THE RESEARCH OF LOW-ULTRASONIC ENERGY AFFECTS TO YEAST GROWTHIN FERMENTATION PROCESS

Jomdecha, C., Prateepasen, A.
Department of Production Engineering
King Mongkut's University of Technology Thonburi
Bangkok, Thailand, 10140

Abstract

This paper presents a study of low-ultrasonic energy that can be affected to increase yeast growth. Forces of the ultrasonic stimulation in the yeast media broth, a liquid nutrient medium for the culture of yeast, produce change of liquid densities and surrounding pressure, which affect to the yeast growth. A utilized ultrasonic system including transducer of 20 kHz was varied powers and applied into the 200 ml. starter broth including *Saccharomyces cerevisiae* yeast. Those powers can be adjusted intensities, repetition times and durations to stimulate yeast cell. For monitoring the yeast growth, a cell direct-count with a Hemacytometer and an optical density using a Spectrophotometer were employed every 1 hour. Experiment results were shown enhancements of the yeast growth which were stimulated by the various ultrasonic powers compared with commonly used methods. Consequently, the ultrasonic can be applied to accelerate the yeast growth in fermentation to increase productivity and decrease the fermentation time.

Keywords: Ultrasonic, stimulation, yeast growth, fermentation

1. Introduction

An ultrasonic, 20 kHz to above 25 MHz is widely used to apply in many applications. In general, there are two points to approach the ultrasonic based on its applications. First, the use of ultrasonic equipment to generate, receive or measure sound wave in medium then, study its behavior or character. Second, a study of the ultrasonic wave energy affect to an irradiation medium from the generated ultrasonic equipments. In addition, powers of the ultrasonic waves can be divided to two steps; there is high and low power. Some applications of high power ultrasonic are welding, cleaning, and chemical or biological processing, which are approached by generated ultrasonic wave effecting the materials or mediums. On the other hand, the low ultrasonic power used for material testing, measuring and communication are considered the behavior or character of the received ultrasonic waves.

For Non-Destructive Testing (NDT) in liquids, the low ultrasonic power is inclusive utilized to investigate the change in liquid by the sound wave analysis such as sound velocity and attenuation. In the recent year, the ultrasonic for liquid characterization is a becoming technique to apply in a biotechnology. Especially in food and biotechnology fields, the advantages of ultrasonic characterization are rapid measuring and non-invasive technique to the products. Many researches had complete results to character or measuring their liquid properties. For example, [1, Savazyan, 1991] The research was reviewed and concluded that the technique can be used to determine the hydration of biological substances and the state of water in hydration shell, molecular transitions and interactions, thermodynamics and the state of biological substances in solutions. The expectation of this review have been agreed that the ultrasonic velocity technique raised by the increased numbers of publications. [2, Self et al., 1992] Measurements of velocity and attenuation have been made in suspensions of carrot cells from the taproot cortex. The velocity showed the frequency difference slightly between one and six megahertz. [3, Resa and Elvira] The research was shown the transform of fermentation process from carbohydrate to ethanol and carbon dioxide by the change of ultrasonic velocities. The important ultrasonic properties to measure material changes are velocity, reflection wave, and attenuation. Therefore, in several fermentation products, changes of liquid phase properties can be comprehensive measured by measuring character of an ultrasonic velocity and attenuation.

Conversely, effects from the ultrasonic powers besides can influence to the liquids property. Since the high power ultrasonic was widely used in many processing applications, some mechanical and

