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Fermentation of *Saccharomyces cerevisiae* in a 7.5 L ultrasound-enhanced fermenter: Effect of sonication conditions on ethanol production, intracellular Ca^{2+} concentration and key regulating enzyme activity in glycolysis

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ABSTRACT

In this study, the effect of sonication on the fermentation process of a single-celled fungus was examined. During the experiment, *Saccharomyces cerevisiae* (*S. cerevisiae*) was used as the starting strain for ethanol fermentation (batch fermentation) in a 7.5 L automated fermentation tank. The fermentation tank connected with a six-frequency ultrasonic equipment. Non-sonication treatment was set up as the control. Sonication treatment with power density of 280 W/L and 48 h of treatment time were set up as trial groups for investigating the influence of different ultrasound frequency including 20, 23, 25, 28, 33 and 40 kHz on the changes in dry cell-weight, glucose consumption rate, and ethanol yield. The results showed that the dry cell-weight, glucose consumption rate, and ethanol content reached the best results under the ultrasonic condition of 28 kHz ultrasound frequency in comparison with other ultrasound frequency. The dry cell-weight and ethanol content of the 28 kHz ultrasonic treatment group increased by 17.30% and 30.79%, respectively in comparison with the control group The residual sugar content dropped to a lower level within 24 h, which was consistent with the change in ethanol production. Besides, the results found that the glucose consumption rate increased compared to the control. It indicated that ultrasound accelerated glucose consumption contributed to increase the rate of ethanol output. In order to explore the mechanism of sonication enhanced the content of ethanol output by *S. cerevisiae*, the morphology, permeability of *S. cerevisiae* and key enzyme activities of ethanol synthesis were investigated before and after sonication treatment. The results showed that after sonication treatment, the extracellular nucleic acid protein content and intracellular Ca^{2+} concentration increased significantly. The morphology of *S. cerevisiae* was observed by SEM and found that the surface of the strain had wrinkles and depressions after ultrasonic treatment. furthermore after sonication treatment, the activities of three key enzymes which catalyze three irreversible reactions in glycolysis metabolism, namely, hexokinase, phosphofructokinase and pyruvate kinase increased by 59.02%, 109.05% and 87.27%, respectively. In a word, low-intensity ultrasound enhance the rate of ethanol output by *S. cerevisiae* might due to enhancing the growth and cell permeability of strains, and increasing the activities of three key enzymes of ethanol biosynthesis.

1. Introduction

Ethanol is vital to industries as a raw material for disinfection and beverage products [1,2]. *S. cerevisiae* as the principal strain to produce ethanol by liquid fermentation. Ethanol production by *S. cerevisiae* fermentation due to its low production cost, fast growth rate and simple steps has also become a common means of ethanol production in industry [3]. However, high level of ethanol concentration and the

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production of metabolic waste could damage the strains and inhibit the synthesis of ethanol during the fermentation [4,5]. In order to enhance the yield of ethanol, traditional methods focused on optimization of the culture medium and increasing the biomass of the strain [6]. Obviously, the high osmotic pressure and cost of the culture medium also need to be considered [7]. Thus, it is imperative to find novel techniques to improve the process and increase ethanol yield.

Recently, many researchers have applied physical techniques such as ultrasound to increase fermentation efficiency. Dai et al. [5] found that under the optimum ultrasonic conditions (28 kHz, 140 W/L, 1 h), the biomass of yeast increased by 127.03% in comparison with non-sonication treatment. In addition, they also found that ultrasound improved the cell activity and growth rate. These results showed that low-intensity ultrasound is able to significantly promote the proliferation of *S. cerevisiae* and increase the yield of alcohol [8–11]. Sulaiman et al. [12] found the ethanol production of yeast *Kluyveromyces marxianus* using lactose fermentation under different ultrasonic conditions (20 W, 11.8 W/cm^2), the final alcohol concentration was 5.20 ± 0.68 g·L^{-1}, which increased by ~ 3.5 times in comparison with the control. Furthermore, Some researchers as well as found that low-intensity ultrasound can change the activity of related enzymes and increase the permeability of cell membrane [13,14]. Liu et al. [15] found that ultrasound treatment increased the content of extracellular protein, nucleic acid and fructose 1, 6-diphosphate (FDP).

Ethanol fermentation is closely related to glycolysis. There are three irreversible reactions in glycolysis. These reactions were regulated by hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). Therefore, this study explored the changes in the activities of key enzyme to explain the changes in ethanol production after ultrasound treatment [16,17]. In a word, this study used low-intensity ultrasound for stimulating *S. cerevisiae* to synthesis ethanol and investigate the changes in biomass, permeability and enzyme activity to explain the mechanism of increasing the ethanol production.

However, most of the previous studies were carried out in the process of small-scale experiments such as shake flask culture. There may be many errors between experiment and actual product. Therefore, in this study, 7.5 L fermenter was used to ferment *S. cerevisiae* for producing ethanol. The ultrasonic frequency (20, 23, 25, 28, 33 and 40 kHz) was screened by single factor based on the outcome of the changes in pH, dissolved oxygen, cell dry weight and residual sugar content. Also, the changes of 3 key enzymes (hexokinase, phosphofructokinase, pyruvate kinase) in the glycolysis process under the optimal frequency treatment were studied to explore the reason why ultrasound treatment enhanced the ethanol production of *S. cerevisiae*.

