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Fermentation of *Saccharomyces cerevisiae* in a one liter flask coupled with an external circulation ultrasonic irradiation slot: Influence of ultrasonic mode and frequency on the bacterial growth and metabolism yield

Zhaoli Zhang[a,b,e], Feng Xiong[a], Yang Wang[a,b], Chunhua Dai[a], Zheng Xing[a], Mokhtar Dabbour[a,c], Benjamin Mintah[a,d], Ronghai He[a,e,*], Haile Ma[a,e]

[a] School of Food and Biological Engineering, Jiangsu University, Zhenjiang, Jiangsu 212013, China
[b] Institute of Applied Chemistry and Biological Engineering, Weifang Engineering Vocational College, 8979 Yunmenshan South Road, Qingzhou, Shandong 262500, China
[c] Department of Agricultural and Biosystems Engineering, Faculty of Agriculture, Benha University, P.O. Box 13736, Moshtohor, Qaluobia, Egypt
[d] Department of Nutrition and Food Science, University of Ghana, POB LG 134, Legon, Accra, Ghana
[e] Institute of Food Physical Processing, Jiangsu University, Zhenjiang, Jiangsu 212013, China

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ABSTRACT

In this study, the influences of ultrasonic treatments with different working modes (fixed frequency and sweeping frequency) and various frequencies on the bacterial growth and metabolism yield of *Saccharomyces cerevisiae* (*S. cerevisiae*) were investigated by employing an in situ ultrasonic irradiation slot coupled with a flask fermentation equipment. The results revealed that the in situ ultrasonic treatment could promote the bacterial growth and metabolism yield, and the effect of fixed frequency ultrasound (FF) was higher than sweeping frequency ultrasound (SF). The content of metabolite in the fermentation broth increased with FF and decreased after SF treatments. On the other hand, when the frequency of FF was more than 33 kHz, the growth of *S. cerevisiae* became weaken, and the mortality rate of *S. cerevisiae* increased in fermentation broth. At FF of 23 kHz and 48 h fermentation-time, ethanol content increased by 19.33%, and the content of β-phenylethanol and other volatile metabolites such as esters also increased. In conclusion, FF could significantly improve the growth of *S. cerevisiae*, and the FF of 23 kHz showed the optimum impact on fermentation process of *S. cerevisiae*.

1. Introduction

Ultrasound technology, as a new non-thermal physical processing technology, is widely used in food industry, especially in fermentation engineering [1,2]. Suitable application of ultrasound has the potential for enhancing the productivity of bioprocesses involving live cells and bioactive enzymes [3–5]. Ultrasonic may improve the permeability of membrane, speed up the transfers of substance and promote cell's growth and propagation [6,7], which indicates that ultrasound have a promising application in fermentation engineering.

Fermentation of food involves the chemical transformation of complex organic compounds into modest compounds by the action of enzymes, organic catalysts produced by microorganisms including yeasts, moulds and bacteria [8]. *Saccharomyces cerevisiae* (*S. cerevisiae*) plays an important role in changing glucose into alcohol, β-phenylethanol, phenethyl acetate, and other fatty acids [9,10]. The alcohol is a significant raw material for alcoholic beverages and modern industrial fuels, and around 95% alcohol is produced by the fermentation process [11]. Moreover, β-phenylethanol is a higher aromatic alcohol with a rose-like odour, which makes it the most-used fragrance chemical ingredient in perfume, cosmetics and food [12]. However, recently there were some restrictions including longer fermentation cycle and lower yield of products in the fermentation of *S. cerevisiae*. Many researchers have applied physical techniques such as ultrasound to microbial fermentation processes to improve the fermentation efficacy. Dai et al. [13] found that the biomass increased by 127.03% when *S. cerevisiae* in latent anaphase was treated with sonication under the conditions of frequency 28 kHz, power density 140 W/L and treatment time 1 h. Sulaiman et al. [14] reported that a final ethanol concentration of 5.20 ± 0.68 g/L was obtained with sonication frequency 20 kHz using 20% duty cycles and nearly 3.5 folds compared to the control fermentation. Nitayavardhana et al. [15] showed that higher ethanol yields were directly related to sonication time. Higher ethanol yields were observed only for sonicated samples at the high-power

* Corresponding author at: School of Food and Biological Engineering, Jiangsu University, Zhenjiang, Jiangsu 212013, China.
E-mail address: heronghai@ujs.edu.cn (R. He).

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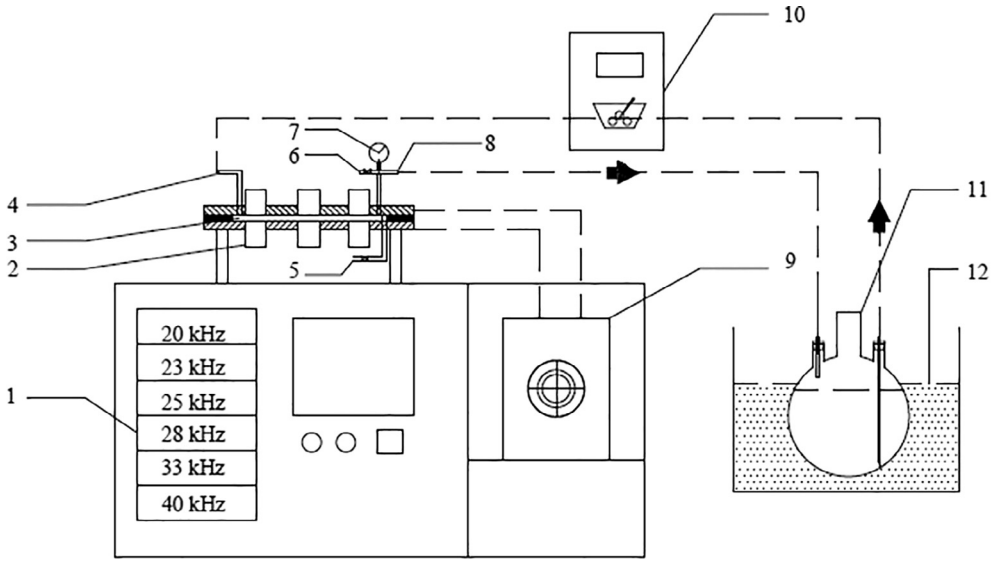

Fig. 1. Ultrasonic device of slot muti-frequency scanning and fermentation system (1: Ultrasonic generator; 2: Ultrasonic transducer; 3: Slot; 4: Inlet; 5: Sample connection; 6: Sterilization vent; 7: Piezometer; 8: Outlet; 9: Condensator; 10: Peristaltic pump; 11: Fermentation flask; 12: Thermostat and agitator water bath).

level. The ethanol yield from the sonicated sample increased by 2.7 folds compared with the control sample. The sonicated samples produced ethanol about 29% higher than the heat-treated samples. Productivity enhancements have been claimed by sonication in some other *S. cerevisiae* fermentations [7,16], due to the enhanced permeability of *S. cerevisiae* cell to proteases [16].