chemical effects are induced from generated ultrasonic in liquid such as, cavitations, irradiation forces, free radicals or streaming effects. In particular of cavitations, they are induced great amount of micro-bubbles from various sites around a transducer due to rarefaction phase of ultrasound. Then, collapse in a compression phase and release the energies through the liquid. In many chemical and biology fields, cavitations become to desire for sonochemistry processing and cell disruption. There are many researches provide the details statement of the affect of high power ultrasound in liquid or matters in liquid. [4, Laborde et al., 1998] The mathematical modeling has been presented to predict acoustic cavitation fields by computing the acoustic equations and fluid dynamics equations. Calculation of pressure fields were obtained in case of cylindrical sonoreactor at 28 and 500 kHz. The comparison with experimental observation and measurement showed good agreement with both theory and calculations. [5, Stack et al., 2005] The ultrasound intensity duration and other important factors such as pH, conductivity and temperature were studied the ability to coagulate grease from waste water. The propose process was capable of removing up to 100% of grease from studying waste water. [6, Parag group et al., 2005] The current status of the hydrodynamic cavitation reactors has been reviewed discussing of the bubble dynamics analysis, optimum design considerations, design correlations for cavitational intensity and different successful of chemical synthesis applications. The assessment of the hydrodynamic cavitation reactors and comparison with the sonochemical has been done by citing the different industrially important reaction.

In biotechnology, both of high and low ultrasonic powers are applied to the products of fermentation in several applications. It is a point of view to categorize the research of the ultrasonic effects into three groups.

First, the high power of ultrasonic are used to destroy the microorganisms to get the product inside them such as enzyme or protein. Several researches play into the different technique to use of the ultrasonic powers, disruption techniques and filtering the products off the dead cell. [7, Cocito, 1995] The aromatic compounds of must and wine were extracted with a new rapid using ultrasound by showing good recovery, linearity and reproducibility for most of the compounds, together with rapidity and simplicity better than those resin extraction method. [8, Limaye et al,. 1996] Microorganism of *S. cerevisiae* and *E. coli* were removed from small batch and flow system in a 5 ml cylindrical tube containing a 3 MHz standing wave ultrasound. The cell concentration dependence *S. cerevisiae* recovery reached to 97% in a 5.5 min and E. coli was 72% in 11 min. [9, Pramodkumar, 2003] Cell disruption of *S. cerevisiae* was carried out with ultra-sonication for isolation of alpha glucosidase in a leached solution. The result was showed the exercise of an optimization effect to get maximize and selective adsorption of alpha glucosidase.

Second, various ultrasonic power are utilized to inactivate the microorganisms for food, beverage or liquid purification by propagation and standing wave technique. [10-11, Tsukamoto et al., 2004] The inactivations using ultrasonic comparing with clorination technique have been investigated. For an ultrasonic, a horn type sonicator with 27.5 kHz, the change in the inactivation showed the behavior in pseudo first-order. The constant rate was as a function of the change of the amplitude on the vibration face. The 90% inactivation of the yeast cells was achieved by ultrasonic for 60 min. Since, the *S. cerevisiae* inactivation by ultrasonic can be inactivated almost like a bactericidal effect (a quantitative change in cell number) in the beginning of sonication. For longer sonication, the quantitative damage of the cell growth activity (bacteriostatic effect) was increased together with bactericidal effect. However, the chlorination using sodium hypochlorite solution (NaOCl) can inactivate only bacteriostatic effect. In addition, an immobilization for stop moving cell or filtering cell in the biotechnology process is another way of research. [12, Gherardini et al., 2004] A cell immobilization technique using ultrasonic has been shown the manipulation and positioning of cells/particles within various nontoxic gel matrices. The specific result showed the viability of cell in ultrasonic standing wave up to 4 day after treatment.

Third, the effects from ultrasonic to the microorganism are studied by some researches, and give details of the reasons and results from ultrasonic effects. [13, Radel, 2000] Viability of yeast cells subjected to ultrasonic standing wave field in acoustic cell retention system was studied by varying the pressure amplitude of ultrasonic. The exposure ultrasonic of slightly above 2 MHz both of propagating and standing wave was utilized in the research. The results was showed that there is no loss of cell viability, but have changed in some morphology by the different types of suspension and ultrasonic treatment. [14, Guerrero, 2001] The resistance of *S. cerevisiae* to 20 kHz ultrasonic

stimulation with different temperature and pH was analyzed. The consequence showed the resistance of cell decrease as ultrasonic amplitude increase. Furthermore, the effects of low ultrasonic to products of fermentation were reported in some researches group. [15, Chang, 2004] The rice alcohol beverage and maize wine maturation were accelerated by different ultrasonic powers and frequencies. The effects of ultrasonic treatment can be shown the good influenced to the rice alcohol aging, and improved the matured maize wine more quickly than standard maturation.

