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Disruption Kinetics of Bacterial Cells during Purification of Poly-β-Hydroxyalkanoate Using Ultrasonication

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Abstract — Poly-β-hydroxyalkanoate (PHA) is the intracellular product of *Haloferax mediterranei*. In order to gain PHA, *H. mediterranei* cells are disrupted by an ultrasonication, and PHA is then purified by a centrifugal separation. The results of purification are observed by SEM. Most sizes of PHA granules range from 0.2 to 0.8 μm. The SEM images demonstrate that the ultrasonication is an efficient mode for the cell disruption of *H. mediterranei*. The Kick's size reduction law is used to describe the mean size variation during an ultrasonication operation. The required energy is in proportional to the size reduction ratio. Based on this size reduction mechanism, the disruption kinetics of *H. mediterranei* cells is studied and a theoretical model is derived to relate the cell survival fraction, acoustic power, size distribution index, and operation time. This model provides a theoretical method to predict the cell disruption fraction by ultrasonication under various operating conditions with no parameter adjustment. The results show that an increase in acoustic power or operation time leads to lower survival fraction of cells. Use of this theoretical model, one can choose the optimum acoustic power and operation time to attain a specific cell disruption fraction.

Key Words : Cell disruption, Ultrasonication, Disruption kinetics, PHA, Bio-separation

INTRODUCTION

Poly(β-hydroxyalkanoate)s (PHAs) are natural, bacterially produced, biodegradable thermoplastic polyesters. Since PHAs can be degraded to carbon dioxide and water under naturally environmental conditions by various bacteria, they have high potential for the applications of environmentally degradeable plastics (Zhang *et al.*, 1997). In recent years, PHAs have attracted much attention to researchers and engineers in the fields of chemical, environmental and polymeric applications. However, a realistically important problem associated with the industrial manufacture of PHAs is their high production cost. Since PHAs can be produced by a wide variety of bacteria in fermentation broths, *e.g.*, *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas*, recombinant *Escherichia coli* or *Haloferax mediterranei* (Ahn *et al.*, 2001; Fernandez-Castillo *et al.*, 1986; Garcia Lillo and Rodriguez-Valera, 1990; Lenz and Marchessault, 2005; Sudesh *et al.*, 2000; Tamer *et al.*, 1998), to choose less expansive substrates, to improve cultivation strategies and to use an economical downstream processing method are essential to reduce the production cost.

Because PHAs are always accumulated in the form of intracellular granules by a variety of bacteria, the bacterial cells should be disrupted in order to obtain pure PHAs. In general, the methods for purification of PHAs involve mechanically mediated cell lysis (*e.g.*, high-speed wet milling, high pressure homogenization, *etc*.) and extraction of PHAs with hot solvents (Hrabak, 1992). The mechanical procedures are often effective for cell disruption; they may compromise the structural integrity of released products (Resch *et al.*, 1998). Chemical procedures that break down envelops include solubilization of cells with detergents or lysozyme/EDTA in combination with osmotic shock procedures. However, some chemical methods have dangerous, toxic and environmental pollution problems due to the use of large amount of organic solvents. In the past, the production of PHAs by *H. mediterranei* was really rare. An organic solvent such as chloroform was generally used to disrupt the *H. mediterranei* cells and simultaneously to dissolve PHAs polymers in few previous studies (Fernandez-Castillo *et al.*, 1986; Garcia Lillo and Rodriguez-Valera, 1990). In order to avoid these disadvantages of chemical procedures, a simple me-

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chanical-physical method, ultrasonication, is tried to use for disruption of bacterial cells in this study.