2. Materials and methods

2.1. Yeast strains and culture conditions

The *S. cerevisiae* 1048 used in this study. It was purchased from China Center of Industrial Culture Collection.

The strain preserved in a 5 mL EP tube was inoculated into YPD medium (2% glucose, 1% yeast extract and 2% tryptone were added into a 250 mL of conical flask and the distilled water was used to reach a constant volume 100 mL. YPD medium was prepared and sterilized at 115 °C for 20 min) at 30 °C for 12 h to activate strain.

Fermentation media was consisted of 20 g/L peptone, 250 g/L glucose, 10 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4$, 25 g/L yeast extract and 0.15 g/L $CaCl_2$ and sterilized at 115 °C for 20 min. After activating, 200 mL of strain solution was transferred into fermentation tank contained with 4 L of fermentation medium and cultured at 30 °C with a shaking ratio of 160 r/min for 48 h.

2.2. Ultrasonic equipment

A self-developed bioreactor consisted with a fermentation tank and a sonication device was employed in this study to increase the ethanol production (Fig. 1). This device is mainly composed of 19 parts, i.e., 1. slit ultrasonic equipment 2. control panel 3. ultrasonic generator 4. ultrasonic transducer 5. material liquid inlet 6. material liquid outlet 7. sterilization vent 8. peristaltic pump 9. fermentation tank 10. DO electrode 11. pH electrode 12. air flow 13. temperature sensor 14. air filter 15. exhaust outlet 16. circulating condensate 17. electric pump 18. fermentation tank main console 19. computer. It is handled as following. 4 L fermentation medium was added into the fermentor and sterilized at 115 °C for 20 min. A suitable ultrasonic generator was chosen and the flow rate was set to 100 mL/min for obtaining stable fermentation conditions (power density). Then the specific parameters (stirring speed, temperature, ventilation) were set in the fermentation tank main console and the device was started by the control panel.

2.3. Determination of pH and dissolved oxygen value

During the fermentation process, the pH and dissolved oxygen values were recorded by using pH and DO electrode every 6 h, respectively.

2.4. Single factor optimization experiment of ultrasonic frequency

The conditions of 30 °C of fermentation temperature, 160 r/min stirring speed, 1.5 SLPM (standard liter per minute) of ventilation,48 h of culture time were set as control. According to the culture conditions of control, under the condition of ultrasonic power density was 280 W/L [18,19], the sonication treatments were utilized to stimulate *S. cerevisiae* fermentation and the effects of different ultrasonic frequency (20, 23, 25, 28, 33 and 40 kHz) on the changes of ethanol yield during the fermentation of yeast were investigated.

2.5. Determination of cell dry-weight of S. cerevisiae, and ethanol production and residual sugar content fermentation broth

2.5.1. Cell dry-weight

At the fermentation of 6, 12, 24, 30, 36 and 48 h, the strain precipitation was obtained by centrifuge (4 °C, 4000 r/min, 10 min). The strain precipitation was washed twice with sterile water, and dried in electric-heating-blast-drying-oven (DHP-9272) to a constant weight for calculating the biomass of strain.

2.5.2. Determination of ethanol content

Ethanol was determined by gas chromatography. Standard ethanol solutions 0.05%, 0.10%, 0.5%, 0.7% and 1% were prepared respectively. Ethanol concentration and peak area were set as the abscissa and ordinate, respectively. The standard curve was established by the least square method. The gas chromatograph (GC-2010 plus), equipped with hydrogen flame detector (FID) and capillary column (30 m × 0.250 mm, INNOWAX, Agilent) was used to obtain the peak areas of samples. The conditions of GC program and FID as follows: the temperature of the inlet and detector are 250 °C and 280 °C respectively, the oven temperature adopts the heating program: the initial temperature is 90 °C for 3 min, the temperature is increased to 165 °C for 0.5 min at a rate of 25 °C/min, and the temperature is increased to 210 °C for 2 min at a rate of 15 °C/min. The flow rates of nitrogen, hydrogen, and air are 30, 40, and 300 mL/min, respectively. The peak areas of samples were substituted into standard curve to calculate the production of ethanol.

2.5.3. Determination of residual sugar content

The content of residual sugar was determined by dinitrosalicylic acid method (DNS) [20]. 1.0000 g glucose was dried to a constant weight, and dissolved in 1 L distilled water to obtain glucose standard solution with a concentration of 1 mg/mL. Then, the mixed solution at different volumes (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL) was transferred into test tubes, and distilled water (1 mL) was added to the tubes. After that 2 mL DNS solution was mixed with the mixture. The tubes were placed in water