From many review literatures, the effects of ultrasonic to microorganisms or products of fermentation have been reported in individually application or research. In general, the quantity of fermentation products is directly depended on the speed of fermented time. By activated microorganisms as a starter fills into a bioreactor to make the activity with the fermented broth for fermentation acceleration. Therefore, the growth of microorganisms is an importance function in fermentation process to increase the fermented products. The starter to fill in bioreactor is required to accelerate growth. Now a day, the employed techniques to culture microorganism for fermentation starter are shaking by lateral and orbital techniques. The microorganism growth is directly related to speed of shaker. However, cells in finally shaking are dead more than growth when they are shaking in the high speed [16]. The growth of cell is reduced then, the quantity of prepared starter is not enough to fill in the bioreactor. In addition, the time to culture starter is required to 24-48 hours depended on the strain of microorganisms, fermentation media broth and techniques to acceleration.

This paper deals with the investigation of yeast growth effected from low-energy ultrasonic technique. A concept idea of a research was stated that the ultrasonic energy transmitted in to liquid possibly affects to accelerate the fermentation process. However, the effects of low ultrasonic energy to the microorganism growths are still indistinct. Hence, the expose to ultrasonic effects to yeast growth are main propose of the research. The valuable of fast growth rate of yeast will be increased the product of the fermentation such as ethanol or alcohol for energy problems solving in the present and the future. By using various powers of 20 kHz ultrasonic transducer to stimulate the fermentation broth included baker's yeast cell strain *S. cerevisiae* were setup the experiment. The change of growth rate was investigated then, the results were analyzed and discussed theory of ultrasonic properties and its characters affect to yeast growth.

2. Theory approach

Acoustic pressure, intensity and radiation force of ultrasonic [17, 18] are the important factors which are utilized to stimulate the yeast growth in this study.

The acoustic pressure, p, is the pressure deviation from the local ambient pressure caused by a sound wave. The compression and rarefaction phases can be described the positive and negative pressure like sinusoidal wave. In the sound wave, the complementary variable to sound pressure is the acoustic particle velocity (v). The local instantaneous sound intensity is the product of the sound pressure and the acoustic particle velocity.

The intensity, I, is an essentially quantity of the traveling plan wave in liquid and can be defined mathematically as

$$I = \frac{1}{T}\int_0^T p(t)\cdot v(t)dt \qquad (1)$$

As the sound power P per unit area A, is the usual context measurement of sound intensity. Therefore, a spherical sound source in liquid, the intensity as a function of distance r is

$$I = \frac{P}{A} = \frac{P}{4pr^2}, \qquad (2)$$

where the intensity decreasing by $I \propto \frac{1}{r^2}$. Subsequently, the sound energy density E is an adequate measure to describe the sound field at a given point as a sound energy value. The sound density for an even-proceeding sound wave is

$$E = \frac{I}{v_C}, \qquad (3)$$

where v_C is the sound speed in m/s. The sound energy density is given in W.s/m^3. In speaking of average energy density E_A, it is necessary to distinguish between the space average at a given instant and the time average at a given point. From the theory of radiation force for plane, the

formula given of its quantity from planar wave fields is

$$F_R = \frac{v_G}{v_C} E \cdot A, \quad (4)$$

where v_G is the group velocity. In this paper, the approached irradiation force is defined by integral of the average energy density in average of time T as

$$F_R = \frac{1}{T}\int_0^T E_A dt, \quad (5)$$

due to the utilized stimulation by pulse technique. Hence, the following source parameters were varied to study the effects to yeast growth.

It is known that the mainly growth of yeasts depends on the media composition, the initial levels of pH, temperature and the airflow rate by shaking or dissolved oxygen [16]. However, in this research, the estimation of the effects of ultrasonic on yeast growth has been studied and practiced. The optimal constant growths or the specific growth rate with nutrient absorption in the culture media were explored the growth kinetic using an apply of monod's model.