Ultrasonication is an easily-installed and widely-used procedure for cell breakage in bio-chemical processes, though often on a laboratory scale. The efficiency of the operation is markedly dependent on the ultrasonic energy source and operation time. Although this method has been frequently used for purification of bio-products in a fermentation tank, the studies on the mechanism or efficiency of cell disruption still limit to empirical correlations among operating parameters. In the past, Doulah (1977) studied the mechanism of cell disruption in ultrasonication using the ultrasonic cavitation. He used Kolmogoroff 's theory of local isotropic turbulence to explain the mechanical energy dissipation in the cell suspension and proposed an empirical equation for the kinetic expression of protein release. Wase and Patel (1985) investigated the effect of cell volume on the cell disruption by ultrasonication using four kinds of bacterial cells. They found that the disruption constant (an empirical constant in their model) increased with mean cell volume. Feliu *et al.* (1998) studied the effects of acoustic power, cell volume, cell concentration and ionic strength on the release of *β-galactosidase* from *E. coli* by ultrasonication. They concluded that cell concentration and ionic strength had trivial effects on the *β-galactosidase* release. Recently, the disruption and protein release kinetics of *Acetobacter peroxydans* by ultrasonication was examined by Kapucu *et al.* (2000). They found that the extent of cell disruption increased with increasing acoustic power, while the disruption rate decreased as the pH of ultrasonic medium increased. The empirical constants in Doulah's equation under various operating conditions were also regressed and discussed.

In this article, the *Haloferax mediterranei* cells are disrupted and PHA is purified by ultrasonication and centrifugal separation. The purification results are observed by SEM. Based on the size-reduction mechanism, the disruption kinetics of *H. mediterranei* cells is studied and a theoretical model is derived to relate the cell survival fraction, acoustic power, size distribution index and operation time. The cell disruption fraction by ultrasonication and the optimum operating condition can then be determined by the proposed model with no parameter adjustment.

DISRUPTION KINETICS OF BACTERIAL CELLS

In this study, PHA is separated and purified from *H. mediterranei* cells by mechanical-physical procedures. The *H. mediterranei* cells are disrupted by ultrasonication, and the cell fragments and intracellular matters are clean up and separated off by a high-speed centrifuge. The disruption efficiency is strongly dependent on the ultrasonic strength (the power and frequency), the cell characteristics and the operation time (Feliu *et al.*, 1998; Kapucu *et al.*, 2000). However, the effects of cell concentration and ionic strength on the disruption can be neglected (Feliu *et al.*, 1998).

Some mechanical size reduction methods, *e.g.*, grinding or milling, have been widely used in many chemical industrial processes. Since the cell disruption by ultrasonication can be reasonably considered as a size reduction operation, the well-established model for size reduction may be applied to an ultrasonication process. Based on the previous theories (*e.g.*, Kick law, Rittinger law, *etc*.), the required energy for which an original size is reduced to a new small size (or a new surface area) can be estimated (Coulson and Richardson, 1993). The previous results showed that the energy efficiency in a size reduction operation is only around 0.1 to 2%. Most energy is wasted on the mechanical friction loss and heat generation. The relationship between the supplied energy and the size variation can be expressed as the following empirical equation (Coulson and Richardson, 1993):

$$\frac{\mathrm{d}E}{\mathrm{d}L_m} = -C \cdot L_m^r \,, \tag{1}$$

where E is the input energy, L_m is the mean particle diameter (or characteristic size), while C and r are empirical constants which is determined by the particle characteristics and the machine type. Equation (1) can be easily integrated to obtain the Rittinger law, that is,

$$E = -\frac{C}{r+1}(L_m^{r+1} - L_{m,o}^{r+1}) \,, \tag{2}$$

where $L_{m,o}$ is the original mean particle diameter. If r is put equal to –1, integration of Eq. (1) gives:

$$E = -C \cdot \ln \frac{L_m}{L_{m,o}} \,. \tag{3}$$

This equation is known as Kick's law (Coulson and Richardson, 1993). This indicates that the energy required is in proportional to the size reduction ratio. If the operation according to Kick's law, the curve in a semi-log plot of E vs. L_m is a straight line, and the empirical constants, C, can be given by the slope of the line. Since E is equal to the input power, P, times the operating time, t, Eq. (3) can be rewritten as:

$$P \times t = -C \cdot \ln \frac{L_m}{L_{m,o}} \,. \tag{4}$$

The relationship between L_m and t can be known from Eq. (4).