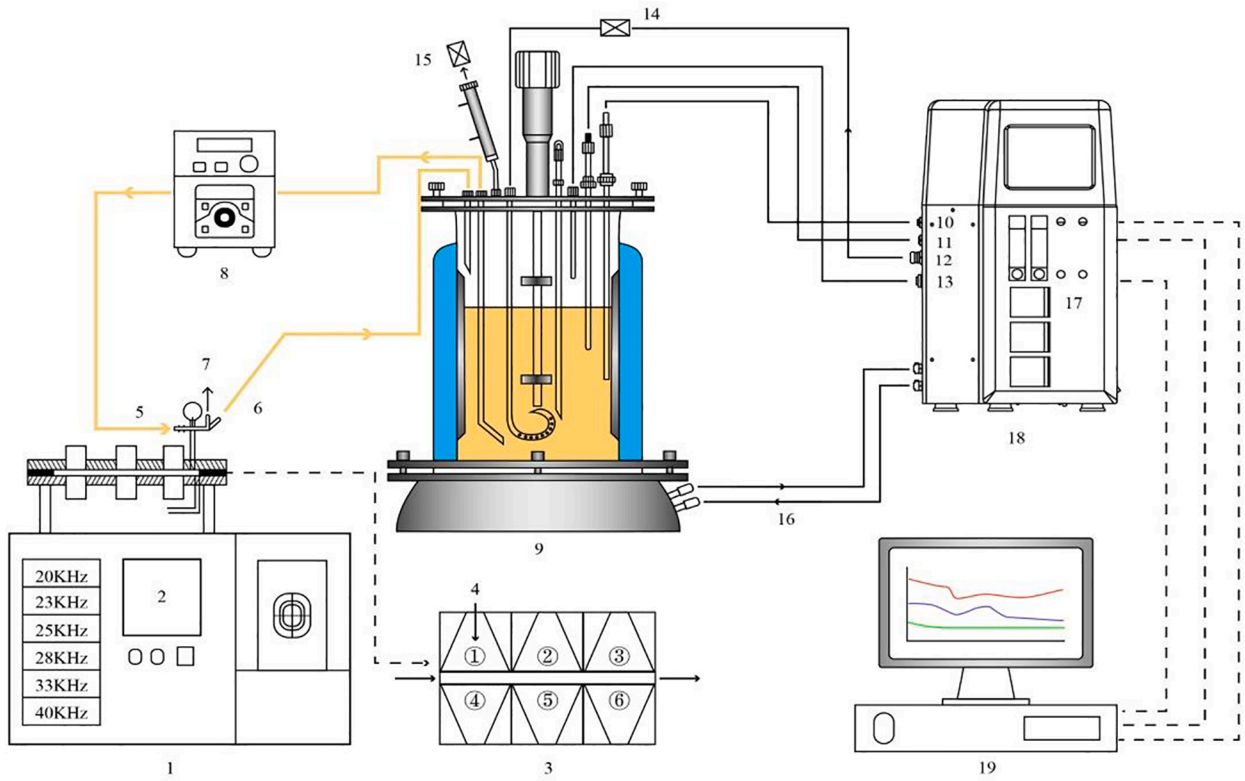

Fig. 1. External ultrasound irradiation of broth in a recycle bioreactor (1. Slit ultrasonic equipment 2. control panel 3. Ultrasonic generator 4. Ultrasonic transducer 5. Material liquid inlet 6. Material liquid outlet 7. Sterilization vent 8. Peristaltic pump 9. Fermentation tank 10. Do electrode 11. pH electrode 12. Air flow 13. Temperature Sensor 14. Air filter 15. Exhaust outlet 16. Circulating condensate 17. Electric pump 18. Fermentation tank main console 19. Computer host).

bath at 100 °C for 5 min, After then the solution was cooled to room temperature. The standard curve of residual sugar content was established by measuring the absorbance at 540 nm and the fitted equation was obtained as follows:

$$y = 1.2999x\text{-}0.0254\ R^2 = 0.9987$$

where y is light absorption value (540 nm), x is glucose concentration (mg/mL).

2.6. Effect of ultrasonic treatment on cell permeability of S. Cerevisiae

2.6.1. Determination of extracellular nucleic acid and protein content of S. Cerevisiae

2 mL of strain solution and 8 mL of distilled water were added into a 15 mL EP tube. The resultant was transferred to centrifugation (4 °C, 5000 r/min, 5 min). According to the Liu's study [15], during the fermentation time of 6, 12, 24, 36 and 48 h, the changes of nucleic acid and protein in the extracellular fluid of yeast were determined at 260 nm and 280 nm, respectively.

Determination of nucleic acid and protein: at 260 nm and 280 nm wavelength, the absorbance values of strain solution were determined. The increasing rate of nucleic acid (R_1) and the improvement rate of protein (R_2) were estimated as follows:

$$R_1 = (b/a - 1) \times 100\%$$

where R_1 is the increasing rate of nucleic acid (%); a is absorbance value of the strain solution without ultrasound treatment; b is the absorbance value of strain solution with sonication treatment.

$$R_2 = (d/c - 1) \times 100\%$$

where R_2 is the improvement rate of protein (%); c is absorbance value of the control; d is the absorbance value of the strain solution after ultrasonic treatment.

2.6.2. Analysis of S. Cerevisiae morphology

The strain with sonication treatment or not were cultured to the later exponential stage, respectively and centrifuged (4 °C, 2000 r/min) to remove the supernatant. The supernatant was fixed with 5% glutaraldehyde. After fixation, it was rinse 3 times with 0.1 M phosphate buffer. The samples were fixed again with 1% osmium tetroxide and washed 3 times in buffer solution. The fixed samples were dehydrated with different concentration gradients of ethanol (30%, 50%, 70%, 90% and 100%). Subsequently, the samples were placed in the sample chamber for ion splashing Shoot. The cell morphology was observed by scanning electron microscope (Hitachi, SU8200).

2.6.3. Changes in intracellular Ca^{2+} concentration of S. Cerevisiae

1 mL of fermentation broth was centrifuged (4 °C,10000 r/min, 10 min), and yeast precipitation was washed 3 times with 0.001 M PBS buffer solution. 0.5 mL of PBS buffer solution and 0.5 mL of 7 μM Fluo-4/AM (Beyotime Biotechnology). were added to the cell precipitation. After incubation at 37 °C for 1 h, the supernatant was removed and the precipitation was washed 3 times with PBS buffer solution. The fluorescence intensity of *S. cerevisiae* was observed by the fluorescent inverted microscope.