Nevertheless, most of the studies focusing on one or several ultrasound frequencies, did not research on the ultrasound working modes and frequencies systematically. Our previous studies have shown that low-intensity ultrasound could significantly increase the growth rate of *Bacillus subtilis*, *Candida tropicalis*, *Aspergillus niger*, etc. in the fermentation process of protein resources. Jiang et al. [17] investigated the number of bacteria (*Bacillus subtilis*), Yeast, and *Aspergillus niger* with sonication treatment in seed liquid, and found the organisms increased by 116 ~ 290%, 63 ~ 152% and 160 ~ 264%, respectively compared to the control. Huang et al. [18] studied the mechanism of ultrasound promoting microbial proliferation from the genetic level, and the results showed that the key genes controlling the proliferation of *Candida tropicalis* were significantly up-regulated after ultrasonic treatment. However, up till the present moment, the most studies of the promotion of ultrasonic irradiation on microbial fermentation were carried out with batch ultrasonic treatment method; there are very few reports using in situ ultrasound irradiation method during the fermentation process. It is no doubt that an in situ ultrasound treatment method can more suitable for industrial fermentation application than the batch treatment method.

Recently, our research group developed a novel ultrasound equipment, viz. a multi -frequency scanning slot ultrasonic equipment, which can work in sweeping frequency (SF) or fixed frequency (FF) working modes, and six different frequencies. The difference between SF and FF depends on their frequency. The frequency of SF is designated as$\alpha \pm \delta$in which δ is much lesser than α (the center frequency). But the frequency of FF is fixed. SF provided ultrasonic effects such as cavitation, mechanical and heating effects as FF did to rupture protein granules and facilitate the disintegration of particles. Furthermore, SF ultrasound could provide a wider range of frequencies than FF ultrasound to make the sound field distribution more uniform, and avoid the appearance of working “dead zone”. The objective of this study was to explore the influences of ultrasonic treatments with different working modes and various frequencies on the bacterial growth and metabolism yield of *S. cerevisiae* using a method of in situ ultrasonic irradiation for fermentation promotion. The study can be divided into two related parts: (1) to investigate the effects of ultrasound working modes on growth and metabolism yield of *S. cerevisiae*; (2) to study the impact of ultrasound with six different frequencies on the growth and metabolism yield of *S. cerevisiae*. This study may provide some valued results for the prospective application of ultrasound promotion technology in fermentation industry.

2. Materials and methods

2.1. Microorganism, maintenance and preparation

S. cerevisiae CICC 1048 was obtained from China Center of Industrial Culture Collection as a freeze-dried powder in a glass vial and was activated in a malt extract medium (prepared by dissolving 130 g malt extract powder and 0.1 g chloramphenicol in 1 L of distilled water and autoclaved at 121 °C for 15 min) and incubated at 30 °C and 160 rpm [13] for 24 h. After a further incubation on agar slant medium at 30 °C for 48 h to form clear colonies, they were transferred to a refrigerator (4 °C) and used for inoculum preparation.

Fermentation medium was prepared by dissolving 0.5 g yeast powder, 1 g peptone, 25 g glucose, 0.5 g ammonium sulfate, 1 g potassium dihydrogen phosphate, 0.05 g Magnesium Sulfate and 0.015 g calcium chloride in 100 mL of distilled water and autoclaved at 115 °C for 20 min. Seed culture of *S. cerevisiae* (under the optimum ultrasonic conditions: frequency (28 kHz), power density (140 W/L) and sonication time (1 h)) was incubated at 30 °C and 160 rpm for 12 h, which would be used for the following seed culture.

2.2. Experiment on in situ ultrasound assisted fermentation of S. Cerevisiae

In this study, the fermentation system consists of the multi-frequency power ultrasonic equipment (left) and the thermostatic fermentation bottle (right) (Fig. 1). The ultrasonic slot is equipped with six frequency generators, which are 20, 23, 25, 28, 33 and 40 kHz. It was employed to study the influence of ultrasound treatment on the bacterial growth and metabolism production of *S. cerevisiae*. The experiment included two parts: i) exploring the ultrasonic working mode, i.e., fixed frequency mode and sweeping frequency mode; ii) screening the optimum frequency at the suitable ultrasonic working mode by comparing different frequencies’ effects on *S. cerevisiae* growth and fermentation production.

2.2.1. Effect of ultrasonic working modes on S. cerevisiae growth and metabolite production

The prepared fermentation *S. cerevisiae* strains seed liquid was added to the fermentation bottle at the ratio of 4%, and the bottle was sealed using the sterilized gauze and fully shaken. Then, the fermentation bottle was placed in the water bath and connected to ultrasonic slot which had been sterilized before connection. The whole fermentation time was set as 48 h (based on pre-experiment of fermentation, in which it was found that the residual sugar content was very low after 48 h fermentation). During the fermentation, a fast circulation of liquid can make the sonication treatment more homogeneously; however, too fast flow rate may cause excessive shear stress on microbial, which might lead to cell disruption. Therefore, a moderate flow rate was suitable in this experiment. Considering those factors, the fermentation sonication and conditions were as following: fermentation temperature 30 ℃, in situ ultrasound treatment sonication time 48 h (pulse durations of on-time 30 s and off-time 10 s), , ultrasonic power density 50 W/600 mL liquid (i.e., around 83.3 W/L) and the liquid flow rate 100 mL/min [13]; the ultrasonic work modes are fixed frequency mode and sweeping frequency mode, i.e., FF of 28 kHz, SF of 28 ± 2 kHz, FF of 33 kHz and SF of 33 ± 2 kHz. Compared with the non-sonicated samples, cell concentration, death rate of cells, residual sugar content, ethanol yield and metabolite yield were recorded as the indexes in the process of fermentation samples, to investigate the effect of fixed frequency and sweeping frequency of two kinds of ultrasonic working modes on the growth of *S. cerevisiae* and its metabolite production.

2.2.2. Effect of ultrasonic frequencies on S. Cerevisiae growth and metabolite production

In situ ultrasound of six different frequencies (20, 23, 25, 28, 33 and 40 kHz) with their suitable ultrasonic working mode (fixed or sweeping frequency mode, according to the research result of Section 2.2.1), were employed respectively in the fermentation process of *S. cerevisiae* to explore the frequency influence on the microbial growth and fermentation production. The fermentation conditions were similar to Section 2.2.1. The samples were processed and analyzed according to the previous method (Section 2.2.1).

2.3. Determination of experimental index

2.3.1. Cell concentration

Cell concentration was determined with turbidimetry method, by adding 0.5 mL of fermentation liquid to the fermentation medium containing 4.5 mL sterilization raise test tube was fully mixed. The absorbance value was analyzed at 600 nm wavelength using the spectrophotometer (UV-1100, Purkinje General Co., Ltd., Beijing, China).

2.3.2. Death rate of S. Cerevisiae

The death rate of *S. cerevisiae* was investigated by methylene blue staining[19,20]. The fermentation broth was diluted to a certain ratio of 1 mL and placed in methylene blue staining solution of 1 mL for 5 min, and the dead *S. cerevisiae* cells were stained blue. The death rate of *S. cerevisiae* was calculated by counting the number of dead yeasts and total number in the unit volume using the cell count plates.