The size distribution of many particular sample produced by size reduction is often approximated by

the Rosin-Rammler distribution; its cumulative fraction of size L can be expressed as:

$$D(L) = 1 - \exp\left[-\left(\frac{L}{\alpha} \right)^n \right], \quad (5)$$

in which $D(L)$ is the cumulative fraction of particle size L, α is a characteristic size parameter for which $D(L)$=0.632, while n^{-1} is an index indicating the dispersion of particle size. If the size distribution according to Eq. (5), a plot of $-\ln[1-D(L)]$ vs. L on a log-log paper is linear. The value of n can then be given by the slope of the straight line, and α corresponds the size for which $-\ln[1-D(L)]=1$.

In a cell disruption operation, a cell can be considered to be disrupted when its characteristic size is smaller than the cell size. Since the PHA granules produced in cells are spherical or spheroidal in shape and the size of each granule generally ranges from 0.2 to 0.8 μm, the size of 0.8 μm is used to determine the achievement of cell disruption. Therefore, the disruption fraction of cells, S, can be defined as:

$$S \equiv \frac{D(L_{PHA}) - D_o(L_{PHA})}{1 - D_o(L_{PHA})}, \quad (6)$$

where $D_o(L_{PHA})$ is the original cumulative fraction of PHA size, L_{PHA}. If the index n in the size distribution function remains constant during a disruption operation, the following equation can be given by substituting Eq. (5) into Eq. (6):

$$1 - S = \exp\left[\left(\frac{L_{PHA}}{\alpha_o} \right)^n - \left(\frac{L_{PHA}}{\alpha} \right)^n \right], \quad (7)$$

in which $1-S$ can be considered as the survival fraction of cells. If the particle mean characteristic size is defined as the size for which the cumulative fraction is equal to 0.5, then the relationship between L_m and α can be obtained from the size distribution function, Eq. (5), that is,

$$L_m = \alpha \cdot \left[-\ln(0.5) \right]^{1/n} = \alpha \cdot 0.693^{1/n}. \quad (8)$$

Therefore, Eq. (7) can be rewritten as:

$$1 - S = \exp\left[0.693 (L_{PHA})^n \left(\frac{1}{L_{m,o}^n} - \frac{1}{L_m^n} \right) \right]. \quad (9)$$

Based on Eq. (4), the variation of L_m during a disruption operation can be described by

$$L_m^{-n} = L_{m,o}^{-n} \cdot \exp\left(\frac{nPt}{C} \right). \quad (10)$$

Therefore, Eq. (9) can be rewritten as:

$$1 - S = \exp\left[0.693 \left(\frac{L_{PHA}}{L_{m,o}} \right)^n \cdot \left(1 - \exp\left(\frac{nPt}{C} \right) \right) \right]. \quad (11)$$

To get a simple model, the exponential function can be expanded using Taylor series, that is,

$$\exp(x) = 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \ldots + \frac{x^i}{i!} + \ldots . \quad (12)$$

Therefore, Eq. (11) can be rewritten in the following form if the higher order terms in the series are omitted:

$$1 - S = \exp\left[-0.693 \left(\frac{L_{PHA}}{L_{m,o}} \right)^n \cdot \frac{nPt}{C} \right] \quad (13)$$

or

$$1 - S = \exp(-K \cdot t), \quad (14)$$

where K is an empirical constant which is a function of cell size distribution and the input power in ultrasonication. This formula is the same as the empirical equation of Wase and Patel (1985) and is similar to those in Doulah (1977) and Kapucu *et al.* (2000).