2.7. Determination of enzyme activity

Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) kits were used for determination according to the instructions of the kits (Beyotime Biotechnology). The HK, PFK and PK activities of the sample with or without ultrasound treatment were detected at different time (20, 24, 28, 32 and 36 h).

3. Results and discussion

3.1. Single factor optimization of ultrasonic frequency

The ethanol yield of *S. cerevisiae* under different ultrasonic frequency conditions is showed in Fig. 2. It showed that there was significant difference ($P < 0.05$) in ethanol yield between non-sonicated and sonicated samples. The production of ethanol without sonication was 8.01% (v/v) and the production reached the top value which was 10.48% (v/v) under the 28 kHz of ultrasonic frequency. Sonication treatment (28 kHz) enhanced the ethanol content by 30.79% in comparison with the control. The ethanol content of the 20, 23, 25, 33 and 40 kHz experimental groups basically reached the maximum at 36 h of fermentation time, and the content remained basically unchanged at the end of fermentation at 48 h, while the ethanol content of 28 kHz increased significantly at 36 ~ 48 h. Therefore, the optimal ultrasonic frequency was 28 kHz.

Ultrasound treatment increased the ethanol production of *S. cerevisiae* might be linked to the fact that after ultrasonic treatment, *S. cerevisiae* produced stress response in response to external stimulation result in promoting the yeast growth and increase of membrane permeability [21] and enzyme activities [22,23].

3.2. Analysis of pH and DO values during fermentation

Changes in pH and DO during fermentation were showed in Fig. 3. The pH value decreased slowly at the beginning of fermentation, indicating the yeast was mainly adapting to the new environment at this growth stage with fewer metabolites. During the fermentation of 20 h, the yeast was in the exponential growth stage, rapidly consuming glucose in the medium to produce large amount of acid, alcohol and other metabolites, thereby reducing the pH value of the fermentation broth. This result was consistent with what has been reported by other researchers [24]. Further, the acid substances produced in the subsequent stages of fermentation and reached saturation. These might the reason why the pH value of the fermentation broth did not continue to decrease.

It can be seen from the Fig. 3. At 0 h, the DO value of medium was 100%. Although 1.5 L/min of sterile air was continuously introduced into the fermenter, the DO value reduced rapidly from 0 to 12 h. It might indicate that at the initial stage, the strain used oxygen quickly in the system for growth lead to reducing the DO content. At the culture time of 36 h, the DO value in the fermentation broth started to increase. This may be due to the fact that as the fermentation time increased, the nutrients in the fermentation medium decreased which caused *S. cerevisiae* to be in a nutritional deprivation, resulting in the amount of consumed oxygen was reduced. Sulaiman et al [12] carried out an ultrasound-aided yeast fermentation study, they found that after a period of fermentation, the amount of dissolved oxygen in the fermentation broth also started to increase.

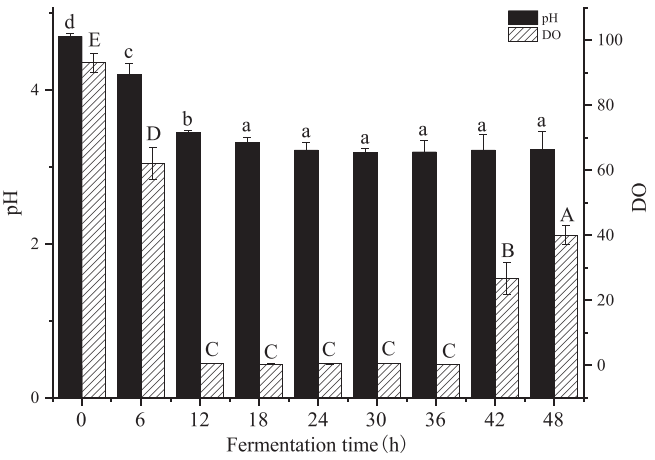

Fig. 3. Changes in pH and DO during fermentation.

3.3. Changes in dry weight of yeast at different ultrasound frequencies

The changes in dry weight of yeast at different ultrasound frequencies were displayed in Fig. 4. From the results, there was no significant difference ($P > 0.05$) in the dry weight of the yeast at 0 h due to the same inoculation amount in the fermentation tank. When the yeast reached the stable growth stage (24 h), the yeast concentration and growth rate of the sonicated sample was significant higher than control group. From 0 ~ 12 h, the growth of all the groups was basically the same, and the yeast concentration of 20, 23, 33 and 40 kHz ultrasound groups was lower than that of the 25 kHz and 28 kHz. This indicated that ultrasound treatment promoted the growth of yeast, but high ultrasonic frequency could hamper the proliferation effect of yeast. This result could possibly be illustrated by the concept that as the appropriate intensity of ultrasound was able to promote the growth of *S. cerevisiae*.