The Monod's model [19] is often used to quantify and predict biomass growth. The model can be utilized to describe the growth of simple microbial organisms because their growth rate is limited by the concentration of a growth-limiting nutrient. In the case of yeast, the growth limiting nutrient is most likely sugar. The specific growth rate of yeast cells is proportional to the sugar concentration. This type of growth pattern is described by a mathematical model known as the Monod equation

$$m = \frac{m_m S}{K_S + S} \quad (6)$$

It was empirically derived from experimental data that followed the curve of the graph shown in Figure 1. The constants m_m and K_S depend on the conditions of the system itself. There are the organisms, the growth-limiting nutrient, the fermentation medium and environmental factors such as pH and temperature. m_m is the maximum specific growth rate achievable, when the concentration of the growth-limiting nutrient is not limiting. K_S is known as the Monod constant, and it is the concentration of the growth-limiting nutrient at which the specific growth rate is half the maximum value.

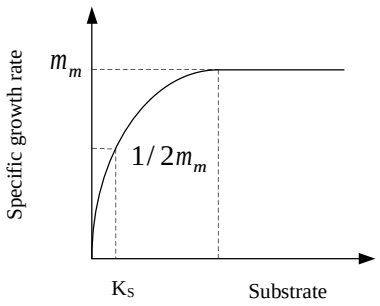

Figure 1. Growth curve following monod's equation

3. Materials and methods

3.1 Materials

In the experiment, The Fermipan baker's yeast strain *S. cerevisiae* was used to culture in the YM media, a kind of liquid nutrient, which included 0.3% yeast extract, 0.3% malt extract, 1% glucose and 0.1% peptone [20].

S. cerevisiae was inoculated in 200 ml YM medium for making a starter in the experiment. Then, they were activated and cultured at 30 degree C and 100 rpm orbital shaking [20]. After 24 hours cultured, 1 ml of about 5×10^7 ml^{-1} yeast cells were collected to employ into prepared Erlenmeyer flasks of 200 ml YM medium for the experiment.

3.2 Methods

The selected ultrasonic powers from tunable power system were employed to stimulate the yeast cell comparing with general culture by non-shaking and 100 rpm orbital shaking. The total intensity in an equation (1) was applied to stimulate yeast cells by decision on the amplitude and duty cycle of generated ultrasonic wave. The chose intensity should not be made the cavitation or heat occurrences. Then, the equation (2) was applied to calculate the power of the sample, which are 0.2, 0.4 and 0.8 W/cm^2 at the transducer/liquid interface.

An employed ultrasonic system was integrated with a generator to supply continuous voltage, an amplifier to gain the amplitude of electrical waveform to a transducer, and the transducer, which is a 20 kHz exponential horn-type, to generate the ultrasonic wave to the sample. The

output powers can be adjusted from 0 to 600 W. The generated pulse can be adjusted the on and off time to transducer. In the experiment, the transducer was 2 cm dipped into the cultured yeast cells in the flask for stimulation. The ultrasonic system of the experiment is demonstrated in Figure 2

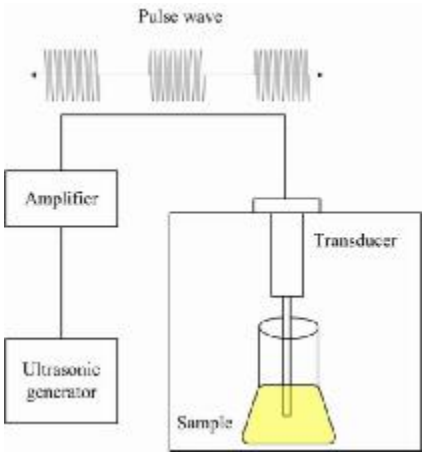

Figure 2. System of ultrasonic stimulation

For measuring growth of yeast, an optical density method using spectrophotometer was utilized to measure the absorbance of light. The optical density of a yeast culture is a measurement device to determine the amount of yeast cells presented in a liquid culture. Optical density value (OD value) is the amount of light that is able to pass through a liquid culture. The more cells in a culture involve the more density. The method of the spectrophotometer is shown as Figure 3