MATERIALS AND EXPERIMENTS

Haloferax mediterranei is a kind of haliphilic bacteria which can produce PHA in a hyper-saline condition. The fermentation broth of *H. mediterranei*, a kind gift from Professor C. Will Chen at the department of bioengineering at Tatung University in Taiwan, was prepared by a bacterial fermentation in a 5 L jar using rice bran and corn starch (1: 8) as carbon source. After 3 days of fermentation, the broth was removed and stirred in a freezer at –20°C . The refrigerated crude product was warmed for 50 min using a water bath at a room temperature before experiments. The solution was mixed well by a magnetic stirrer. 25 mL of the solution was sampled and put into a test tube with 200 mL distilled water. The test tube was then centrifuged by a centrifuge manufactured by *Hermle-Labortecnik* (#Z200A) in Germany in order to separate impurities. Repeat the centrifugal operation for three times. The sampling solution was then diluted with distilled water to 300 mL and was put in a 6 cm-inner-diameter cylindrical container for the next experiments.

The bacterial cells in the prepared solutions were broken by a *Sonics & Materials* (VCX750) high intensity ultrasonic processor for different operation times. A 19 mm probe of the processor was put at the center of the solution prepared previously, and the amplitude and the output power was kept at 20 kHz and 525 W, respectively. The temperature of the solution was kept at 25°C during ultrasonication using the thermostatic jacket of the container. After each experiment, the solution was sampled to measure the size distribution of particles in the solutions using a *Horiba LA*-910 particle sizer. The residual

solution was put on a filter paper and air-dried naturally. A small part of the dry sample was observed and photoed by a *LEO*1530 Scanning Electronic Microscope.

In order to measure the disruption faction of *H. mediterranei* cells, a set of ultrasonication experiments were carried out the same as mentioned before. However, when the ultrasonication is terminated, the residual solution was dilute and clean-up by adding distilled water. The cell fragments and the undisrupted cells were separated out by a centrifuge since their densities were lighter than PHA granules. The product was dissolved in chloroform after clean-up, filtered and then dried under vacuum. The final dry product was weighed to obtain the PHA mass from cell disruption. The structure of the obtained PHA was analyzed by NMR and found to be a poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV) copolymer.

RESULTS AND DISCUSSION

Figure 1 shows the SEM image of the original *Haloferax mediterranei* sample under a zoom of 10,000. Most original samples are observed in agglomerates due to the drying step before SEM measurement, and some impurities (mostly salts) exist in the chinks among agglomerates. A few PHA granules are also observed in this photo. This implies that a little amount of PHA has been released during the centrifugal and sampling procedures. This result can be indicated by the size measurement and taken into consideration in the calculation of cell disruption fraction.

The SEM image of *H. mediterranei* sample after 30-min ultrasonication is shown in Fig. 2 using the same zoom as in Fig. 1. The amount and size of cell agglomerates have been markedly decreased after 30 min ultrasonication. Compared with Fig. 1, the fraction of roundness PHA granules is much more than the original state. This means the ultrasonication is efficient for the cell disruption of *H. mediterranei*.

Figure 3 shows the SEM image of PHA after 60-min ultrasonication and centrifugal clean-up. This photo shows distinctly the shape and size of PHA granules. Most sizes of PHA granules range from 0.2 to 0.8 μm. This photo also demonstrates that the undisrupted cells and cell debris can be almost separated out by the mechanical-physical procedures and then pure PHA can be obtained.

Figure 4 shows the size frequency distributions of *H. mediterranei* sample at different ultrasonication times. The experiments have been carried out repeatedly in some typical conditions, and the deviations are less than 5%. In this figure, all of undisrupted cells, PHA granules and cell debris are taken into account. It can be seen that the original sample has a wider and larger size distribution. The size distribution of *H. mediterranei* sample decreases with time and becomes narrower during the ultrasonication. This indicates that the *H. mediterranei* cells are disrupted by ultrasonication operation. The mean sizes of *H. mediterranei* cells at different ultrasonication times can be calculated from the size distributions shown in Fig. 4. The ultrasonic energy in ultrasonication is plotted against the dimensionless mean

