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Mild Sonication Accelerates Ethanol Production by Yeast Fermentation

Pulidindi Indra Neel,[†] Aharon Gedanken,*[,†] Rakefet Schwarz,[‡] and Eleonora Sendersky[‡]

[†]Department of Chemistry and Kanbar Laboratory for Nanomaterials at the Bar-Ilan University Center for Advanced Materials and Nanotechnology, and [‡]The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

ABSTRACT: Ethanol is a renewable and environmentally benign substitute for the current fossil-fuel-based transportation fuels. Fermentation of sugars, which is, in general, a slow process, is an essential step in the production of ethanol from renewable sources. This paper reports a successful attempt to accelerate the well-known sugar fermentation process by applying soft sonication. Fermentation of glucose was carried out using *Saccharomyces cerevisiae* under continuous mild ultrasonication conditions. The kinetics of the fermentation reaction was monitored by ^{13}C nuclear magnetic resonance spectroscopic analysis and weight loss measurements of the fermentation broth. The reaction rate constant was enhanced by 2.3 ± 0.2 and 2.5 ± 0.2 times as a result of sonication at 30 and 20 °C, respectively, as compared to a stirred reaction, and was about 10 times faster than non-stirred fermentation. The acceleration in the fermentation of glucose was observed for both 20 and 40 wt % concentrations of the glucose solution.

1. INTRODUCTION

"Noah planted a vineyard, produced wine and drank of the wine." Thus, the fermentation of sugars to ethanol is one of the oldest technologies known to mankind. Ethanol production refers to a process employing starch or cellulose as a starting material and culminating in the formation of ethanol.[1−3] This process is based on the chemical or enzymatic breakdown of the raw material to simple sugars followed by fermentation.[4−9]

Fermentation is the most vital and unavoidable process among several steps (pretreatment, hydrolysis, fermentation, and distillation) involved in the production of ethanol from plant materials.[10] It is, however, a time-consuming process and not free from microbial contaminations as well.[11,12] This is one of the challenges faced by the upcoming ethanol industries. Several attempts have been made to accelerate the fermentation process.

Saita et al. increased the fermentation rate of sugars (D-glucose and maltose) by a factor of 2 by changing the concentration of NH_4^+ ions in the fermentation broth from 50 to 250 mg of nitrogen (N) L^{-1}. Such an increase in the fermentation rate was attributed to the improved protein synthesis by *Saccharomyces cerevisiae*.[13] SivaRaman et al. reported the reduction of the fermentation period from 24 to 16 h for the fermentation of sugar cane molasses (total sugar concentration of 150 g L^{-1}) in the presence of synthetic zeolite (20 g L^{-1}). The acceleration in the fermentation process was attributed to the removal of the components inhibiting the fermentation process and also to the changes in the flocculation behavior of the yeast.[14] Matsuura et al. succeeded in reducing the fermentation periods by 50−64% for the fermentation of saccharified rice solution employing weak ultrasonic waves (intensity, 30 mW/cm^2; frequency, 43 kHz). The acceleration of the fermentation was attributed to the acceleration of cell growth as well as the decrease in dissolved CO_2 in the broth.[15] Nakanishi et al. have increased the sugar (10% glucose) consumption and ethanol production by a factor of 2 by employing an electric current as a stimulant for the growth of *S. cerevisiae* OC-2 (wine yeast). The increase in ethanol production was attributed to the increase in the cell growth rate.[16]

The above-mentioned reports suggest that the obstacle of the slowness of the fermentation reaction can be surmounted. The objective of this research is to evaluate the effect of mild ultrasound on the kinetics of the fermentation of glucose, a model carbon source for the yeast, *S. cerevisiae*. The current paper reports the successful acceleration of the fermentation of glucose and also the possibility of fermenting a 40% glucose solution at an accelerated speed.