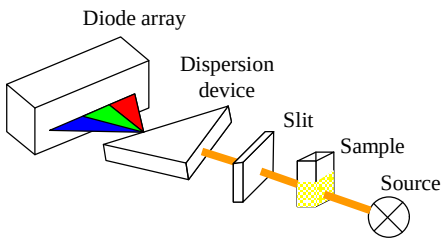

Figure 3. Optical density measuring technique

A sample to appropriate concentrations as needed was measured the absorbance of the sample with the spectrophotometer at 600 nm [20]. For the culture sample, a good OD value should yield linear relationship between the number of cells and the absorbance. However, the optical density is also a function of cell morphology such as size and shape, because the amount of transmitted or scattered light depends strongly on these factors. Therefore, to produce a calibration curve of the relation between the absorbance and cell amounts was examined by an equipment for counting the number of cells in a measured volume namely Hemacytometer. Consequently, the relation between OD values and cell amounts can be utilized to calibrate the growth curve of the yeast in the experiment.

4. Results and Discussions

4.1 Standard curves

Firstly, the standard curves of yeast growth were produced from non-shaking and 100 rpm rotational shaking fermentation. The *S. cerevisiae* baker's yeast was cultured in the flask containing 200 ml YM broth media at temperature of 30 degree C. Then, the OD and direct counting cells were utilized to measure the growth about every 1 hour for 7 hours culture. After that, the specific growth curves can be generated as Figure 4.

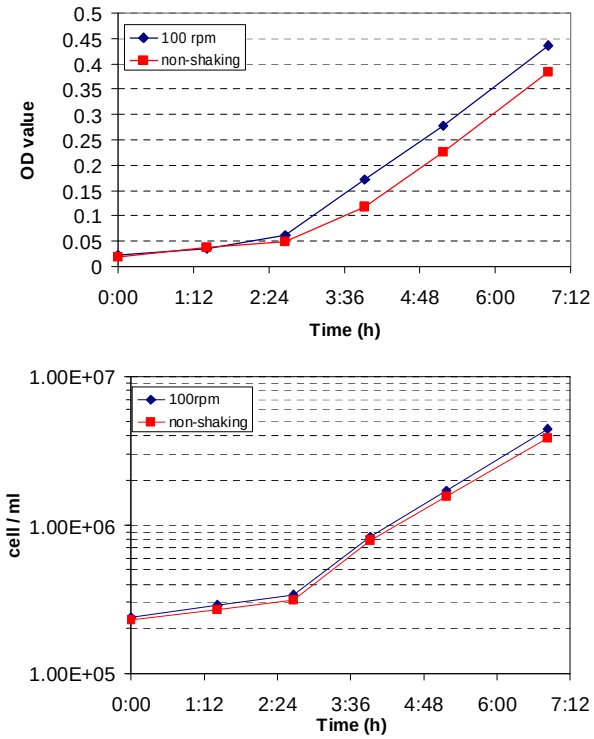

Figure 4. Standard growth curves of yeast from controls

Under this condition as mentioned above, the growth of yeast was shown the relation between the OD values and the number of cells. In the first 2-3 hours from the beginning, the growth was slow because the cells were adapted to the new

surrounding media. The volume of cell was swelling and the metabolism of cell was increased, but the proliferation of cell was slow. This phase was called lag phase. After lag phase, the growth was become to increase rapidly because cell can adapt to the surrounding media, and the substances can be passed through cell than in the lag phase. So, the growth of yeast was increase by budding cell in this phase called exponential phase. The budding cell cycle is the succession of events, whereby a cell grows and divides into two daughter cells that each contains the information and then, repeats the process. From the results of the standard curves, the linear relation between the cell numbers and the OD values was illustrated in Figure 5.