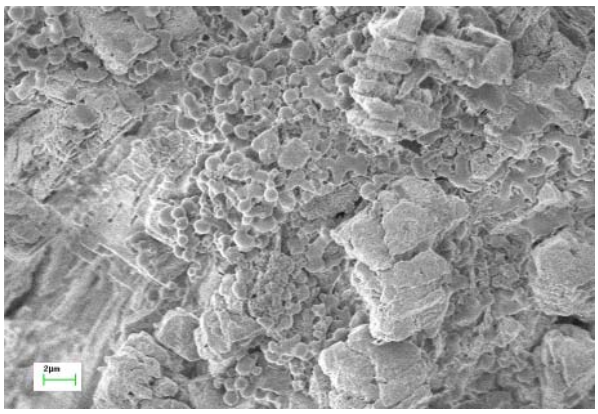

Fig. 1. SEM image of original *Haloferax mediterranei* sample. (×10,000).

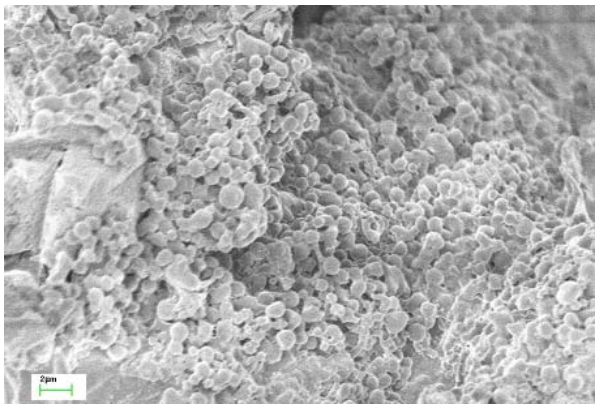

Fig. 2. SEM image of *Haloferax mediterranei* sample after 30-min ultrasonication (×10,000).

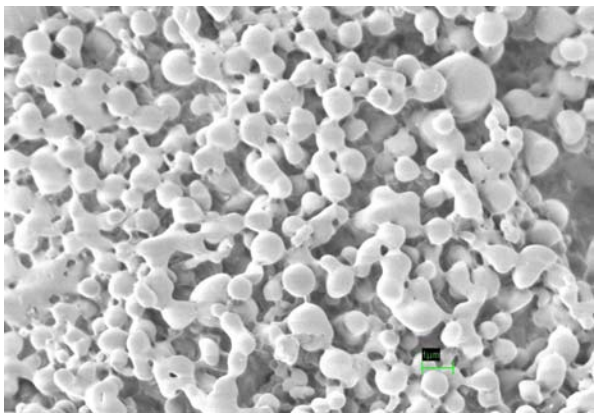

Fig. 3. SEM image of PHA granules after 60-min ultrasonication and centrifugal clean up (×20,000).

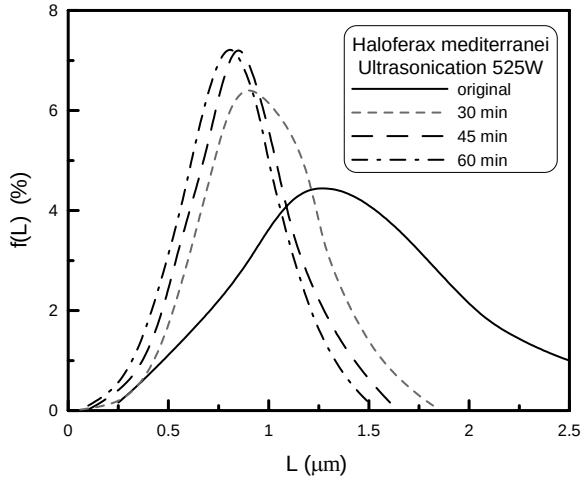

Fig. 4. The size distributions of *Haloferax mediterranei* sample at different ultrasonication times.