2.3.3. Residual sugar content

Residual sugar content in fermentation broth was measured by 3,5-two nitro salicylic acid colorimetric (DNS) method [21]. Glucose standard solution (1 mg/mL, w/v) was prepared by dissolving 1 g of glucose (dry basis) in 1 L distilled water. Glucose standard solution (ranged from 0 to 1.0 mL) was respectively mixed with distilled water in 1 mL test tube. 2 mL of DNS solution was added into the test tube using the pipette, and allowed to stand for 5 min at a temperature of 100 ℃ (water bath, SHZ-88A, Taicang Laboratory Equipment Factory, Jiangsu, China). The samples were cooled to room temperature. The absorbance was measured at wavelength of 540 nm using the spectrophotometer (UV-1100, Purkinje General Co., Ltd., Beijing, China). Based on the results, the standard equation ($y = 1.025x$-0.02133) was concluded between absorbance values (y) and glucose concentration (x) ($R^2 = 0.9962$). The fermentation liquid was taken out at the fermentation time of 0 h, 4 h, 8 h, 12 h, 16 h, 24 h, 28 h, 32 h, 40 h and 48 h. The samples were diluted to an appropriate concentration after centrifugation at 4000 rpm for 5 min. 1 mL of supernatant was mixed with distilled water in 25 mL scale test tube. The absorbance was estimated at 540 nm by the spectrophotometer, and the content of residual sugar was calculated according to the standard equation.

2.3.4. Ethanol content

Ethanol content was determined by potassium dichromate method with the reduction of alcohol [22]. The standard ethanol solution (0.5%, v/v) was prepared by mixing 5 mL of absolute ethanol with distilled water into 1 L ethanol volumetric flask. Ethanol standard solution (ranging from 0 to 4.0 mL) was respectively taken into the test tube, and 3 mL of potassium dichromate solution was added. The solutions were allowed to stand for 15 min, and then were diluted to 10 mL with distilled water. The value of absorbance was measured at 610 nm by the spectrophotometer (UV-1100, Purkinje General Co., Ltd., Beijing, China). From the results, the equation was concluded between absorbance values (C) and ethanol content (A). The standard equation was $C = 0.2213A + 0.0079$, and the coefficient of determination (R^2) was 0.9988.

During the fermentation process, 50 mL of fermentation broth was taken from the sampling port of the fermentation system at 24, 32, 40 and 48 h, and then the sample was added to the distillation bottle with 50 mL of distilled water. The sampled liquid (45 mL) was mixed with distilled water in a 50 mL volumetric flask. 1 mL of the mixture was mixed with 3 mL of potassium dichromate solution in the volumetric flask (10 mL). The absorbance was measured at 610 nm using the spectrophotometer, and ethanol content was calculated by the obtained equation.

2.3.5. β-phenylethanol content

Detection of β-phenylethanol was investigated as outlined by Yang et al. [23] with slight alterations. The mobile phase of methanol–water (v/v = 1:1) was prepared, by dissolving 500 mL of chromatographic methanol in 500 mL of ultrapure water. The β-phenylethanol concentration (1 μL/mL) was obtained by dissolving 100 μL of β-phenylethanol in mobile phase with 100 mL volumetric flask. The concentration of the standard solution was diluted into 0.05, 0.0125, 0.01, 0.005 and 0.001 μL/mL, respectively. The diluted solutions were injected into a Kromasil C18 reversed-phase column (4.6 × 250 mm, 5 μm) of RP-HPLC (LC-20AT, Shimadzu Corporation, Japan). Elution was as follows: temperature 30 ℃, detection wavelength 210 nm, the injection volume 20 L, time 30 min. The standard chromatogram of β-phenylethanol was obtained. The standard equation was $Y = 9 \times 10^7 X + 24162$, ($R^2 = 0.9962$).

1 mL Of samples were drawn from the sampling port in a capacity bottle of 10 mL, and then fixed with ultrapure water, then filtered by using 0.22 μm organic membrane.

2.3.6. Volatile substances

2 mL of fermentation broth was mixed with 4 mL of ultrapure water in a volumetric flask. 3 mL of the mixture was extracted in the headspace solid-phase extraction flask, and styrene standard 0.069 mg/mL (10 μL) was added into the extraction bottle as the internal standard, and then NaCl solid (2 g) was added into the extraction bottle. The sample was allowed to stand at the temperature of 50 ℃ using water bath magnetic stirring (600 rpm, 5 min). The extraction head (50/30 μm DVB/CAR on PDMS) was put into the extraction bottle and the extraction time was continued for 40 min. After extraction, the fiber absorption head was retracted, and then the extraction needle was inserted into the inlet (250 ℃) of the GC/MS analyzer (Agilent

6890–5973, Agilent Technologies Co. Ltd., USA). SPME-GC/MS analysis was conducted after desorption for 5 min.

Gas chromatography conditions were as follows: injection port temperature for 250 ℃; initial temperature of column temperature for 40 ℃, standing for 3 min; temperature of 220 ℃ at a flow rate of 6 ℃/min, retention time for 5 min; Carrier gas (He); the flow rate 1 mL/min; the injection split ratio of 10:1.

Mass spectrometry conditions were as follows: EI ion source; ionization voltage of 70 eV; ion source temperature of 250 ℃; transmission line temperature of 220 ℃; scanning range of 33 ~ 450 amu. The composition of volatile compounds in the fermentation broth was determined by comparing the mass spectra of the sample with the automatic mass spectrometry library.

2.4. Statistical analysis

The experiments were performed in triplicate, and results were expressed as the means ± standard error. Tukey's test was used to indicate significance level of $P < 0.05$. All graphs were carried out with the Origin Pro15.0 (OriginLab Corporation, MA, USA) software.

3. Results and discussion

3.1. Effects of ultrasonic working modes on the growth and metabolite production of S. Cerevisiae

3.1.1. Effects of FF and SF on the growth of S. Cerevisiae

The growth of *S. cerevisiae* with the fermentation time from 0 to 24 h was investigated by ultrasound working modes as shown in Fig. 2. The results showed that *S. cerevisiae* grew rapidly at fermentation time of 2 ~ 14 h, then slowed down and reached its stable growth phase during fermentation time of 16 ~ 24 h. The cell growth rate and the final cell concentration by sonication were significantly higher than non-sonicated samples. The growth of *S. cerevisiae* depends on the media composition, the initial levels of pH, temperature and the airflow rate by shaking or dissolved oxygen [24]. This may be linked to the fact that sonication treatments may promote the transportation of small molecules in an either stagnant or relatively slow moving fluid medium, as well as the exchange of materials between intracellular and extracellular, leading to the acceleration of material and energy metabolism of *S. cerevisiae* [25,26]. Therefore, a reasonable improved mass transfer as a consequence of sonication [27] may potentially explain the observed increase in the cell growth rate and the final cell concentration of *S. cerevisiae*. Fig. 2 showed that cell concentration reached the highest value and the fastest growth rate at FF 28 kHz, compared with SF 28 ± 2 kHz. Additionally, the result of cell concentration for FF 33 kHz treatment was higher than SF 33 ± 2 kHz. Therefore, sonication FF was more obvious to promoting the growth of *S. cerevisiae*. SF sonication enabled the sample material resonate to limit the bacterial growth. However, another study showed that SF sonication appeared to improve the growth of microorganism. Huang et al. [18] showed that both cellular permeability and cell growth rate of *Candida tropicalis* were enhanced after SF treatment.