2. EXPERIMENTAL SECTION

2.1. Strain and Fermentation Conditions. Glucose fermentation was carried out with the aid of yeast, *S. cerevisiae*, a strain of commercial baker's yeast procured from a supermarket. D-Glucose was obtained from Sigma Aldrich. Fermentation was carried out in 100 mL Erlenmeyer flasks. The fermentation medium comprises of 10.0 g (or 20.0 g for the 40% solution) of glucose dissolved in 50 mL of water to which 1.0 g of yeast is added. The flasks were closed with a cotton plug. Fermentation reactions were carried out under continuous irradiation with mild ultrasonic waves of 40 kHz frequency generated in an ultrasonic bath [MRC, Clean-01 ultrasonic cleaner (dental) of 4.5 L volume with an input current of 220−240 VAC and a frequency of 40 kHz]. The fermenter flasks were suspended in the water medium with the aid of stands in the bath sonicator with an input power of 120 W. Both the test and the control flasks were held in the bath sonicator, containing water as the sound transfer medium, with the aid of a stand in such a way that the whole of the fermentation broth in the flask is exposed to the sound irradiation. The sonicator (of 4.5 L volume) is filled with 4 L of water. The bath has a built-in temperature control. To cross-check the exactness of the temperature, a thermocouple is inserted in the fermentation broth throughout the fermentation

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reaction and the required temperature (20 and 30 °C) is maintained. In addition, to maintain the exact values of the required temperatures, a Julabo (FT901) was placed in the bath sonicator throughout the fermentation reaction. All of the conditions for the control experiments were identical to those of the test experiments. The control experiments were carried out on regular stirring (magnetic stirrer, model MH-4, Fried Electric, 1000 rpm) with a provision to maintain the required temperature with a Julabo immersion cooler (FT 901 series). The same lot of yeast is used for both the control and test experiments. The experimental time periods of the fermentation reaction include the lag phase. The kinetics of the fermentation process was monitored by ^{13}C nuclear magnetic resonance (NMR), as well as a weight decrease of the fermentation broth because of CO_2 release.

2.2. ^{13}C NMR Analysis. In addition to weight decrease measurements, aliquots of samples were collected from the fermentation broth at regular intervals and were analyzed by ^{13}C NMR spectroscopy. D-Glucose resulted in a characteristic spectrum with typical peaks at 60.6 (C6), 69.5 (C4), 72.7 (C2), 75.7 (C3), 75.8 (C5), and 95.8 (C1). Ethanol yielded two characteristic peaks at 16.8 and 57.4. The relative intensity of the peaks of ethanol (16.8) and glucose (95.8) has been found to increase with time during the fermentation process and is employed as a measure of the conversion of glucose, as shown in eq 1

$$\text{conversion of glucose (wt \%)} = (I_{E,16.8\ \text{ppm}}/2I_{G,95.8\ \text{ppm}}) \times 100 \quad (1)$$

where I_E is the intensity of the ethanol peak at 16.8 ppm and I_G is the intensity of the glucose peak at 95.8 ppm.

^{13}C NMR spectra were recorded on a Bruker Avance DPX 300.

2.3. Monitoring the Decrease in Weight of the Fermentation Broth as a Function of Time. A total of 1 mol of D-glucose produces 2 mol of CO_2 that escape the system according the following stoichiometric equation (eq 2):

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (2)$$

This phenomenon is reflected in a weight decrease, which can be correlated to the amount of ethanol produced.[18]

Theoretically, every 1 g of glucose can yield 0.51 g of ethanol. Nearly 50% of glucose is used to produce ethanol, and 50% is used to produce CO_2. Thus, there is a weight decrease as a result of the amount of CO_2 formed and removed from the system, and this is a direct reflection of the amount of ethanol produced. The conversion of glucose to ethanol is deduced from the weight decrease according to the following equation (eq 3):

$$\text{conversion of glucose (wt \%)} = (\text{observed decrease in weight}/\text{total decrease in weight expected}) \times 100 \quad (3)$$

The weight loss because of the evaporation of water during the course of the fermentation process has been corrected. An Erlenmeyer flask with water alone has also been subjected to sonication along with the flask with the fermentation broth. The loss in the weight in the case of the flask with water alone is due to evaporation, and this value is subtracted from the weight loss observed in the case of the fermentation reaction vessel. Thus, the gravimetric assay exclusively accounts for the loss of CO_2.

The weight of the fermentation broth was measured at regular intervals on an electronic balance Precisa 205 ASCS.

2.4. Evaluation of the Reaction Rate Constant. The glucose conversion values as a function of time, obtained from both weight loss measurements and ^{13}C NMR analysis, were inserted into the first-order reaction rate equation, as shown below, to obtain the reaction rate constant as a function of time

$$k = (2.303/t)\log[a/(a - x)]$$

where t is the time, a is the initial concentration of glucose, and x is the conversion of glucose at time t. The rate constant values cited are at some specific time period based on the conversion values of glucose to ethanol during the period of time.