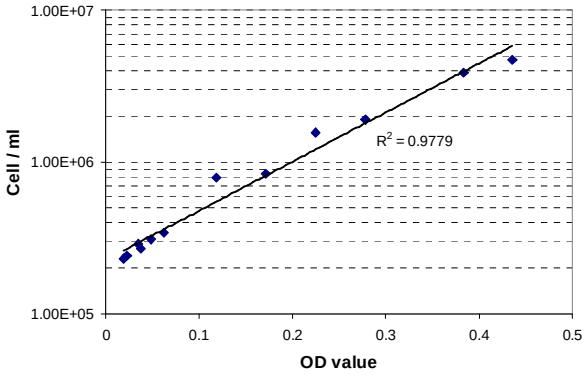

Figure 5. Relation between the OD value and the number of yeast cells

The result of $R^2 = 97.79\%$ was illustrated the percentage of total variation in the response of cell/ml to the factors of the OD value. The linearity and the accurate relation between the OD value measured by spectrophotometer and the cell number of direct counting cell were matched acceptably.

4.2 Ultrasonic stimulations

To deal with the general growth of yeast, an experiment to explore the ultrasonic effect to yeast growth, the low-ultrasonic powers were adjusted after calculation the power per unit area, which are 0.2, 0.4 and 0.8 W/cm^2 output powers at the transducer/liquid interface. The ultrasonic techniques were stimulated 15 minutes after the fill the 1 ml starter cells (3×10^7 cells ml^{-1}) into the flasks. The stimulation period was 10% duty cycle, 1 second pulse on and 9 seconds pulse off, and the duration of stimulation was 10 minutes. After stimulations, the sample flasks were not to be shaking and placed in the incubator controlled the temperature at 30 degree C. The samples were collected to measure the living cell by OD method and direct counting about every 1 hour for 7 hours to observe the change. The result of the OD value and cell numbers of different ultrasonic intensities comparing with standard curves were shown in the Figure 6

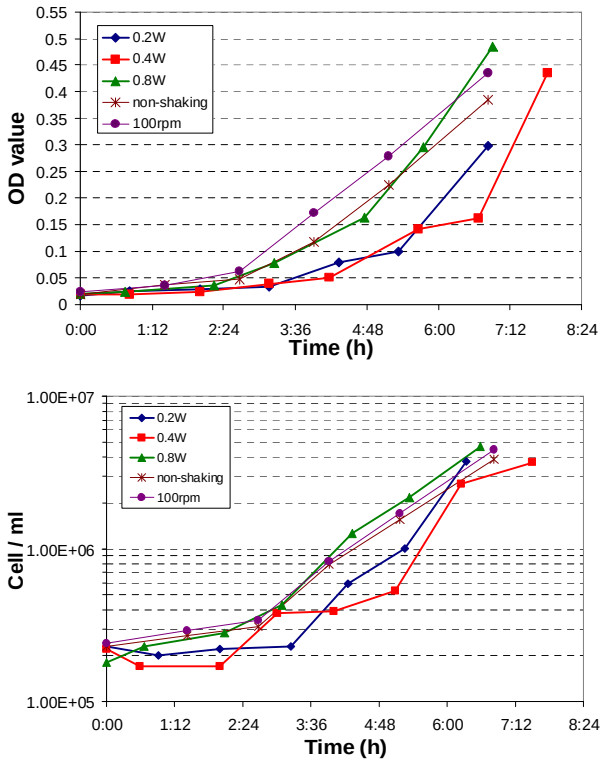

Figure 6. Yeast growth curves of ultrasonic stimulation comparing with the controls

After measuring data for 7 hours of ultrasonic treatments, the results of the OD values and the numbers of cell were illustrated the effects to yeast growth. At the first few hours, all of the growths from stimulated flasks in different powers were lower than those controls. The ultrasonic may restrained the cell growth at the lag phase then, the growth raised rapidly. Especially, the growth of power 0.8 W /cm^2 stimulation increased slightly upper than the non-shaking flask then, the final measurement result was higher than both of controls. Although the summary results of 0.2 and 0.4 W/cm^2 stimulations were low, but they had movement or trend to high comparing with the controls. Due to the stimulated cells sensitive to receive the substances and useful nutrients in the exponential phase, the growths were accelerated to increase high values. There is the result of nutrients and substances easily passed through the cells.

