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Molasses fermentation to ethanol by *Saccharomycescerevisiae*M30 using low ultrasonic frequency stimulation

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Abstract

The effect of low-frequency ultrasonic power (20-30 kHz) on ethanol production from molasses by *Saccharomycescerevisiae* M30 at 30-35°C was studied. Ultrasonic power was continuously supplied to the fermentation systems. Fermentation environment and process performance were evaluated during bioreactor operation. The bioreactor operation with supplying of 20-25 kHz of ultrasonic power could perform under normal pH (4.6-5.0) and liquid temperature (≤ 38°C), while at 30 kHz a high liquid temperature (≤ 40°C) wascreated. Thus, the ultrasonic power at 30 kHz affected to yeast activity in this bioreactor. Models of specific ethanol production rate (v) and specific ethanol production time (T) were used to evaluate the efficiency of ultrasonic power in enhancing ethanol production. The results confirmed that ultrasonic power could enhance the ethanol production by enhancing yeast activity. The optimum ultrasonic frequency to stimulate the fermentation system was achieved at 25 kHz. Under this condition, the maximum v and T were obtained at 1.55 and 1.9 g/g.h, respectively.

Keywords: ethanol production, low-power ultrasonic, specific ethanol production rate, specific ethanol production time, *Saccharomycescerevisiae* M30

1. Introduction

The advantages of ultrasonic characterization are rapid measurement and noninvasive techniques. In biotechnology, both high and low ultrasonic powers are applied to the products of fermentation in several applications (1-2). The stimulation of optimum low-intensity ultrasonic power may improve permeability membrane, speed upsubstance transfer, and promote cell growth and propagation(3, 4). Ultrasonic power is generally used to examine a change in liquid via sound wave analysis, such as sound velocity, attenuation, monitoring the kinetics of invertase hydrolysis and improvement processes (5-7). However, the research into the ultrasonic effect on microbial characteristics has been conducted by categorization into three main

groups. These three groups were destruction using high ultrasonic power, inactivity of microorganism for food or beverage using propagation and the standing wave technique, and enhancement of cell growth and activity of microorganisms using an optimum range of ultrasonic power (8-9). This study investigates how the ethanol production rate via yeast activity is affected by low-frequency (20-30 kHz) ultrasonic techniques using a mathematical model to identify the specific ethanol production time. The results can be used to identify the optimum ultrasonic power level to obtain the maximum yeast growth and ethanol production rate during a short time of fermentation.

2. Materials and methods

Molasses characteristics

Raw molasses was collected from molasses tanks of a sugar production plant in Thailand. Molasses is a thick, dark substance that is a by-product of cane sugar processing. The physical and chemical characteristics were analyzed according to the standard method (10), as summarized in Table 1. The quality of molasses depends on the maturity of the sugar cane, the amount of sugar extract and the method of extraction (11). Before ethanol fermentation, the molasses was prepared by diluting with distilled water to establish the final concentration of total sugar at 15 g/l.

Table 1. Molasses characteristics

Characteristics	Raw molasses	Molasses using in this study
pH	7.5±0.2	7.8±0.2
Total solid (g/l)	76.3 ± 2.5	2.1 ± 0.5
Moisture content (g/l)	133.0±18	97.8±28.0
Reducing sugar (g/l)	114.7±25	3.1±1.0
Total sugar (g/l)	740.6±40	15.0 ±1.4
Total nitrogen (g/l)	8.8±2.8	0.8±0.2

Microorganism

*S.cerevisiae*M30, which is capable to grow and ferment at and below 40°C, was used throughout this study. It was kindly provided by the Yeast laboratory, Department of Microbiology, Kasetsart University, Thailand. A starter culture was prepared by enrichment and sub-culturing on Yeast Peptone Dextrose (YPD)agar plates (pH 6.5) two times. Then, a loop of the starter culture was transferred to 100 ml of medium with 0.5% (v/v)yeast extract at pH 5.0 before being incubated at 37°C with shaking at 100 rpm for 24 hours. These tubes of the starter were stored in the refrigerator at 4°C. Each tube of the starter was inoculated in a 200 ml erlenmeyer flask of the molasses containingtotal sugar concentration of 15 g/l. These flasks were incubated at 37°C with shaking at 100 rpm for 24 h and used as the starterof ethanol fermentation.