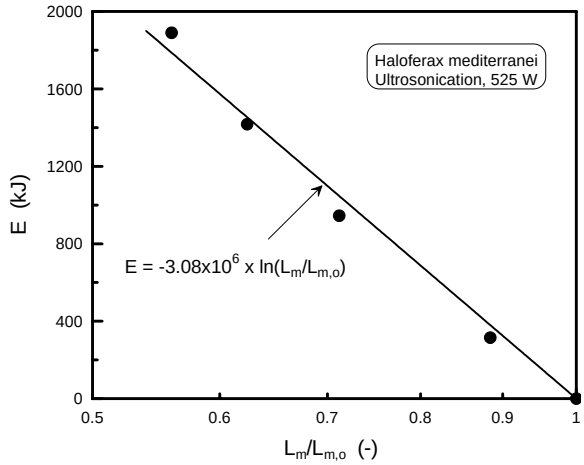

Fig. 5. The relationship between acoustic power of ultrasonication and the mean size of *Haloferax mediterranei* sample.

size of *H. mediterranei* sample in a semi-log figure, Fig. 5. The experimental data can be regressed as a straight line in this figure. This means the Kick's size reduction model, Eq. (3), can be used to describe the size variation during an ultrasonication operation. The relationship between required energy and the natural logarithm of size reduction ratio was linear. The empirical constant, *C*, can be obtained from the slope of the line.

Figure 6 shows the variation of the mean diameter of *H. mediterranei* sample with operation time. The mean diameter decreases with time be cause of the ultrasonication disruption of cells and the decreasing rate is quicker at the early period. This trend is similar to most size reduction operations (Coulson and Richardson, 1993). Furthermore, this figure shows that the plot of $\ln(L_m/L_{m,o})$ against time is a straight line. This means that the size reduction model, Eq. (4), can be used to describe the variation of mean size during an ultrasonication operation.

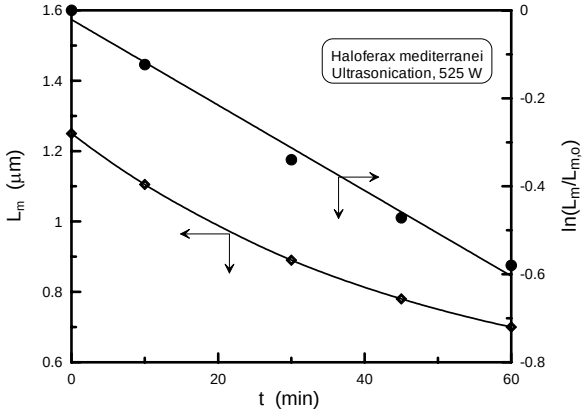

Fig. 6. The mean size of *Haloferax mediterranei* sample during ultrasonication.

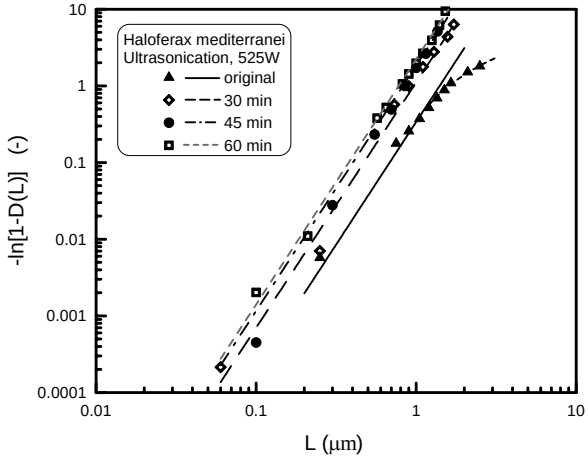

Fig. 7. A plot of $-\ln[1-D(L)]$ vs. L at different ultrasonication times.

In order to understand the size distribution function of *H. mediterranei* cells during an ultrasonication operation, the values of $-\ln[1-D(L)]$ are plotted against L at different times in Fig. 7. The size distributions can be modeled as Rosin-Rammler function, Eq. (5), since the plots are straight lines. The size distribution index, n, can then be obtained from the slope of the straight line, and the characteristic size, α, at different times can be given accordingly. An interesting phenomenon is that the straight lines in Fig. 7 are almost parallel to each other. The values of n at different times are therefore the same, *i.e.*, n=3.2. However, the larger particles in the original sample have slight deviations from the Rosin-Rammler function. This fact has been taken into consideration in calculation of the mean size of original sample, while the mean size after ultrasonication can be estimated theoretically by the Rosin-Rammler function.