2.5. Microscopic Studies. The microscopic images of the yeast from the fermentation broth were recorded using Axio Imager Z1.

3. RESULTS AND DISCUSSION

3.1. Evaluation of Kinetics of the Fermentation of Glucose. The fermentation reaction of glucose was carried out at 20 and 30 °C under bath sonication and control (stirring) conditions. Control experiments were carried out under regular stirring with other conditions remaining identical. The values of the conversion of glucose, under sonication and control conditions, as a function of time, deduced from the weight decrease[17] in the fermentation broth and also by ^{13}C NMR[19] are represented pictorially in Figure 1. A good correlation

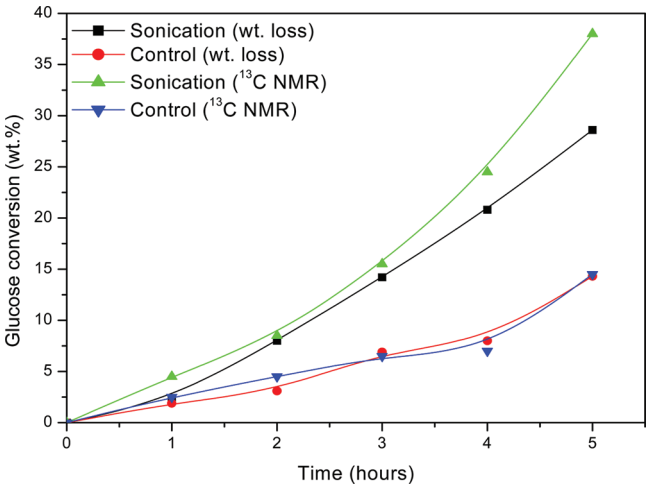

Figure 1. Effect of ultrasonic sound on the conversion (wt %) of glucose at 30 °C.

between the glucose conversion values deduced by both methods is obtained. Substitution of the experimental data on the glucose concentration values at different time periods, in the first-order reaction rate equation, yielded nearly constant values of k, indicating that the reaction of fermentation of glucose follows first-order reaction kinetics.

The mean of the rate constant values obtained from weight loss measurements (13.4×10^{-6} s^{-1}) and ^{13}C NMR analysis (17.3×10^{-6} s^{-1}) are in reasonable agreement and are considered as the rate constant for the reaction of fermentation of glucose under bath sonication at 30 °C. Thus, the reaction rate constant for the fermentation of glucose under sonication at 30 °C ($k_{\text{sonication, 30 °C}}$) is 15.35×10^{-6} s^{-1}. The reaction rate constant for the fermentation of glucose under control (stirring) conditions at 30 °C ($k_{\text{stirring, 30 °C}}$) is 6.67×10^{-6} s^{-1}. Thus, the use of continuous mild (frequency, 40 Hz; input power, 120 W) ultrasonic waves has accelerated the glucose fermentation by 2.3 times at 30 °C.

Similarly, the effect of bath sonication on the reaction rate of glucose fermentation was evaluated at 20 °C. The reaction rate constant values under sonication ($k_{\text{sonication, 20 °C}}$) and control ($k_{\text{stirring, 20 °C}}$) conditions were found to be 6.31×10^{-6} and 2.46×10^{-6} s^{-1}, respectively. Thus, ultrasonic sound has an accelerating effect on the glucose fermentation reaction at both 30 and 20 °C by a factor of 2.3 and 2.5, respectively.[19]

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The glucose conversion (wt %) values under non-stirred conditions (30 °C) at 11, 14, and 21 h are 10, 16 and 54%. Under stirring and bath sonication conditions, the glucose conversion values at 11 h are 65 and 100%, respectively. Thus, the fermentation under stirred and bath sonication conditions were 6.5 and 10 times faster than the unstirred reaction.

3.2. Monitoring the Fermentation (Glucose) Reaction until Completion. The potential of ^{13}C NMR for monitoring the glucose fermentation reaction has been elucidated for the first time. The fermentation reaction has been monitored until completion by ^{13}C NMR spectroscopy by collecting aliquots of samples from the fermentation broth at regular intervals.