3.4. Analysis of residual sugar content in fermentation broth at different ultrasonic frequencies

The changes in residual sugar content of the fermentation broth with

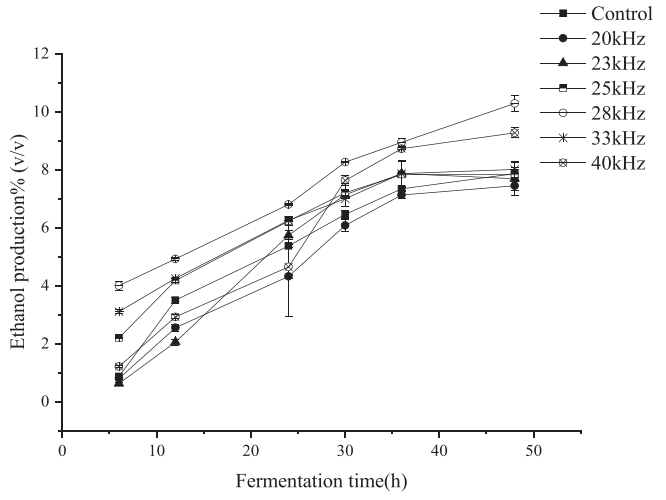

Fig. 2. The effect of six ultrasonic frequencies on ethanol yield of *Saccharomyces cerevisiae*.

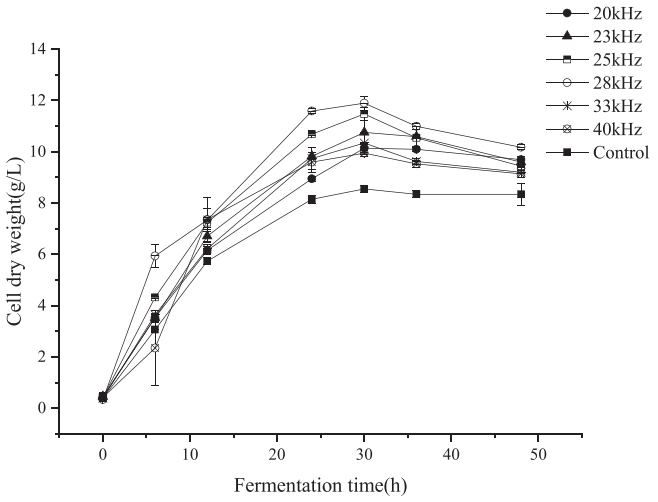

Fig. 4. Changes in cell dry-weight at different ultrasound frequencies.

different ultrasonic frequencies was showed in Fig. 5. The figure showed that sonication promoted the consumption of glucose in the fermentation broth and the residual sugar content drops to a lower level fast. At frequency of 23 and 28 kHz, glucose consumption rate decreased rapidly in 24 h. Same trend was also observed for other ultrasonic frequencies, but the glucose consumption rate was higher than that of the control. The samples with sonication treatment, the residual sugar content decreased to 36 h, which was consistent with the ethanol production curve (Fig. 2). This reduction may be correlated with the changes in the permeability of *S. cerevisiae* cells following sonication, thus speeding up glucose transport and increasing ethanol production through glycolysis. Matsuura et al. [25] found that the use of ultrasound in treating samples was helpful in producing the ethanol. In summary, ultrasound-assisted fermentation of *S. cerevisiae* could increase the growth rate of yeast contributed to enhancing the production of ethanol.

3.5. Effect of ultrasound treatment on cell membrane permeability

3.5.1. Changes in extracellular nucleic acid and protein

The changes in extracellular nucleic acid and protein with sonication treatment were showed in Fig. 6. After ultrasonic treatment (28 kHz), the increase rate of extracellular nucleic acid and protein of *S. cerevisiae* was significantly higher than the control. In the process of 0 ~ 36 h ultrasonic treatment, the extracellular nucleic acid and protein of *S. cerevisiae* increased with ultrasonic treatment time increased. Liu [15] showed that Ultrasonic frequency and duration can affect the increase rate of extracellular proteins and nucleic acids. When sonicated for 36 h, the increase rate of extracellular nucleic acid and protein of *S. cerevisiae* was 8.8% and 12.8%, respectively. The results showed that ultrasound improved the permeability of *S. cerevisiae* cell membrane, which was conducive for movement of substances in and out of the yeast. After 36 h, the increase rate of extracellular nucleic acid and protein decreased, which may have been caused by extended ultrasound treatment of *S. cerevisiae* cells. This finding was consistent with the observation of dry weight of cells treated by ultrasound.

3.5.2. Effect of ultrasound treatment on the morphology of S. Cerevisiae

The morphology of *S. cerevisiae* was observed by scanning electron microscopy (Fig. 7). The result showed that the surface of the control strain was smooth and round. However the surface of the strain after the ultrasonic treatment showed dents and wrinkles. This may be due to the fact that the force generated by the ultrasonic waves on the strain may have deformed the cell membrane lead to changing the permeability of the cell membrane. The cavitation effect of ultrasound changed the permeability of the cell plasma membrane, resulting in the release of intracellular or extracellular substances into the cells [26].

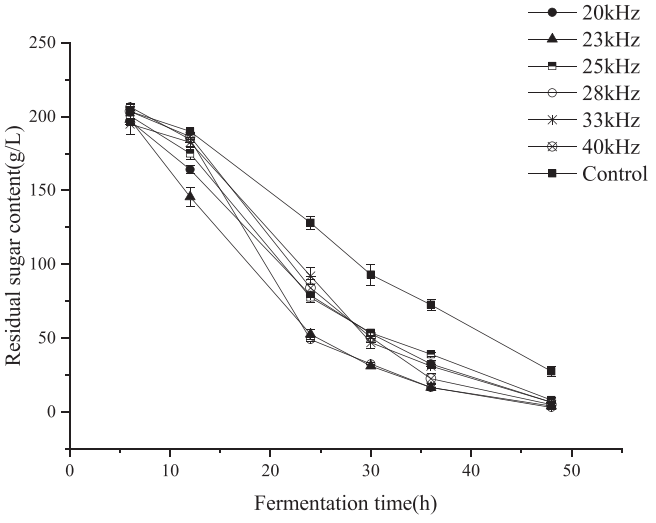

Fig. 5. Change in residual sugar content of fermentation broth with different ultrasonic frequencies.