Batch fermentation startup and operation

Batch fermentation experiments were carried out in two bioreactors. The molasses containing 15% (w/v) of total sugar solution was used as a carbon source for *S.cerevisiae*M30. Experiments were performed in a stainless steel tank with a total volume of 10 liters, using 8 liters of total liquid volume. One bioreactor

was operated without ultrasonic supply and used as the control sample. The other bioreactor was supplied with ultrasonic frequency in the range of 20-30 kHz from an ultrasonic transducer (NEC TOKIN., Co. Ltd). The experiments were initiated by transferring the starter culture to both reactors at a concentration of 5% (v/v) with a speed mixing of 1 rpm. The ultrasonic transducer was placed under the bottom of the bioreactors and the supplied ultrasonic power passed through the liquid inside the bioreactor, with a total liquid interface area of 550 cm^2. Ultrasonic power was fixed at 23.4-26.7 Watts or 0.045 W/cm^2 of the power transducer/liquid interface. The bioreactor was operated with continuous ultrasonic supply at 20, 25, and 30 kHz throughout the bioreactor operation. These bioreactors were operated for 48 hours under ambient temperatures (30-35°C). During fermentation processing, the fermentation performance was evaluated in terms of the ethanol production rate and the maximum growth rate of yeast. The environments of the fermentation systems were monitored via pH, liquid temperature, and ethanol concentration.

Environmental condition monitoring

The environments of the fermentation systems were monitored by measuring liquid pH and temperature. These values were measured in real time by pH and temperature portable meters with accuracy at ± 0.01 and ± 0.3°C, respectively. The determination of ethanol concentration was analyzed by using Shimadzu, Class-GC 14Bwith a flame ionization detector (FID) and Carbowax B-DA column.

Fermentation performance monitoring

The performance of ethanol fermentation was determined by evaluating the doubling time of *S. cerevisiae*M30 and specific ethanol production time. A concept of doubling time evaluation was incorporated in which the growth rate of *S. cerevisiae*M30 was related to the doubling time of the budding cell cycle. The budding index represents the fraction of budded cells in an exponentially growing *S. cerevisiae* culture and the budding yeast cell cycle could provide an excellent example of the need for modeling stochastic effects in mathematical modeling of biochemical reactions (12). The maximum growth rate and optimum doubling time were obtained at the same time of maximum ethanol production rate. The time of the maximum production rate was recorded at the shortest fermentation duration time that offered the maximum ethanol concentration. Therefore, the effects of different ultrasonic frequencies could be confirmed by evaluating the doubling times. This value can be calculated using the following equation (Eq.1) (5).

$$\mu = \frac{n}{t} \qquad \text{(Eq.1)}$$

Where μ represents the specific constant growth rate (the specific constant growth rate of *S.cerevisiae*M30 was 0.14), n is maximum doubling time, and t is the time offering the maximum ethanol production rate.

The specific ethanol production time was evaluated to determine the optimum ultrasonic frequency offering both maximum ethanol production rate and lowest doubling time of budding yeast. Ethanol production rate was calculated following equation (Eq. 2), which was based on the mathematical model of ethanol production developed by Muenduen et al. 2006 (13).

$$\frac{dCp}{dt} = vCx \qquad \text{(Eq.2)}$$

Cp and Cx were ethanol concentration (g/l) and cell concentration (g/l), respectively. In this study, Cx was fixed at 0.48 g/l and Cp varied depending on the fermentation performance of each batch (13). The v was the specific ethanol production rate (h^{-1}) and calculated

according to the equation above by substituting maximum ethanol concentration, fermentation time, and yeast cell concentration from the experiment. The specific ethanol production rate (v) and the doubling time were then evaluated by specific ethanol production time (T) using the following equation (Eq. 3).