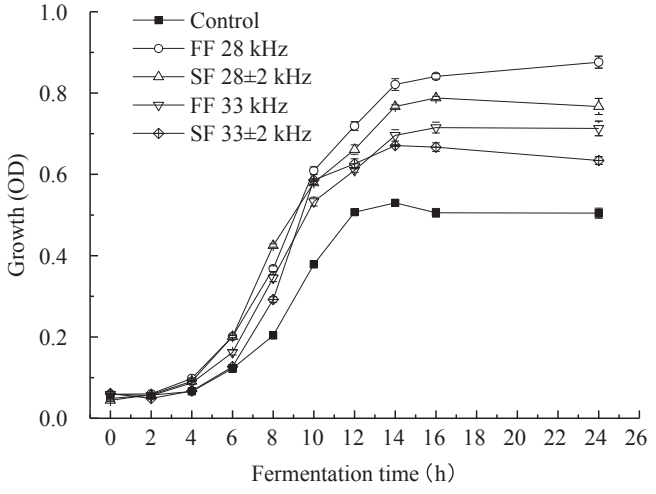

Fig. 2. Effects of ultrasound fixed frequency and sweeping frequency on growth of *Saccharomyces cerevisiae* (FF: Fixed frequency; SF: Sweeping frequency.)

3.1.2. Effects of FF and SF on death rate of S. Cerevisiae

The death rate of *S. cerevisiae* was determined, as shown in Table.1. Compared to the control group, FF 28 kHz had no significant difference at the other five time periods except for 16 h. The death rate of SF 28 ± 2 kHz was higher than the non-sonicated samples, and there was a significant difference between the four stages (4, 8, 16 and 24 h) ($P < 0.05$). The death rate was also higher at frequency of 33 kHz. Therefore, SF mode has a greater impact on the death rate of *S. cerevisiae*, and the death rate significantly improved at fermentation time from 0 to 24 h. The reason was that the frequency of resonance was greatly induced, and cavitation bubble [28] was more likely to collapse by SF, which would aggravate the damage of *S. cerevisiae* and lead to higher mortality. So FF mode has less damage to *S. cerevisiae*.

3.1.3. Effects of FF and SF on residual sugar content of S. Cerevisiae

The effect of FF and SF on the residual sugar content of *S. cerevisiae* fermentation broth at 0 ~ 48 h stage was shown in Fig. 3. After ultrasonic treatment, the residual sugar content in the fermentation broth was significantly lower than non-sonicated sample at the same phase. The content of residual sugar dropped to below 6 g/L at the culture time of 48 h, indicating that the fermentation was closing to the end. With the increasing of the cell concentration, the growth of *S. cerevisiae* was marked. Thus, the lower the residual sugar contents in the fermentation broth, the faster the rate of consumption (Fig. 2 and Fig. 3). Ultrasound treatment could promote the transfer of oxygen in the liquid [14,27], which might be the reason for the rapid consumption of sugar after ultrasound treatment. In conclusion, ultrasound was beneficial to the growth and fermentation of *S. cerevisiae*, and FF was better than SF.

3.1.4. Effects of FF and SF on ethanol yield of S. Cerevisiae

As shown in Fig. 4, when the sugar content was sufficient, the ethanol yield improved with the increasing of fermentation time, and the final ethanol yield by ultrasound treatment was higher than the control group. The ethanol yield at FF 28 kHz was higher than SF 28 ± 2 kHz and SF 33 ± 2 kHz, and the maximum value of ethanol yield was 12.05% (v/v) at the fermentation time of 40 h, which increased by 20.98% in comparison with the classical fermentation (48 h) yield of 9.97% (v/v); and the fermentation time was shortened by 16.67% (from 48 h to 40 h). During the fermentation process, the whole electricity energy consumption (including stirring, pumping, water bath heating and cooling) was around 0.6 kW; the power of ultrasonic equipment was 0.05 kW, which increased the electricity cost by 8.33%. However, considering the fermentation time reduction of 16.67%, the actual cost of electricity decreased 9.72% ((0.6 kW × 48 h-0.65 kW × 40 h) / (0.6 kW × 48 h) × 100%). And because the ethanol yield increased by 20.98%, the total profit of this ultrasonic assisted ethanol fermentation would increase more than 21%. However, the ethanol yield of SF 33 ± 2 kHz was higher than that of FF 28 kHz and FF 33 kHz at fermentation time of 48 h. This may be due to the fact that growth and reproduction of *S. cerevisiae* were faster and the metabolism was vigorous, leading to a lower sugar concentration from 40 to 48 h, and the decrease of ethanol yield and the volatilization of alcohol under sonication treatments at 28 and 33 kHz [29]. On the other hand, FF facilitated the metabolism of *S. cerevisiae* to produce other metabolic substances in the lower sugar concentrations [30].

Table 1
Effects of ultrasound fixed frequency and sweeping frequency on death rate of *Saccharomyces cerevisiae.*

Fermentation time (h)	Control	FF 28 kHz	SF 28 ± 2 kHz	FF 33 kHz	SF 33 ± 2 kHz
0	7.38 ± 1.95^{ab}	9.01 ± 0.88^{b}	6.56 ± 0.51^{a}	8.98 ± 0.60^{b}	7.00 ± 0.49^{ab}
4	8.85 ± 1.49^{a}	12.31 ± 2.42^{ab}	19.78 ± 0.82^{c}	13.79 ± 0.73^{b}	16.49 ± 4.18^{bc}
8	11.62 ± 2.73^{a}	11.57 ± 4.47^{a}	18.56 ± 1.74^{b}	12.30 ± 2.28^{a}	14.17 ± 0.92^{ab}
12	10.11 ± 1.54^{a}	11.62 ± 1.21^{a}	12.89 ± 3.47^{a}	14.27 ± 2.08^{a}	14.28 ± 3.23^{a}
16	14.08 ± 0.91^{a}	22.26 ± 3.10^{c}	35.81 ± 1.46^{d}	25.34 ± 4.27^{c}	28.16 ± 3.11^{c}
24	21.49 ± 5.49^{a}	25.38 ± 1.17^{c}	37.11 ± 2.07^{c}	25.74 ± 2.75^{ab}	30.69 ± 5.44^{bc}

Means ± SD (n = 3).
Within columns, mean values with same superscript are significantly different ($P < 0.05$).

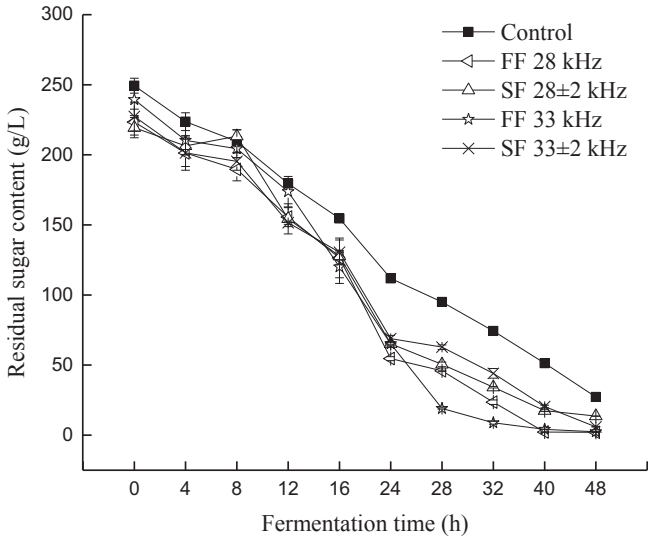

Fig. 3. Effects of fixed frequency and sweeping frequency ultrasound on residual sugar content.