3.5.3. Changes in intracellular Ca^{2+} concentration of strains

The changes in intracellular Ca^{2+} concentration of the strain following sonication were showed in Fig. 8. The result showed that the fluorescence of the strain (control) was concentrated but the brightness was weak. After the ultrasonic treatment, the fluorescence of the strain was relatively scattered but high brightness. This observation may be linked to the increase in membrane permeability caused by the ultrasound treatment, which may result in an increase in the fluorescence intensity of intracellular Ca^{2+}. The enhancement of cell membrane permeability improved the rate of nutrient transport and harmful metabolites excretion from the cell, providing sufficient nutrients for the production of ethanol. Wang et al. [27] investigated the effect of low intensity ultrasound on the intracellular Ca^{2+} concentration of *S. cerevisiae*, and found that sonication promoted cell membrane permeability and helped transport passively diffused Ca^{2+}. Therefore, with the improvement of cell permeability, the intracellular Ca^{2+} content was promoted.

3.6. Changes in key enzyme activity during glycolysis

Glycolysis is an indispensable metabolic pathway in organisms [28]. It was the main energy supply pathway in the process of ethanol synthesis [29]. Irreversible reactions in the glycolytic pathway are regulated by HK, PFK and PK. In order to analyze the effect of sonication on the ethanol yield of *S. cerevisiae*, the activities of 3 key enzymes in glycolysis were detected.

3.6.1. Evaluation of hexokinase (HK) activity

Changes in hexokinase activity following ultrasound treatment were showed in Fig. 9. The HK activity of the sonicated samples was significantly higher than control. At the end of fermentation, the activity of HK in strain with or without sonication treatment were 288 and 458 U/mgprotein, respectively. This finding showed that sonication improved the activity of HK by 59.02% compared to the control. HK was able to catalyze the synthesis of glucose-6-phosphate from glucose. Glucose-6-phosphate is an important intermediate of pentose phosphate pathway. Pentose phosphate pathway was able to further catalyze the synthesis of NADPH by G6PDH to provide reductive power [30,31].

3.6.2. Determination of phosphofructokinase (PFK) activity

The changes in phosphofructokinase activity after sonication were showed in Fig. 10. At the fermentation time of 20 h, the activity of PFK in ultrasonic group was 90.47% higher than control group. At the end of fermentation, sonication improved the activity of PFK by 109.05% in comparison with control. Fructose phosphate kinase catalyzes fructose-6- phosphate to produce fructose-1,6-phosphate [32]. This reaction is an irreversible one. The catalytic reaction of PFK plays a central role in glycolysis. Its catalytic efficiency is very low. The whole rate of glycolysis is controlled by its activity level, which is the rate limiting step of glycolysis [33]. The activity of PFK in the ultrasound treatment sample was higher than the control group indicated that ultrasound could improve the activity of PFK and improve the efficiency of glycolysis process.

3.6.3. Evaluation of pyruvate kinase (PK) activity

The PK activity (Fig. 11) of the sample with sonication treatment was significantly lower than the control. At the end of fermentation, the PK activity of the ultrasound samples was increased by 87.27% in comparison with the control. PK is one of the main rate limiting enzymes in glycolysis [34]. It catalyzes phosphoenolpyruvate to pyruvate [35]. The increase in PK activity indicated that the rate of pyruvate formation was increased, resulted in increasing the content of pyruvate. Pyruvate is the

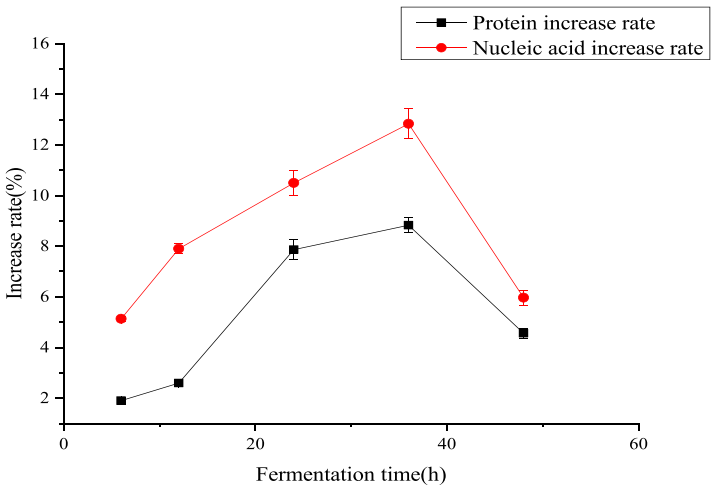

Fig. 6. The changes of extracellular nucleic acid and protein before and after ultrasound (Note: Ultrasonic frequency 28 kHz).