The ^{13}C NMR spectra of the aliquots of samples collected from the fermentation broth maintained under continuous bath sonication at 30 °C and the corresponding control maintained under stirring at 30 °C are shown in panels A and B of Figure 2,

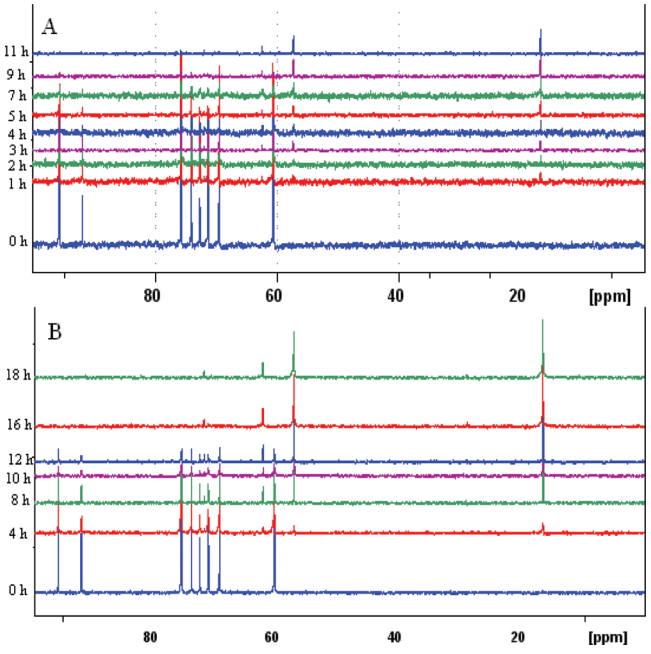

Figure 2. ^{13}C NMR spectra of aliquots from the fermentation broth under (A) bath sonication and (B) control (stirring) conditions at 30 °C collected at regular time intervals.

respectively. In Figure 2A, it is observed that, as a function of time, the intensity of the peaks corresponding to ethanol (16.8 and 57.4 ppm) increases and the intensity of peaks corresponding to glucose [60.6 (C6), 69.5 (C4), 72.7 (C2), 75.7 (C3), 75.8 (C5), and 95.8 (C1)] decreases. In the 11 h sample, no trace of glucose is observed, indicating the completion of the fermentation reaction. Close examination of the spectra reveals that, in addition to ethanol, a small amount of glycerol is formed as a secondary metabolite during the fermentation process, as indicated by the two peaks located at 62.5 and 72.0 ppm. ^{13}C NMR spectra of the aliquots of samples collected from the control (stirring, 30 °C) are depicted in Figure 2B. The spectral features of the reaction products are analogous to those obtained under bath sonication conditions. The intensity of peaks corresponding to glucose decreases as a function of time, and no glucose is detected in the sample after 18 h, indicating the completion of the fermentation reaction. Thus, the time required for the completion of the fermentation reaction has been reduced, from 18 to 11 h, by the use of continuous mild ultrasonic waves at 30 °C.

The glucose fermentation reaction has also been carried out at 20 °C under bath sonication conditions and monitored by ^{13}C NMR spectroscopy until completion. The corresponding control reaction was carried out at 20 °C under stirring conditions. ^{13}C NMR spectra of aliquots of samples were collected from the fermentation medium, maintained under continuous sonication as well as control conditions. In the case of the fermentation broth maintained under bath sonication, no trace of glucose is observed in the aliquot of the sample collected at 19 h, whereas the fermentation reaction under stirring conditions took 36 h for the complete disappearance of glucose.

To further evaluate if the yeast could be reused even after exposure to the ultrasonic sound, the yeast was separated from the fermentation broth maintained under bath sonication at 20 °C for 19 h by centrifugation. The yeast thus separated was washed with distilled water and recentrifuged to remove traces of ethanol that were carried over from the previous fermentation medium. The washed yeast cells were inoculated into a fresh fermentation medium (10.0 g of glucose + 50 mL of water), followed by sonication at 20 °C. The fermentation ability of these cells was monitored by ^{13}C NMR. With the reused yeast, the fermentation is completed in 21 h, as observed from the absence of glucose peaks in the aliquot of the sample collected at 21 h. This indicates that the yeast is reusable.