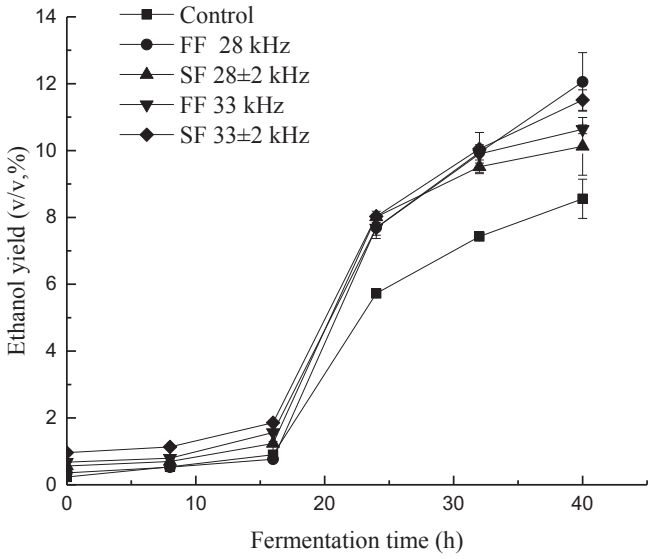

Fig. 4. Effects of fixed frequency and sweeping frequency ultrasound on ethanol yield of *Saccharomyces cerevisiae.*

3.1.5. Effects of FF and SF on metabolites yield of S. Cerevisiae

The typical chromatography about the metabolites was shown in Fig. 5. The effect of ultrasound on the yield of metabolites was evaluated by the ratio of the peak area of metabolites to the peak area of internal standard β-phenylethanol in fermentation broth, as shown in Table. 2. The highest content of β-phenylethanol was found in alcohol

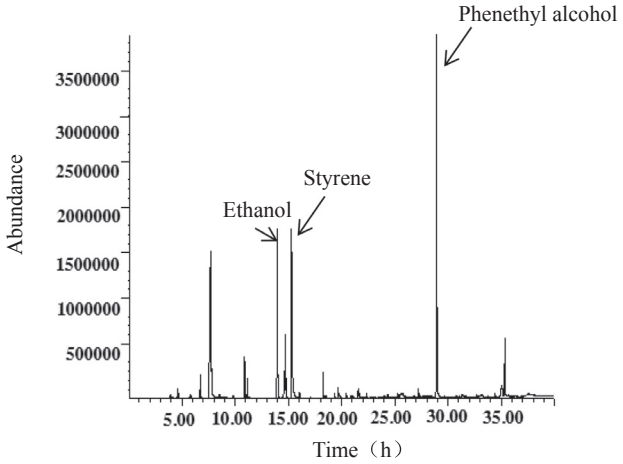

Fig. 5. Total chromatograms of volatile substances in fermentation liquid of fixed frequency ultrasound.

products. The β-phenylethanol, as a widely used in food flavor, has an important influence on the quality of liquor. But its boiling point was 219 ℃, which was not suitable for solid phase microextraction. Among other alcohols, 3-Methyl butanol content treated by FF was higher than that by SF, and the glycerol was only produced after FF treatment at 33 kHz. Ethyl caproate and phthalic acid two butyl ester are the main esters. The content of ethyl caproate by FF was similar to control, but it was relatively low in the fermentation broth after SF treatment. Phthalic acid two butyl ester was not found in non-sonicated sample, this because of ultrasound could stimulate the production of two butyl phthalate in *S. cerevisiae*. The acetic acid content had the maximum value in the acid compared with the other acids. The content of acetic acid after FF 33 kHz was significantly higher than SF 33 ± 2 kHz. Overall, the yield of metabolites after FF was higher than the SF, and the effect of FF 33 kHz was better than SF 33 ± 2 kHz. Thus, FF could enhance fermentation of *S. cerevisiae* and aid to produce more metabolites.

3.1.6. Effects of FF and SF on β-phenylethanol content

The effect of FF and SF on β-phenylethanol content in *S. cerevisiae* was shown in Fig. 6. The content of β-phenylethanol with FF group was significantly higher than SF group at the same frequency ($P < 0.05$). Cantagrel et al. [31] showed that β-phenylethanol derived from phenylalanine, and therefore its content was related to phenylalanine concentration. FF improved ($P < 0.05$) the content of β-phenylethanol compared to SF, and the reason may be that FF was helpful to derivatization of phenylalanine. Compared to the non-sonicated sample, the content of β-phenylethanol in fermentation broth did not significantly increase. Briefly, the effect of FF on *S. cerevisiae* fermentation was better than SF in the whole fermentation process. So, the sonication treatments were carried out with the mode of FF.

Table 2
Effects of ultrasound fixed frequency and sweeping frequency on metabolites yield of *Saccharomyces cerevisiae.*

