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Study on Fermentation Kinetics for Accelerated Production of Bioethanol from Glucose, Sucrose and Molasses

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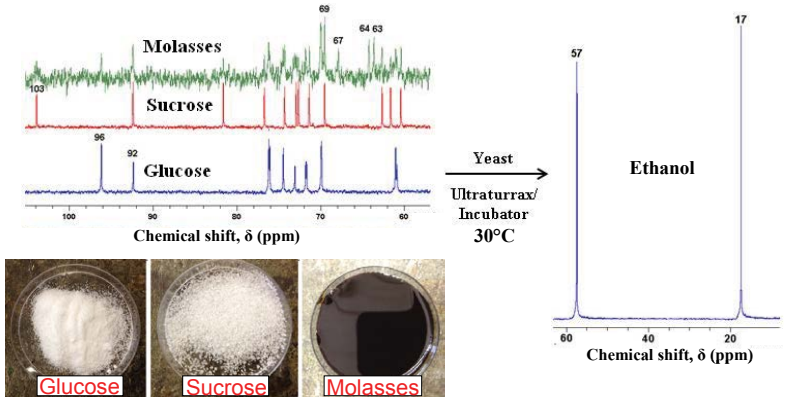
Abstract

Currently, fermentation is the only available pathway for converting carbohydrates to ethanol and it is often time-consuming. The focus of the current research is to expedite ethanol production from carbohydrates using *Saccharomyces cerevisiae*. This study evaluated fermentation using a high-speed stirrer (ultraturrax) where glucose was the model carbohydrate. The reaction kinetics of fermentation was evaluated using ^{13}C NMR spectroscopy. The effect of stirring speed on the kinetics of glucose fermentation was studied and two times enhancement in the rate of fermentation was observed using an ultraturrax ($3.80 \pm 0.40 \times 10^{-5}$ s^{-1}) compared to the incubation ($1.90 \pm 0.71 \times 10^{-5}$ s^{-1}) at 30°C. The acceleration of the fermentation was also observed using other carbohydrates such as sucrose and molasses. We report the effect of varying a few parameters such as: stirring speed, the effect of shelf-life of yeast, pH of the broth, and effect of activated carbon on the fermentation kinetics. The optimum pH range for the fermentation reaction was found to be 4-6.8. Yeast with a six-month shelf-life was found to slow down the fermentation reaction by 5.6 times relative to fresh yeast (up to one month). Our results indicate an efficient method for bioethanol production from carbohydrates.



Keywords: Biofuel; Bioethanol; Fermentation; *Saccharomyces cerevisiae*; Ultraturrax

Introduction

Worldwide decrease in the major sources of fossil fuels has been a cause of concern. With the depletion of these fuels, efforts are being directed to renewable sources such as solar, wind, and biomass [1]. Bioethanol is regarded as potential biofuel from renewable sources. Bioethanol is widely recognized as one of the most unique transportation fuels with powerful economic, environmental, and strategic benefits [2]. High octane value and high combustion efficiency makes bioethanol one of the most promising alternatives to conventional transportation fuels. Furthermore, bioethanol is carbon neutral and the use of it as a transport fuel can reduce CO_2 buildup [3,4]. Apart from fuel applications, production of bioethanol is increasing every year because of its use in medicine, cosmetics, and industrial materials [5]. First-generation bioethanol is already being used as a transportation fuel in Brazil and is blended into petroleum in the US [6]. Second-generation bioethanol from lingo-cellulosic materials is still under research or demonstration stage [7].

The conversion of biomass to ethanol includes two processes: the degradation of starting plant material into fermentable sugars (hydrolysis) and the conversion of sugar into alcohol (fermentation) [8]. Fermentation is a crucial stage in bioethanol production where selection of suitable feedstock for fermentable sugars is a challenge. Homogenous crop materials are easily metabolized to sugars (e.g. molasses from sugar cane, starch from corn kernels) [8,9]. However, fuel-ethanol production from lignocellulosic materials is complicated due to the recalcitrant nature of the molecules present in these materials. In order to make cellulose and hemicellulose more accessible to the catalytic site, a pretreatment is required [10].

Bioethanol production using various bacteria (*Clostridium* sp.) or yeasts (*Saccharomyces* sp., *Zymomonas* sp.) has been studied intensively over the past two decades [11]. One of the most effective

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ethanol-producing microorganisms for hexose sugars (e.g. glucose, mannose, and galactose) is the yeast *Saccharomyces cerevisiae* which has high ethanol productivity, high tolerance to ethanol, and tolerance to inhibitory compounds present in the hydrolysate of lignocellulosic biomass. Native strains of *Saccharomyces cerevisiae* are unable to utilize xylose for growth or fermentation. Some yeast strains have been reported to ferment xylose into ethanol, but the rate and yield of ethanol production are considerably low compared to their glucose fermentation [12].