Figure 8 shows the survival fraction of *H. mediterranei* cells during an ultrasonication using an acoustic power of 525 W. The solid symbols represent experimental data, while the curves are obtained from model calculations. The solid curve represents

the calculated results using the theoretical model proposed in this study, Eq. (11). It can be observed that the survival fraction decreases with time. This means more and more cells are disrupted during the ultrasonication. Although the calculated results are always larger than those experimental data, the deviations are less than 30%. Because the largest size of PHAs is 0.8 μm observed in Fig. 3, it is reasonable to ensure that the *H. mediterranei* cells are disrupted if the measured size is smaller than the characteristic size of 0.8 μm. A possible reason for the inaccuracy shown in Fig. 8 is because of the choice of the characteristic size. However, this model provides a theoretical method to predict the cell disruption fraction by ultrasonications under various operating conditions with no parameter adjustment. Furthermore, if the simplified model, Eq. (14), is employed, the deviation becomes as large as 78%. However, the calculated accuracy can be improved if more higher-order terms in Taylor series (*i* increase) are used.

Since the calculated accuracy of the proposed model has been demonstrated in Fig. 8, the effect of acoustic power on the cell disruption can be comprehended through this model. The relationship between the supplied energy and the size variation have been well discussed in many literatures, *e.g.*, Doulah (1977), Feliu *et al.* (1998), Kapucu *et al.* (2000), *etc*., the effects of power input and operation time in ultrasonication can be well understood though only one acoustic power is used in experiments. Figure 9 shows the calculated results of survival fractions of *H. mediterranei* cells during ultrasonications using different acoustic powers. The parameters used for model calculation are listed in Table 1. The curve shapes in this figure are somewhat hyperbolic, especially under a higher acoustic power; this trend is similar to those experimental results of Kapucu *et al.* (2000). Because the curves are hyperbolic, the cell disruption rate (the slope of the tangent line) becomes quicker and then slower during ultrasonication. This is possible due to different mechanisms of cell disruption, *e.g.*, cavitation, shear force, *etc.*, during the operations or to the characteristics of ultrasonic energy conversion into mechanical energy. This is much worthy of further study. Moreover, it can be seen in this figure that an increase in acoustic power or operation time leads to lower survival fraction of cells. This is because the mechanical energy converted from ultrasonic energy increases with the increase of acoustic power and operation time. When acoustic power increases from 100 to 800 W, the survival fraction decreases from 0.93 to 0.04 in a period of 1 h. Use of this theoretical model, only a set of size measurement after ultrasonication should be carried out; one can choose the optimum acoustic power and operation time to attain a specific cell disruption fraction.

Table 1. The parameters of *Haloferax mediterranei* ultrasonication used to calculate the disruption ratio in Fig. 9.

C (J)	L_{PHA} (μm)	n (-)	$L_{m,o}$ (μm)
3.08×10^6	0.8	3.2	1.23

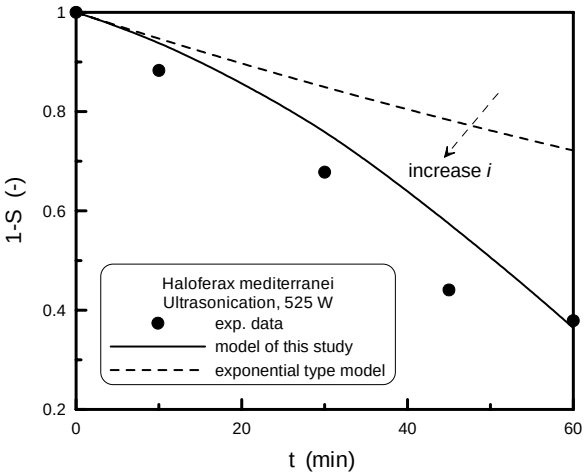

Fig. 8. The survival ratios of *Haloferax mediterranei* cells during ultrasonication.