Metabolites	Control	FF 28 kHz	FF 33 kHz	SF 28 ± 2 kHz	SF 33 ± 2 kHz
2-methyl propyl alcohol	4.86 ± 0.52^{bc}	3.25 ± 0.35^{a}	2.94 ± 0.69^{a}	5.00 ± 0.34^{c}	4.52 ± 0.67^{b}
3-methylbutanol	61.91 ± 2.28^{b}	81.38 ± 5.97^{c}	82.37 ± 9.24^{c}	53.70 ± 6.29^{a}	50.87 ± 2.94^{a}
2- hexyl hexyl alcohol	0.91 ± 0.09^{b}	—	—	—	0.69 ± 0.08^{a}
2,3-butanediol	7.37 ± 1.28^{b}	11.20 ± 0.97^{c}	22.88 ± 2.39^{d}	5.08 ± 0.94^{a}	4.52 ± 0.91^{a}
3- methyl propyl alcohol	2.33 ± 0.84^{c}	1.42 ± 0.34^{b}	2.25 ± 0.31^{c}	0.93 ± 0.34^{a}	1.29 ± 0.37^{b}
β-phenylethanol	168.19 ± 11.07^{b}	163.23 ± 12.19^{b}	206.76 ± 13.41^{c}	142.92 ± 10.69^{a}	142.24 ± 9.17^{a}
Glycerol	—	—	13.34 ± 1.31	—	—
Propanol	1.63 ± 0.16^{a}	1.33 ± 0.12^{a}	—	—	—
Ethyl acetate	15.93 ± 0.91^{b}	—	1.36 ± 0.11^{a}	—	—
Ethyl hexanoate	2.09 ± 0.24^{a}	15.66 ± 0.96^{c}	16.75 ± 1.97^{c}	10.64 ± 1.04^{b}	10.57 ± 1.46^{b}
Octanoic acid ethyl este	4.23 ± 0.49^{c}	0.30 ± 0.02^{a}	1.58 ± 0.16^{b}	1.22 ± 0.04^{b}	1.33 ± 0.09^{b}
Ethyl phenylacetate	1.83 ± 0.27^{a}	5.29 ± 0.57^{c}	7.07 ± 0.81^{d}	3.04 ± 0.27^{b}	4.58 ± 0.23^{bc}
Palmitic acid ethyl ester	—	1.40 ± 0.08^{b}	1.38 ± 0.09^{b}	0.98 ± 0.09^{a}	0.90 ± 0.06^{a}
Phthalic acid two butyl este	—	9.36 ± 1.09^{ab}	11.31 ± 0.68^{b}	8.38 ± 0.69^{a}	8.09 ± 1.21^{a}
Hexyl butyrate	7.35 ± 0.75^{c}	1.53 ± 0.81^{a}	1.53 ± 0.34^{a}	—	3.79 ± 0.97^{b}
Propionic acid 2- methyl este	8.08 ± 0.91^{b}	—	—	—	2.66 ± 0.39^{a}
Acetic acid	1.23 ± 0.19^{a}	15.47 ± 1.27^{c}	20.88 ± 1.39^{d}	6.06 ± 0.91^{b}	5.47 ± 0.64^{b}
Phenylacetic acid	3.18 ± 0.34	—	—	—	—
Caproic acid	1.18 ± 0.12	—	—	—	—
Bitter	4.86 ± 0.52^{c}	2.32 ± 0.19^{b}	5.44 ± 0.35^{c}	—	0.64 ± 0.02^{a}
Acetaldehyde	61.91 ± 2.28^{c}	5.51 ± 0.68^{b}	2.16 ± 0.19^{a}	2.30 ± 0.11^{a}	3.46 ± 0.19^{ab}
Nonaldehyde	0.91 ± 0.09^{a}	1.13 ± 0.09^{ab}	1.41 ± 0.17^{b}	—	—
Capraldehyde	7.37 ± 1.28^{b}	—	—	0.35 ± 0.07^{a}	0.37 ± 0.06^{a}

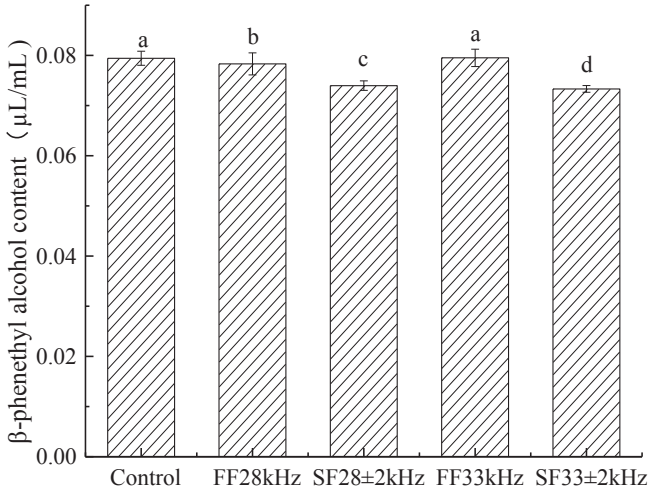

Fig. 6. Effects of fixed frequency and sweeping frequency ultrasound on β-phenylethanol of fermented liquid Vertical bars indicate standard deviations of the mean (n = 3).

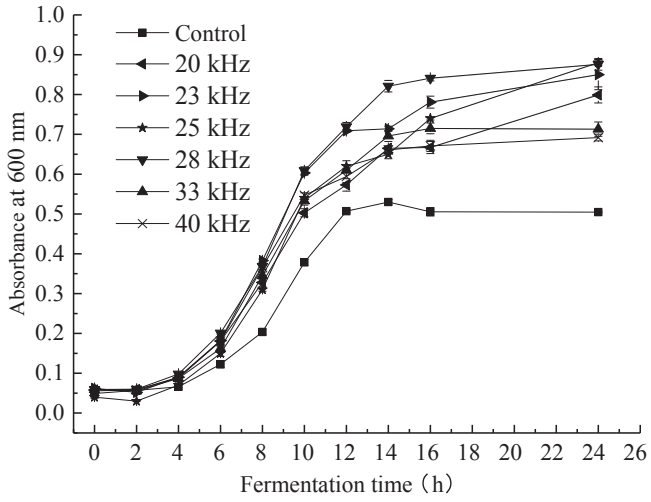

Fig. 7. Effects of various ultrasound frequencies on the growth of *Saccharomyces cerevisiae.*

3.2. Effects of different ultrasonic frequencies on the growth and metabolite production of S. Cerevisiae

3.2.1. Effects of ultrasonic frequency on the growth of S. Cerevisiae

In the fermentation process, the effect of different ultrasonic frequencies on the growth of *S. cerevisiae* was shown in Fig. 7. Cell concentration of *S. cerevisiae* after ultrasound treatment increased, and the growth rate was faster when the stable growth phase of *S. cerevisiae* reached up to 24 h in comparison with the traditional fermentation. The growth rate and cell concentration of 0 ~ 10 h were similar at different sonication frequencies. After fermentation time of 24 h, the final concentration with sonication frequency at 33 and 40 kHz were lower than that of other sonication frequencies (20, 23, 25 and 28 kHz). This indicated that when the ultrasound frequency was higher than 33 kHz, the growth of *S. cerevisiae* was weakened. This may be because of the maximum energy absorption, which was obtained at maximum ultrasonic frequency and fixed power density [32], which made the cell more stimulated and inhibited the growth of some cells.

3.2.2. Effects of ultrasonic frequency on death rate of S. Cerevisiae

The effect of six ultrasonic frequencies on the death rate of *S. cerevisiae* during fermentation process was shown in Table. 3. During fermentation time from 0 to 12 h, sonication had less effect on the death rate of *S. cerevisiae*. Higher death rate was observed by increasing the sonication time and frequency. When the fermentation time reached up to 24 h, there was no significant difference between sonication treatment (23 kHz) and non-sonicated sample (P greater than 0.05). Li et al. [33] studied the effects of different ultrasonic frequencies on the growth of Zhanjiang golden algae, and found that with the increase of ultrasonic frequency, the growth rate of the algae decreased. The reason may be that with the increase of ultrasonic frequency, the expansion of the air bubble in the liquid medium caused the severe tearing of the liquid when the expansion of the air bubble was large enough, leading to an increase in mortality of *S. cerevisiae*. Also, Sulaiman et al. [14] found that as the interval of ultrasound became shorten at the frequency of 20 kHz, the death rate of *S. cerevisiae* increased. Therefore, the frequency of 23 kHz was regarded as the optimal sonication frequency which had the least damage to *S. cerevisiae*.