Several studies were focused on overcoming the limitations in practical bioethanol production. Wargacki et al. [13] engineered a microorganism (*Vibrio splendidus*) capable of metabolizing alginate, the major polysaccharide in brown algae, and Takeda et al. [14] developed a bacterial strain (*Sphingomanas sp.* A1) that could assimilate alginate in brown algae. The utilization of wide variety of feedstock has also been of immense scientific interest. Wang et al. [15] demonstrated the technical and economical feasibility of bioethanol production from waste paper. Rapeseed straw, oil-palm fronds, and wheat straw were recently used for bioethanol production and they yielded 39.9 g/L, 18.2 g/L and 14.0 g/L of ethanol, respectively, under optimal reaction conditions [16-18].

Currently, there is only one available pathway for the conversion of carbohydrates into ethanol, i.e., the biological pathway which is often time-consuming. Ultraturrax is a high-speed stirrer that could generate cavitation, collision, sheer and impact which in turn causes dispersion, homogenization, emulsification and disintegration of particles in the reaction medium. The potential of the device is used for the first time for bioethanol production in this study. There have been recent reports on the acceleration of delignification, enzymatic hydrolysis and fermentation using sonication technique. Pulidindi et al. [19] reported 2.3 times acceleration in the rate of the fermentation of glucose after exposure to soft sonication. Subhedar and Gogate [20] reported two times increase in the delignification of waste newspaper in an ultrasound assisted alkali pretreatment. 2.4 times increase in the release of reducing sugars from waste newspaper was also reported using ultrasound assisted enzymatic hydrolysis of waste newspaper [21]. Ofori-Boateng and Lee [17] reported four times higher bioethanol yield and shorter reaction time in sonication assisted simultaneous saccharification and fermentation (SSF) of oil palm fronds compared to a non-sonication SSF process. Korzen [22] reported bioethanol yield of 6.2 wt.% (dry weight basis) in 3 h from SSF of *Ulva rigida* using sonication compared to 4.9 wt.% yield in incubator for 48 h.

The focus of the current research is to evaluate different carbohydrates and fermentation conditions for accelerated bioethanol production. In the present study, ethanol production was performed with ultraturrax device using glucose as a model carbohydrate. In addition to glucose, the feasibility of sucrose and molasses as carbon sources for the production of bioethanol was also evaluated.

Materials and Methods

Fermentation conditions

For all experiments, fermentation was performed with *Saccharomyces cerevisiae*, commercial Baker's yeast bought from the supermarket. The substrate D-Glucose was obtained from Sigma-Aldrich and molasses and sucrose were obtained from the supermarket. The activated carbon (Activated charcoal Norit®) was purchased from Sigma-Aldrich. Fermentation reactions were performed in 250 mL Erlenmeyer flasks and the flasks were closed with cotton plugs. The fermentation broth for both glucose and sucrose fermentation comprised 20 g (or 40 g for the 40 wt.% solution) of glucose or sucrose dissolved in 100 mL of water to which 2 g (or 4 g for the 40 wt.% solution) of yeast was added. For molasses fermentation, the broth comprised 10 g molasses dissolved in 100 mL of water with 1 g of yeast. As control experiments, the fermentation reactions were performed in an incubator (MRC, LM-570, orbital shaker incubator) without shaking. To evaluate the effect of stirring speed on fermentation, the flasks were placed in a high-speed stirrer ultraturrax device (Leroy Somer, Digidrive SK, make ESCO-LABOR) at 30°C.

^{13}C NMR analysis

The kinetics of fermentation reactions were studied using ^{13}C NMR spectroscopy (Supplementary data Section 1). Aliquots were collected from fermentation broths at regular time intervals and analyzed by ^{13}C NMR spectroscopy. ^{13}C NMR spectra were recorded on Bruker Avance DPX 300. D_2O was used as a solvent.

^{1}H NMR analysis

^{1}H NMR spectroscopy was used for the quantification of ethanol produced in glucose fermentation (Supplementary data section 1.2). Aliquots were collected from the fermentation broth at regular time intervals and analyzed by ^{1}H NMR spectroscopy. D_2O was used as a solvent and HCOONa was used as an internal standard. ^{1}H NMR spectra were recorded on Bruker Avance DPX 300.

Results and Discussion

Effect of stirring speed on the rate of glucose fermentation

Glucose (20 wt.%) fermentation was performed using ultraturrax at different stirring speeds. Mechanical stirring was found to have an accelerating effect on the rate of fermentation. Relative to a stand-still reaction in incubator ($k=1.90 \pm 0.71 \times 10^{-5}$ s^{-1}) or mechanical stirring at 5,000 rpm ($k=1.80 \pm 0.30 \times 10^{-5}$ s^{-1}), stirring at 10,000 rpm ($k=3.80 \pm 0.40 \times 10^{-5}$ s^{-1}) showed two times enhancement ($k_{ultraturrax,10,000}/k_{incubator}=2$) in the reaction rate constant value (at 30°C). Among different stirring speeds tested for 20 wt.% glucose fermentation using the high-speed stirrer ultraturrax, 10,000 rpm was found to be the optimum stirring speed (according to the k values) since increasing the speed further to 20,000 rpm ($k=4.20 \pm 0.40 \times 10^{-5}$ s^{-1}) did not double the reaction rate constant. The values of the reaction rate constants (k) as a function of stirring speed are summarized in Table 1.