Because of the ultrasonic intensity as function of distance in the medium, the intensity distribution was decreased which related to $I \propto \frac{1}{r^2}$. The un-uniform distribution was occurred in this area due to near filed of sound propagation. As a consequence of low intensity ultrasonic, which no effect from cavitation produced in the medium, the growth results of ultrasonic stimulation give an impression to be a combination of many interaction effects such as compression/rarefaction, irradiation force and acoustic streaming. To discuss the results of those effects, the yeast cells in medium were disturbed the local pressure environment as result from the ultrasonic propagation. There are rapidly change of compressibility and rarefaction effects when the sound propagates through the medium. The medium including useful nutrients for yeast cell was also experienced directly those effects. At the same time, irradiation forces produced from sound propagation can be affected beside the structure of cell which is due to absorbed energy of the force. In addition, this force can be induced the acoustic streaming. These streaming flows were the result of absorption of energy in the bulk phase and energy loss at interface of transducer/liquid. Streaming at solid/liquid surfaces can transform mass transfer to the volume, and can induce non-invasive circulation in the flask including cells. Moreover, the cells were affected of vibration from the irradiation force and streaming flow which can dissolve oxygen in the medium and degas carbon dioxide from metabolism of yeast cell.

To confirm the results of ultrasonic stimulation affect to the yeast growth, the specific growth rate (m) of yeast in equation (6) was used to find out the numbers of budding cell (n). The budding yeast from one cell to two cells or 2^n is a cycle of yeast growth and can be calculated by equation (7) and (8) as

$$N_t = N_0 \times 2^n, \tag{7}$$

$$m = \frac{n}{t} \tag{8}$$

where N_t = number of total cells.
N_0 = number of initial cells.
t = culture time.

The results of calculation can be shown that the growth rate from the stimulation flask with 0.2 and 0.8 W/cm^2 was higher than the controls. Subsequently, the generation time or doubling time of budding yeast cell (g) of 2^n were related the growth rate constant by $g = 1/m$. In Table 1, the growth rate and the generation time of yeast in sample flasks were proved the affected results of the low ultrasonic stimulation.

Table 1. growth rate and doubling time of yeast

Condition	Growth rate	Doubling time
0.2 W / cm^2	0.627	96 min
0.4 W / cm^2	0.526	114 min
0.8 W / cm^2	0.652	92 min
100 rpm	0.617	98 min
Non-shaking	0.586	103 min

The treatment by 10 minutes ultrasonic stimulation with 0.2 and 0.8 W / cm^2 were confirmed the results that have higher growth than both controls. However, the result of stimulation of 0.4 W/cm^2 was poor. The yeast cell in this condition may not be appropriated or surrounding effects may affect the yeast growth. However, it may be grown higher if it is increased the culture time. Consequently, the growth of yeast may not to be convinced to relate with the ultrasonic power. The optimal stimulation condition and the relation between ultrasonic and yeast growth are still unclear and should be studied in the future.

5. Conclusion

The ultrasonic stimulation that affects to *S. cerevisiae* yeast growth was studied at the surrounding temperature of 30 degree C. The growth curves were generated by the cell direct-count and an optical density measurement. The results were proved the statement, and shown the different growths from ultrasonic stimulations at intensities of 0.2, 0.4 and 0.8 W/cm^2. Their growth curves were low at the beginning, but trended to higher at the end of measurement than the controls by 100 rpm shaking and non-shaking. In particular, the growth of 0.8 W /cm^2 stimulation raises slightly advanced than the controls. Afterward, the low-ultrasonic effects in liquid including compression/rarefaction, irradiation force and acoustic streaming were discussed to explore the consequences of affecting to the yeast growth.

6. Future works

The growth of yeast may not to be influenced to relate with the ultrasonic powers or

other conditions. Therefore, the relation between ultrasonic and growth of yeast should be realized by the theories and mathematical model. The optimal stimulation condition, growth prediction and yeast activity during ultrasonic stimulation should study in the future.

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