$$T = \frac{n}{v} \quad \text{(Eq.3)}$$

3. Results and discussion

Environmental fermentation

The environment of the ethanol fermentation systems were monitored by measuring pH and temperature of the liquid fermentation. The liquid temperature of the control bioreactor was detected in the range of 28-32°C, which was lower than that of all ultrasonic power-supplied bioreactors. Increasing liquid temperature was detected in three bioreactors that were supplied with ultrasonic power at 20, 25, and 30 kHz. Liquid temperatures of these bioreactors are shown in Table 2. These values were in the range of 30-36°C, 30-38°C, and 30-42°C, respectively. The liquid temperature which ultrasonic power supplied at 30 kHz was higher than optimum fermentation temperature of *S. cerevisiae*M30.This yeast strainis characterized as a high-performance yeast on ethanol production, high protein content in living cells, high resistance to stress environments as low as pH 4.2, and high temperature (38°C) (14). Therefore, high liquid temperature might affect the activity of *S. cerevisiae* M30.Liquid maintained its pH stable in the range of 4.6-8.0 under normal temperature detected in the systems of ultrasonic stimulation at 20 and 25 kHz. These results indicated that *S. cerevisiae* M30 performed under normal conditions in both liquid temperature and pH, whereas ultrasonic power at 30 kHz affected the increasing of liquid temperature via the cavitation effect. Cavitation occurs more readily at a frequency of 20-40 kHz. Bubble implosion and fragmentation produce micro-regions of extreme conditions with estimated temperatures as high as 5000°K and pressures up to 100MPa, which could induce many physical-chemical effects. Water also undergoes thermolysis in the bubbles to release radical species (15). The cavitation effect could also occur with 20-25 kHz of ultrasonic power, but it might be lower than 30 kHz due to low ultrasonic power. High liquid temperature detected in 30 kHz ultrasonic power supplies affected yeast activity and led to a maximum ethanol concentration decrease. Ethanol production increased in proportion to the increase of ultrasonic power. The maximum ethanol concentrations at 13.8%, 15.6%, and 13.1% (v/v) were achieved under ultrasonic power supplied at 20, 25, and 30 kHz, respectively, while the control system was maintained at 12.0%(v/v). At the highest ultrasonic power (30 kHz), not only might an unsuitable liquid temperature affect yeast activity, but also a high level of ultrasonic power might affect yeast performance on ethanol production. A previous study reported that the cavitation effect of high ultrasonic power is often accompanied by the emission of light and can break apart relatively robust small molecules and bioactive macromolecules, and thus a living cell does not survive cavitation for long (16, 17).

Table 2. Environments of fermentation systems and maximum ethanol productions

Sample	pH	Liquid temperature (°C)	Maximum concentration of ethanol (% v/v)	Time offering max-concentration of ethanol (h)
Control	4.6-4.7	28-32	12.0	27
20 kHz	4.6-4.8	30-36	13.8	21
25 kHz	4.6-4.8	30-38	15.6	21
30 kHz	4.6-5.0	30-42	13.1	18

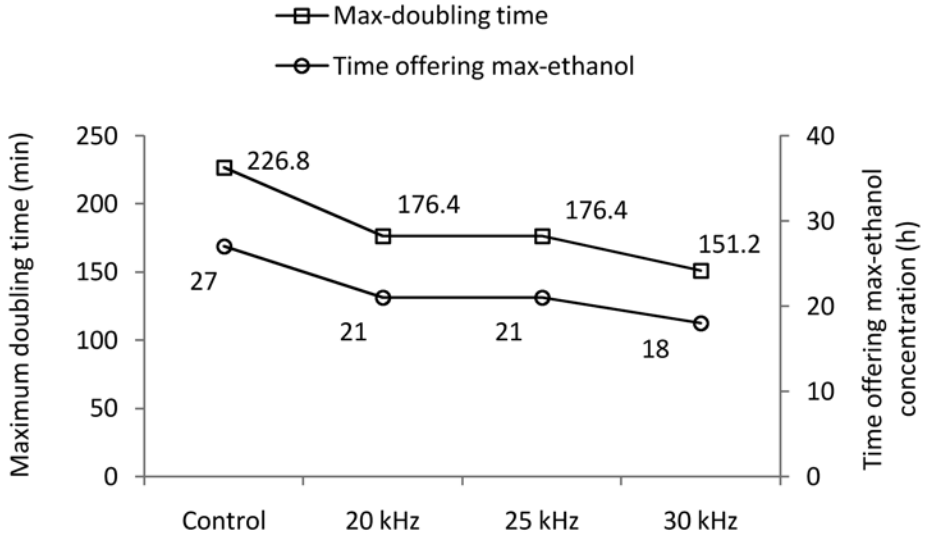

Figure 1. Maximum doubling time and ethanol production

Fermentation performance

The maximum doubling time was evaluated by substituting the specific constant growth rate of *S. cerevisiae*M30 (μ) and the time offering the maximum ethanol production rate (*t*) in equation (Eq.1). The results of the maximum doubling time are illustrated in Figure 1. According to the results, ultrasonic power could enhance ethanol production and reduce the doubling time of *S. cerevisiae*M30. The lowest doubling time of 151.2 min was obtained from ultrasonic power supplied at 30 kHz, while 176.4 min was obtained from ultrasonic power supplied at 20 and 25 kHz. Doubling time was calculated based on the time offering maximum ethanol concentration; therefore, the highest value was achieved at 30 kHz. However, for long-duration ethanol fermentation, this value will be higher than ultrasonic power supplied at 20 and 25 kHz because yeast activity is inhibited by increasing the liquid temperature. Moreover, 30 kHz did not offer the maximum ethanol concentration (Table 2). Therefore, the maximum doubling time could not be used to clarify the optimum ultrasonic power to enhance ethanol fermentation of this study. Doubling time cycles of all ultrasonic supplied bioreactors were lower than those of the control bioreactor by