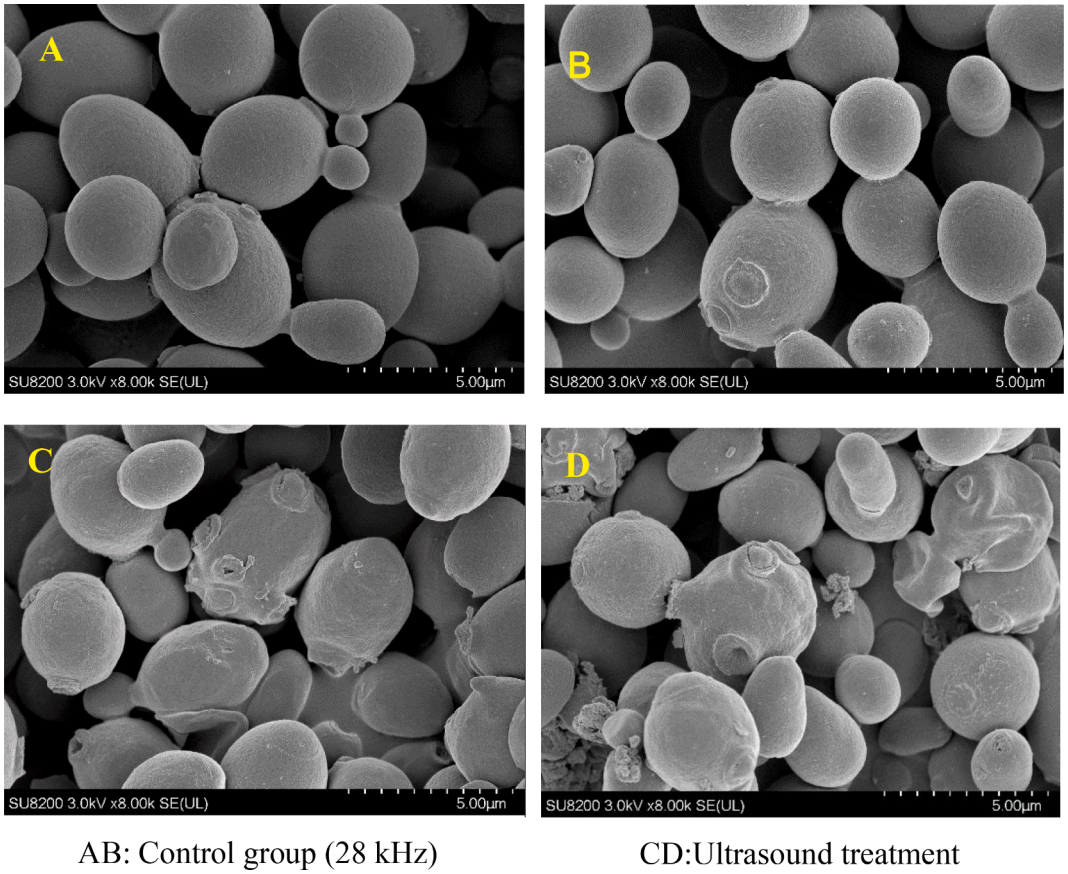

Fig. 7. Scanning electron microscopic observation of the effect of ultrasound on the morphology of *Saccharomyces cerevisiae*.

important precursor for acetaldehyde synthesis. Furthermore, acetaldehyde is the precursor of ethanol. The increase in activity of PK enzyme might indicate that pyruvate was increased, leading to increasing the production of ethanol [36].

4. Conclusion

The current work demonstrated that ultrasound treatment improved the ethanol content by *S. cerevisiae* during fermentation. Under the 28 kHz of ultrasonic frequency, the ethanol production and the biomass increased by 30.79% and 24.10%, respectively compared to non-sonicated sample. The growth rate and final cell concentration of the 28 kHz ultrasound group were higher than the control and other trial groups. The residual sugar consumption rate was also faster than other groups. Thus, the best ultrasonic frequency is 28 kHz. Additionally, ultrasound treatment improved the permeability of *S. cerevisiae* cell membrane. The increase rate of extracellular nucleic acid and protein of *S. cerevisiae* increased by 8% and 12% after ultrasonic treatment. The surface of *S. cerevisiae* after ultrasonic treatment was observed by scanning electron microscope and found that the surface became wrinkled and sunken. Moreover, the activity of HK, PFK and PK increased by 59.02%, 109.05% and 87.27% respectively under the condition of 28