In addition to carrying out the fermentation reaction at a glucose concentration of 20%, we have also examined the fermentation process at higher concentrations of glucose. The fermentation reaction was carried out with 40% glucose concentration (20 g of glucose in 50 mL of H_2O). The fermentation reaction was monitored for 23 h at 30 °C. The glucose conversion values deduced from the weight decrease in the fermentation system both under sonication and control (stirring) conditions are shown in Figure 3. With the use of continuous mild ultrasonic waves, the glucose conversion rates are nearly 2 times faster than the conventional fermentation (control), even at a higher glucose concentration of 40%.

It is worth mentioning that the bath sonicator could not be operated for more than 23 h because of some mechanical problem. However, the control stirred reaction was followed for

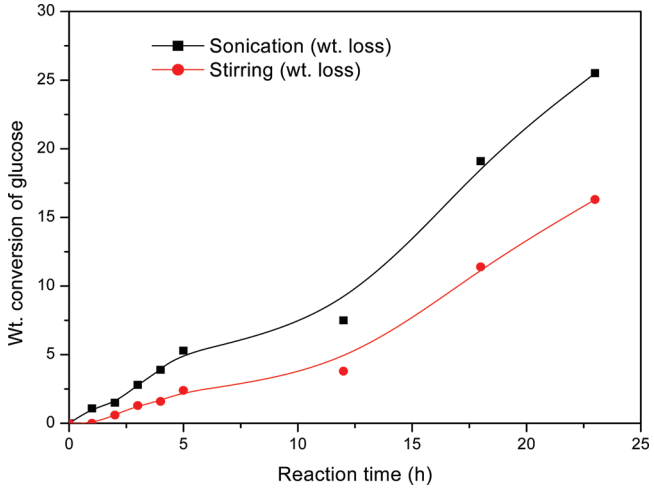

Figure 3. Effect of ultrasonic sound on the conversion (wt %) of glucose (40 wt % concentration).

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the 40% solution until completion (268 h). The ability to ferment 40 wt % glucose solution is demonstrated clearly in Figure 3.

3.3. Microscopic Analysis of the Yeast. The yeast cells from the fermentation broth maintained at 20 wt % glucose concentration under bath sonication and control (stirring) at 30 °C after 30 min of reaction were collected and examined under microscopy. The microscopic images of the yeast cells under control (stirring) and bath sonication conditions are shown in panels A and B of Figure 4, respectively. In the case of

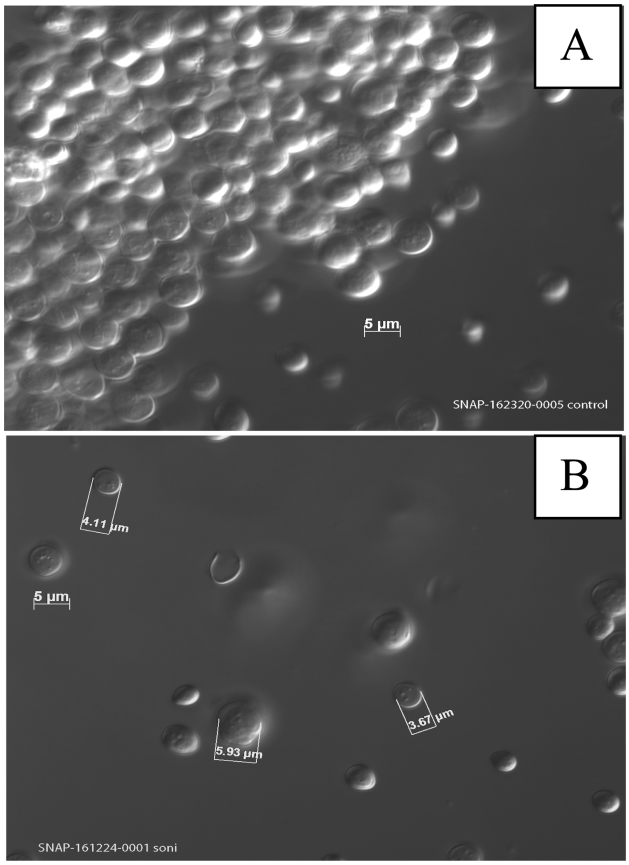

Figure 4. Microscope images of yeast in the fermentation broth (20 wt % glucose) after 30 min for (A) control (stirring) and (B) bath sonication at 30 °C.

the control reaction, aggregates of yeast cells are observed (Figure 4A), whereas in the bath-sonicated samples, well-dispersed individual yeast cells are observed (Figure 4B). Thus, bath sonication is effective in dispersing the yeast cells in the fermentation medium.

