrated that only the operating pressure and the design of the valve unit are important parameters. However, since the content in the cell cytoplasm could be broadly different due to various factors, for instance, growing environment, the measurement of such biochemical substance would be very sensitive to the investigated samples. Moreover, denaturation of those biochemical compounds would also be possible to provide substantially deviated results.