Table 3
Effects of various ultrasound frequencies on death rate of *Saccharomyces cerevisiae.*

Frequency (kHz)	0 h	4 h	8 h	12 h	16 h	24 h
Control	7.38 ± 1.95^{a}	8.85 ± 1.49^{a}	11.62 ± 2.73^{a}	10.11 ± 1.54^{a}	14.08 ± 0.91^{a}	21.49 ± 5.49^{ab}
20	9.62 ± 1.15^{b}	14.02 ± 1.59^{ab}	14.22 ± 1.14^{b}	18.48 ± 1.68^{c}	25.12 ± 2.45^{c}	26.91 ± 3.55^{bc}
23	7.10 ± 1.62^{a}	17.57 ± 1.29^{b}	10.59 ± 2.81^{a}	12.69 ± 1.61^{ab}	20.39 ± 3.05^{bc}	20.75 ± 2.21^{a}
25	7.24 ± 1.61^{a}	11.27 ± 3.66^{ab}	12.78 ± 2.62^{ab}	13.10 ± 0.57^{ab}	15.90 ± 1.26^{ab}	27.15 ± 0.91^{c}
28	9.01 ± 0.88^{ab}	12.31 ± 2.42^{ab}	11.57 ± 4.47^{a}	11.62 ± 1.21^{ab}	22.26 ± 3.10^{c}	25.38 ± 1.17^{abc}
33	8.98 ± 0.60^{ab}	13.79 ± 0.73^{ab}	12.30 ± 2.28^{ab}	14.29 ± 2.08^{b}	25.34 ± 4.27^{c}	25.74 ± 2.75^{abc}
40	9.01 ± 0.88^{ab}	17.63 ± 6.27^{b}	12.13 ± 1.79^{ab}	12.65 ± 1.91^{ab}	24.05 ± 1.16^{c}	26.68 ± 1.25^{bc}

Means ± SD (n = 3).
Within columns, mean values with same superscript are significantly different (P < 0.05).

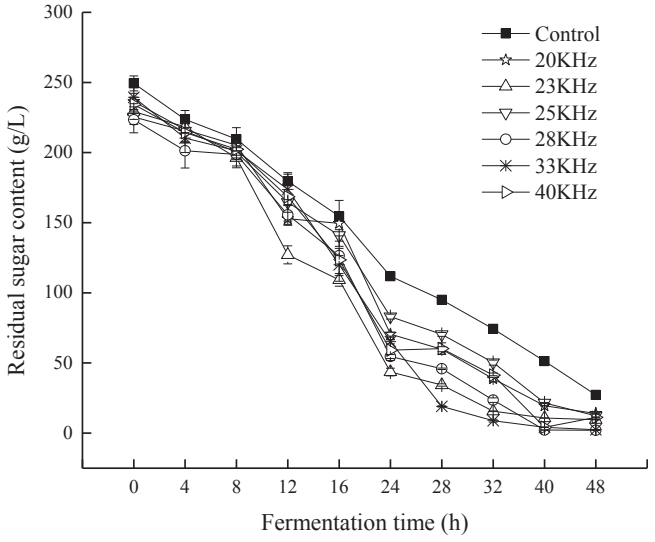

Fig. 8. Effect of various ultrasound frequencies on residue sugar content of fermented liquid.

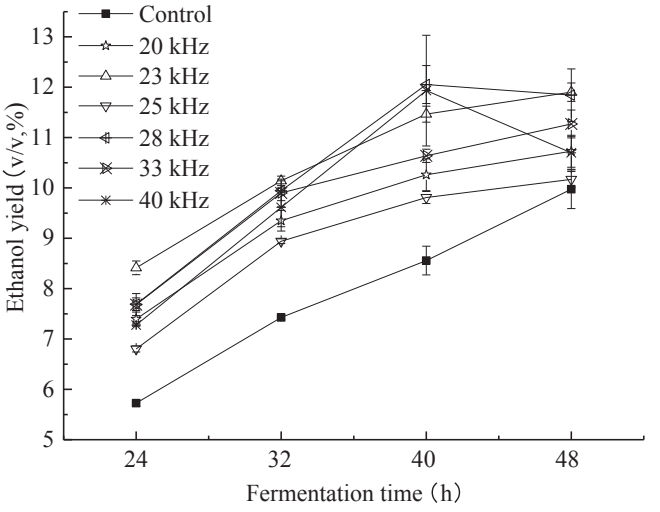

Fig. 9. Effects of various ultrasound frequencies on ethanol yield of *Saccharomyces cerevisiae.*

3.2.3. Effects of ultrasonic frequency on residual sugar content of S. Cerevisiae

The effect of different ultrasonic frequencies on residual sugar content in fermentation broth was shown in Fig. 8. The ultrasonic treatment could accelerate the consumption of sugar and shorten the fermentation time of *S. cerevisiae*. The sugar content decreased to a low level at fermentation time of 28 h at frequencies of 33 and 23 kHz. However, there was no corresponding trend between the fermentation rate and ultrasonic frequency. Matsuura et al. [34] found that ultrasound could shorten the fermentation time for wine. Thus, ultrasonic assisted fermentation of *S. cerevisiae* could accelerate the utilization rate of nutrient.

3.2.4. Effects of ultrasonic frequency on ethanol yield of S. Cerevisiae

Fig. 9 showed the effect of different ultrasonic frequencies on the ethanol yield from *S. cerevisiae*. Compared with the control, the production of ethanol in the fermentation broth by ultrasonic treatment significantly improved. This was consistent with the sugar consumption rate in Fig. 8. After fermentation time of 40 h, the ethanol yield by ultrasonic treatment was slower than that of the control, because the sugar content for sonicated sample was less than the control at 40 h fermentation time. The highest ethanol yield (11.90%) was obtained at ultrasonic frequency of 23 kHz at fermentation time of 48 h, which increased by 19.33% compared with the control group. Wood [35] found that ultrasound assisted fermentation of waste paper to produce alcohol could increase alcohol yield by nearly 20%. Sulaiman et al. [14] found that the ethanol yield from the yeast *Kluyveromyces marxiamus* fermentation after ultrasonic treatment was 3.5 times higher than the control.

3.2.5. Effects of ultrasonic frequency on the metabolite production of S. Cerevisiae

The effects of various ultrasonic frequencies on the production of metabolites of *S. cerevisiae* were shown in Table 4. The main alcohols contained 3 - methyl butanol, β- phenethyl alcohol and 2, 3- butanediol. The contents of 3 - methyl butanol and 2,3- butanediol increased with ultrasonic treatment except the frequency of 20 kHz. The content of ethyl caproate at the frequency of 23 kHz was increased by 40.74%, while the other frequencies had lower effects on ethyl caproate content. This may be that the frequency of 23 kHz was more suitable for promoting the metabolism of *S. cerevisiae* cells. The acetic acid in fermentation broth was mainly acetic acid, and acetic acid yield had the highest value at frequency of 33 kHz. In conclusion, sonication at 23 kHz was the best ultrasonic frequency from the perspective of the content of alcohols and lipids.