To evaluate if the sonication effect is due to effective deagglomeration of the yeast cells, the dry yeast cells were inoculated into H_2O without glucose and subjected to bath sonication for 45 min. To this solution, containing well-dispersed yeast cells, glucose was added and the fermentation reaction was carried out under normal control (stirring) conditions without any further sonication after glucose addition. In this case, no enhancement in the fermentation rate was observed. Thus, the observed acceleration of the kinetics of the fermentation reaction is not due to the disaggregation of the yeast cells.

We can also exclude any sonochemical effect arising from the collapse of acoustic bubbles. This was revealed when conducting an experiment with a horn-type sonicator, where after 30 min, the yeast cells were ruptured and no ethanol was obtained.

As expected, yeast proliferation did not occur during the fermentation reaction, as indicated by the constant optical density at 600 nm. This is in accordance with the particular experimental setting used here because the fermentation reaction included only glucose but none of the other nutrients essential for growth. Similar results were observed when sucrose was fermented using the same yeast and the soft sonication.

In our view, the soft sonication introduces in the system a strong stirring and this causes the acceleration of the fermentation. Clearly, the acceleration of fermentation shown in this study is not proceeding via growth acceleration of the yeast cells, contrary to previous studies that explained enhanced ethanol production by a growth effect.[5,6] The stirring may help to remove the ethanol from the yeast surface and, thus, facilitate the fermentation process. Additionally, it may be suggested that the strong stirring under sonication helps in the removal of CO_2 (reducing the latter in the broth was shown to accelerate fermentation[15,20]). We base this interpretation on the comparison of the conventional stirred reaction and the non-stirred reaction. Regular stirring is shown in this study to speed up the fermentation rate by a factor of 6.5, as compared to the non-stirred reaction. The sonication provides a more effective stirring and, thus, may augment the effects of regular stirring. We do not rule out, however, additional effects of sonication, which may affect the fermentation process, e.g., changes in membrane permeability and an increase in mass transfer.

3.4. Estimation of Energy Efficiency of the Ethanol Production Process Based on Soft Sonication. An estimate of the energy return on energy invested (EROEI) has been made. The ratio of the heating value of the ethanol produced divided by the sum of the heating values of the input glucose and the sonication energy is a measure of the EROEI and is a reflection of the energy efficiency of the process. On the basis of the calculations shown below, the value of EROEI was found to be 0.898.

Heating value of ethanol = 1300 kJ/mol.

Heating value of glucose = 2888 kJ/mol.

Sonication energy consumed per hour = 55 W.

Time of sonication for 100% conversion of glucose = 11 h.

Total amount of glucose used = 10 g.

Yield of ethanol upon complete conversion of glucose = 0.51 g.

Heating value of ethanol/(heating value of glucose + sonication energy) = $(5.1 \times 1300/46.07)$ kJ/$[(2888 \times 10/180.16) + (11 \times 55 \times 2.7778 \times 10^{-4} \times 10^{-3})]$ kJ = 143.9 kJ/$(160.3 + 0.17 \times 10^{-3})$ kJ = 0.898.

Even though the value of EROEI is slightly lower than 1, the current process of ethanol production based on sonication is still attractive, owing to the scope for further improvements in the efficiency.

4. CONCLUSION

Use of continuous mild ultrasonic waves generated by a bath sonicator accelerated the glucose fermentation reaction by a factor of 2.3 and 2.5 at 30 and 20 °C, respectively, as compared to a conventional stirred reaction. The enhancement in the speed of the reaction is observed at 20 wt % glucose concentration and also at a higher concentration of 40 wt % glucose. The yeast, *S. cerevisiae*, employed for the fermentation

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of glucose under sonication conditions is reusable. Exposure to mild ultrasound has not decomposed the yeast cells. The current sonochemical process accelerating the fermentation of glucose is useful not only to the wine industry but also to the upcoming ethanol industries.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 972-35318315. Fax: 972-7384053. E-mail: gedanken@mail.biu.ac.il.

Notes

The authors declare no competing financial interest.

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