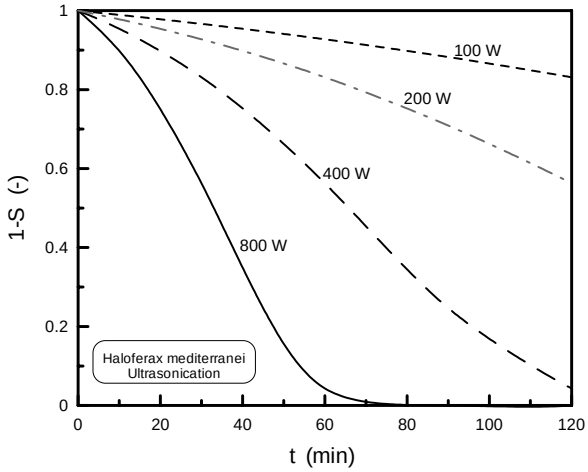

Fig. 9. Simulated survival ratios of *Haloferax mediterranei* cells during ultrasonications using various acoustic powers.

CONCLUSION

Haloferax mediterranei cells were disrupted by ultrasonication in order to purify intracellular PHA. The SEM images showed that the size of PHA granules ranged from 0.2 to 0.8 μm and the ultrasonication was an efficient mode for the cell disruption of *H. mediterranei*. The Kick's size reduction law could be used to describe the mean size variation during an ultrasonication. The relationship between required energy and the natural logarithm of size reduction ratio was linear. A theoretical model has been derived to relate the cell survival fraction, acoustic power,

size distribution index, and operation time. This model provided a theoretical method to predict the cell disruption fraction by ultrasonication under various operating conditions with no parameter adjustment. An increase in acoustic power or operation time led to lower survival fraction of cells. Although the calculated results were always larger than experimental data, but the deviations were less than 30%. Use of the theoretical model in this study, one could choose the optimum acoustic power and operation time to attain a specific cell disruption fraction. For example, when acoustic power increased from 100 to 800 W, the survival fraction decreased from 0.93 to 0.04 in a period of 1 h.

ACKNOWLEDGEMENT

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NOMENCLATURE

C	an empirical constant defined in Eq.(1), J
$D(L)$	cumulative fraction of particles
E	the energy for size reduction, J
$f(L)$	frequency distribution function of particles, %
K	an empirical constant defined in Eq. (14)
L	particle diameter, m
n	an size distribution index defined in Eq. (5)
P	power, W
r	an empirical constant defined in Eq. (1)
S	disruption fraction of cells
t	operation time, s

Greek symbol

α	a characteristic size parameter defined in Eq. (5), m

Subscripts

o	original state
m	mean value

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於純化聚羥基烷酯時使用超音波破碎細菌細胞之動力學

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摘　要

聚羥基烷酯（PHA）是嗜塩菌之細胞內產物。爲了獲得 PHA，可以將嗜塩菌之細胞以超音波破碎，再以離心分離純化 PHA。純化的結果以 SEM 進行觀察，發現多數 PHA 微粒的尺寸介於 0.2 至 0.8 微米之間。SEM 的影像亦証明了超音波可以將嗜塩菌細胞有效地破碎。Kick 的減積理論被用來描述在超音波破碎的過程中樣品平均尺寸的變化，所需的能量與減積比率成正比。本研究並基於所提出的減積機制來探討嗜塩菌細胞之破碎動力學，並且推導一理論模式來關聯細胞殘存分率、音波功率、粒徑分布指標與操作時間之間的關係。這個模式在沒有調整參數的情況下，可以提供一理論方式來預測各操作條件下之細胞破碎分率。結果顯示：提高音波功率或操作時間皆會降低細胞的殘存分率。使用這個理論模式，吾人可以選擇最適的音波功率與操作時間來達到特定的細胞破碎分率。