3.2.6. Effects of ultrasonic frequency on β-phenylethanol content

The effect of six ultrasonic frequencies on the content of β-phenylethanol in fermentation broth was shown in Fig. 10. The content of β-phenylethanol had the lowest value at frequency of 40 kHz, and there was a significant difference between the results treated with sonication of the other frequencies (P < 0.05). Compared with the control, sonication led to increase of β-phenylethanol content in fermentation broth, and there had the significant difference between the results (P < 0.05). This may be that the sugar was largely consumed at a frequency of 23 kHz, leading to the increase of metabolism content of β-phenylethanol. The content of β-phenylethanol was closely related to yeast strain and fermentation material [36,37]. Fukuda et al. [38] found that the sake K-9 yeast was treated by chemical mutagenesis, and the content of sake β-phenylethanol produced by the mutant strain was

Table 4
Effects of various ultrasound frequencies on metabolites of *Saccharomyces cerevisiae.*

Metabolites	Control	20 kHz	23 kHz	25 kHz	28 kHz	33 kHz	40 kHz
2-methyl propyl alcohol	4.86 ± 0.52^{b}	5.03 ± 0.74^{b}	5.19 ± 0.53^{b}	3.27 ± 0.46^{a}	3.25 ± 0.25^{a}	2.94 ± 0.19^{a}	9.46 ± 0.98^{c}
3-methylbutanol	61.91 ± 2.28^{a}	57.87 ± 3.19^{a}	110.36 ± 6.94^{c}	77.68 ± 6.81^{b}	81.38 ± 8.91^{b}	82.37 ± 6.47^{b}	84.62 ± 7.29^{b}
2- hexyl hexyl alcohol	0.91 ± 0.09^{b}	0.58 ± 0.08^{a}	2.03 ± 0.29^{c}	—	—	—	—
2,3-butanediol	7.37 ± 1.28^{ab}	5.36 ± 0.98^{a}	19.80 ± 1.91^{c}	19.21 ± 1.29^{c}	11.20 ± 0.99^{b}	22.88 ± 2.09^{d}	12.68 ± 1.91^{b}
3- methyl propyl alcohol	2.33 ± 0.84^{c}	1.36 ± 0.65^{a}	2.00 ± 0.91^{b}	1.85 ± 0.34^{b}	1.42 ± 0.28^{a}	2.25 ± 0.19^{c}	1.47 ± 0.24^{a}
β-phenylethanol	168.19 ± 11.07^{ab}	144.31 ± 8.92^{a}	216.39 ± 10.34^{c}	182.01 ± 11.27^{b}	163.23 ± 9.21^{ab}	206.76 ± 11.39^{c}	204.25 ± 10.03^{c}
Glycerol	—	—	1.47 ± 0.27^{a}	—	1.33 ± 0.19^{a}	—	—
Ethyl acetate	1.63 ± 0.16^{c}	0.88 ± 0.06^{a}	1.75 ± 0.19^{c}	2.56 ± 0.32^{d}	—	1.36 ± 0.19^{b}	1.30 ± 0.11^{b}
Ethyl hexanoate	15.93 ± 0.91^{b}	9.93 ± 0.71^{a}	22.42 ± 1.06^{c}	16.17 ± 1.20^{b}	15.66 ± 0.98^{b}	16.75 ± 1.07^{b}	17.89 ± 2.09^{b}
Octanoic acid ethyl este	2.09 ± 0.24^{d}	1.53 ± 0.19^{b}	2.49 ± 0.31^{e}	1.84 ± 0.35^{c}	0.30 ± 0.09^{a}	1.58 ± 0.13^{b}	1.74 ± 0.18^{bc}
Ethyl phenylacetate	4.23 ± 0.49^{a}	4.82 ± 0.31^{b}	6.83 ± 0.59^{c}	6.80 ± 0.42^{c}	5.29 ± 0.34^{b}	7.07 ± 0.61^{c}	5.40 ± 0.51^{b}
Palmitic acid ethyl ester	1.83 ± 0.27^{d}	1.35 ± 0.28^{a}	1.73 ± 0.19^{cd}	1.69 ± 0.07^{c}	1.40 ± 0.06^{ab}	1.38 ± 0.16^{ab}	1.42 ± 0.26^{b}
Phthalic acid two butyl este	—	10.13 ± 0.94^{b}	7.27 ± 0.78^{a}	9.35 ± 1.29^{ab}	9.36 ± 0.79^{ab}	11.31 ± 0.91^{b}	18.28 ± 1.29^{c}
glycerol	—	—	9.82 ± 0.97^{a}	15.35 ± 1.04^{c}	—	13.34 ± 1.31^{b}	12.05 ± 0.79^{ab}
Acetic acid	7.35 ± 0.75^{a}	6.27 ± 0.81^{a}	19.42 ± 1.26^{c}	14.41 ± 1.48^{b}	15.47 ± 2.19^{b}	20.88 ± 2.07^{c}	15.05 ± 1.24^{b}
Bitter	8.08 ± 0.91^{e}	1.00 ± 0.09^{a}	3.12 ± 0.39^{c}	2.88 ± 0.27^{bc}	2.32 ± 0.21^{b}	5.44 ± 0.43^{d}	3.19 ± 0.31^{c}
Acetaldehyde	1.23 ± 0.19^{a}	1.71 ± 0.16^{ab}	6.28 ± 0.73^{e}	4.35 ± 0.61^{c}	5.51 ± 0.51^{d}	2.16 ± 0.19^{b}	5.53 ± 0.37^{d}
Nonaldehyde	3.18 ± 0.34^{d}	1.63 ± 0.29^{b}	2.11 ± 0.21^{c}	2.14 ± 0.19^{c}	1.13 ± 0.17^{a}	1.41 ± 0.13^{ab}	—
Capraldehyde	1.18 ± 0.12^{c}	0.59 ± 0.09^{a}	—	0.75 ± 0.07^{b}	—	—	0.71 ± 0.06^{b}

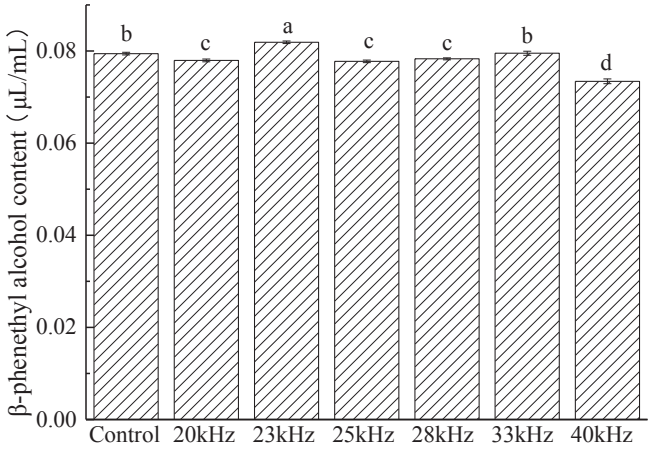

Fig. 10. Effects of various ultrasound frequencies on β-phenylethanol content of *Saccharomyces cerevisiae* Vertical bars indicate standard deviations of the mean (n = 3).

remarkably improved. Thus, sonication at 23 kHz had the best effect on fermentation process among all frequencies.

4. Conclusion

This work have shown that FF could significantly promote the growth and metabolism yield of *S. cerevisiae*. Sonication at FF of 23 kHz showed the optimal effect on fermentation process of *S. cerevisiae*. At FF of 23 kHz and fermentation time of 48 h, the yield of ethanol increased by 19.33%, and the content of β-phenylethanol and other metabolites such as esters also increased. However, the mechanism of FF on promoting the growth of *S. cerevisiae* is still not clear, which will be performed in our future research work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2019.02.017.

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