approximately 50-75 minutes. This indicated that yeast growth was stimulated and its growth was faster than that without ultrasonic power. The maximum doubling time could confirm the efficiency of low ultrasonic power (20-30 kHz) in that it enhanced the cell growth or yeast budding. Other researchers have reported that ultrasonic power at 20 kHz affects yeast growth by enhancing the growth rate and reducing the doubling time of *S. cerevisiae* at 92-98 min(5). Moreover, this result was supported by Dai Chuanyun et al. (2003)(18), whose studied low ultrasonic stimulants on fermentation and revealed that the optimum power of ultrasonic power wasabout 24 kHz by reducing fermentation time from 72 hours to 36 h and increasing the productivity rate of riboflavin to about 5 times thoseof control groups.

The specific ethanol production rate was also determined to find the optimum ultrasonic frequency. Following equation (Eq.2), the specific ethanol production rate was calculated and reported in Table 3. The specific maximum ethanol production rate confirmed the results of fermentation performance of the control system and applied ultrasonic power systems that could improve the ethanol production rate. All applied ultrasonic power systems showed higher specific maximum ethanol production rates than the control system. Themaximum ethanol production rate at 1.55 g/g.hwas achieved at 25 kHz of ultrasonic power. This value of the control systems, 20 and 30 kHz of ultrasonic power supplied, was obtained at 0.93, 1.37, and 1.52 g/g.h, respectively. The specific ethanol production rate has been studied by some researchers using different yeast strains and bacteria without ultrasonic power stimulation. *Zymomonasmobilis* and *S. uvarum* were evaluated with a specific ethanol production rate using glucose 250 g/L at 30°C and pH 5.0. The results were obtained at 2.5 and 0.87 g/g.h, respectively. In the case of *Sacharomycesuvarum*, this value of *S.cerevisiae*M30 even used glucose as the substrate, which was more easily used and converted to ethanol than sucrose in molasses (19). The results confirmed that *S. cerevisiae*M30 is a high-performance yeast for ethanol production and that ultrasonic power stimulation can enhance ethanol production efficiency. According to all results, ethanol fermentation stimulation with 25 kHz of ultrasonic power seems to be the optimum condition offering the highest specific ethanol production rate in the optimum pH and liquid temperature environment.

Table 3. Specific ethanol production values

Sample	Max-doubling time (h)	Specific ethanol production rate (g/g.h)	Specific ethanol production time
Control	3.78	0.93	4.1
20 kHz	2.94	1.37	2.1
25 kHz	2.94	1.55	1.9
30 kHz	2.52	1.52	1.7

To assert these results, the specific ethanol production times of each fermentation system were evaluated. Ultrasonic power resulted in the changing of ethanol production by reducing fermentation time, as mentioned above. Therefore, calculating the time of specific ethanol production according to equation (Eq.3) could help identify the optimum ultrasonic power supply. These values are reported in Table 3. The results matched up with the maximum doubling time and specific ethanol production rate. These values were 2.1, 1.9 and 1.7 at the ultrasonic power of 20, 25, and 30 kHz, respectively, whereas for the control bioreactor this value was obtained at 4.1. The ultrasonic power of 30 kHz was eliminated because of high liquid temperature's influence during long periods of ethanol fermentation. Moreover, the specific ethanol production times of 30 and 25 kHz were quite similar; thus, 25 kHz of ultrasonic power should be the best option for ethanol fermentation enhancement. Therefore, overall fermentation performance under ultrasonic stimulation at 20 and 25 kHz indicated that the effect of ultrasonic power at 25 kHz wasthe best condition to offer the highest ethanol production rate with the lowest specific ethanol production time.

According to theresults, this research can be concluded thatthe range of ultrasonic power between 20, 25, and 30 kHz could stimulate*S. cerevisiae*M30 activity.This range enhanced the ethanol production rate by reducing fermentation about 6 to 9 h compering to the control bioreactor. The ultrasonic power of 30 kHz provided the lowest maximum doubling time at 151.2 min, but it offered specifictheethanol production lower than that of 25 kHz due to high liquid temperatures inhibiting yeast activity. The highest value for the specific maximum ethanol production rate (1.55 g/g.h) was achieved at the bioreactor stimulation by ultrasonic power of 25 kHz.Under this condition, the specific ethanol production time of 1.9 was also obtained.

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