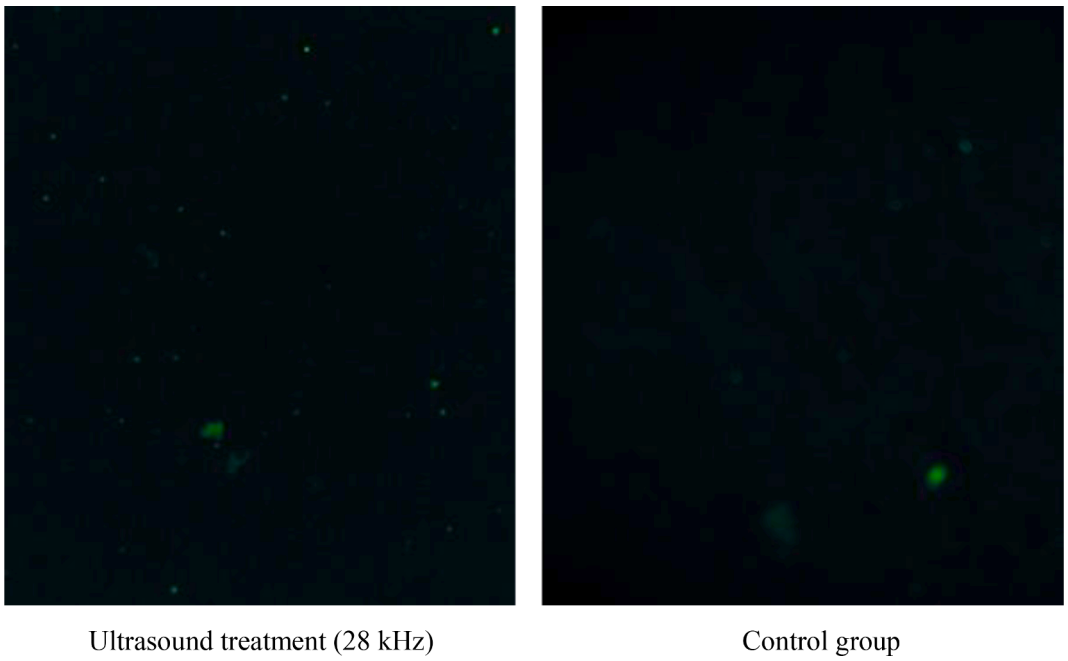

Fig. 8. The changes of intracellular Ca^{2+} concentration of the strain before and after ultrasonic treatment.

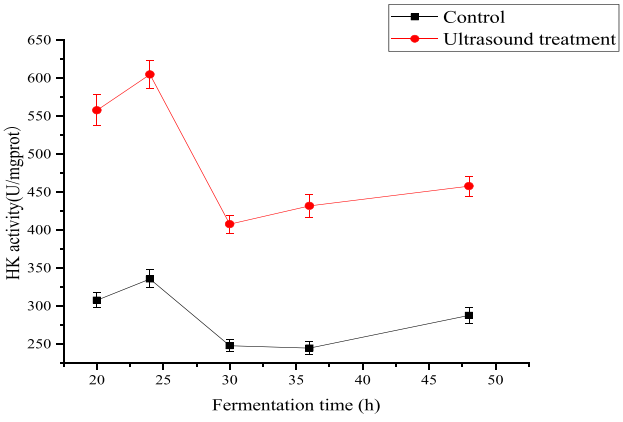

Fig. 9. Effect of *Saccharomyces cerevisiae* hexokinase activity before and after 28 kHz ultrasonic treatment.

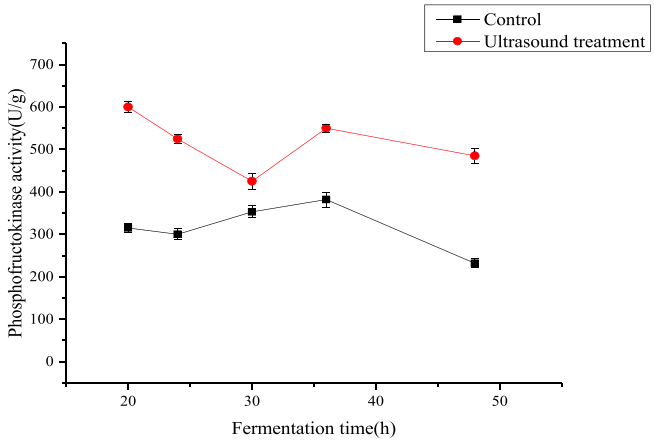

Fig. 10. Effect of *Saccharomyces cerevisiae* phosphofructokinase activity before and after 28 kHz ultrasonic treatment.

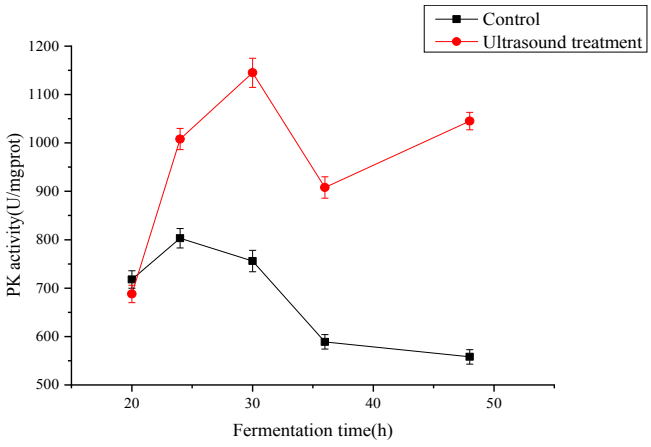

Fig. 11. Effect of *Saccharomyces cerevisiae* pyruvate kinase activity before and after 28 kHz ultrasonic treatment.

kHz ultrasonic frequency. In conclusion, the outcomes of the ethanol production, biomass, residual sugar, pH, DO, extracellular nucleic acid and protein, morphology and Key enzyme activity confirmed that ultrasonic treatment can promote *S. cerevisiae* growth and improve the permeability of *S. cerevisiae* cell membrane, and increase the activity of 3 key enzymes in the process of glycolysis. These results might be the reason why the production of ethanol was increased after sonication treatment.

CRediT authorship contribution statement

Ronghai He: Conceptualization, Funding acquisition, Writing - review & editing. **Wenbin Ren:** Investigation, Methodology, Software, Writing - original draft. **Jiahui Xiang:** Visualization. **Mokhtar Dabbour:** Writing - review & editing. **Benjamin Kumah Mintah:** Data curation. **Yihe Li:** Writing - review & editing. **Haile Ma:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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