Monitoring complete glucose fermentation

Glucose (20 wt.%) fermentation was monitored until completion. ^{13}C NMR spectra of aliquots collected at regular time intervals from the fermentation broth maintained in the ultraturrax and in the incubator (at 30°C) are shown in Figure 1. As a function of time, the intensity of ethanol peaks (17 and 57 ppm) increased and the intensity of glucose peaks (60.9 (C6), 69.9 (C4), 71.7 (C2), 74.4 (C3), 76.0 (C5), 92 (C1, β) 96 (C1, α) ppm) decreased. No trace of glucose was observed in 12 h and 16 h samples collected from the broth in ultraturrax and incubator, respectively, which indicated the completion of the fermentation

Stirring speed (rpm)	Reaction rate constant, k (× 10^{-5} s^{-1})
0	1.90 ± 0.71
5,000	1.80 ± 0.30
10,000	3.80 ± 0.40
20,000	4.20 ± 0.40

Note: k values are mean ± standard deviation, replicate no. *n*=4.

Table 1: Effect of mechanical stirring on kinetics of glucose fermentation.

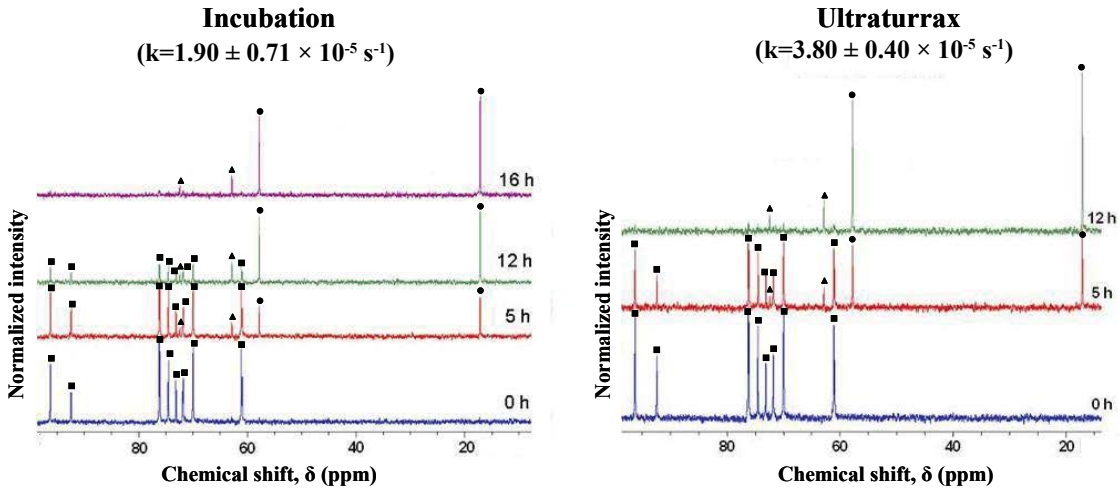

Figure 1: ^{13}C NMR spectra of aliquots from glucose (20 wt.%) fermentation broth (maintained in ultraturrax and incubator at 30°C) collected at regular time intervals until completion. The symbol ■ shows glucose peaks, ▲ shows glycerol peaks, and ● shows ethanol peaks. The ratio of the intensity of peaks of ethanol (at 17 ppm) to that of glucose (at 96 ppm) was used to monitor the reaction kinetics. Reaction rate constant, k, values are mean ± standard deviation, replicate no. *n*=4.

reaction. As shown in Figure 1, the time required for the completion of the fermentation was reduced from 16 h (incubation) to 12 h with the use of the ultraturrax at 10,000 rpm (at 30°C). In addition to ethanol, glycerol (62.8 and 72.3 ppm) was also formed as a secondary metabolite during the fermentation.

Effect of substrate (glucose) concentration on the rate of fermentation

In addition to 20 wt.% glucose fermentation (Figure 1), the fermentation was also performed with 40 wt.% glucose (Supplementary data Figure S1). The objective of the experiment was to verify whether in high glucose concentrations, which usually inhibit yeast performance, the fermentation rate was also accelerated using ultraturrax. Although the substrate concentration was doubled, the reaction rate was almost the same for 20 wt.% ($k=3.80 \pm 0.40 \times 10^{-5}\ s^{-1}$) and 40 wt.% ($k=3.60 \pm 1.16 \times 10^{-5}\ s^{-1}$) glucose fermentation. It is known that, ethanol concentration greater than 4% will have a poisoning effect on yeasts and induces stress by retarding their productivity of further ethanol production [23]. The ratio between the reaction rate constant value ($k_{ultraturrax}/k_{incubator}$) for the fermentation of glucose (40 wt.%) in ultraturrax and incubator (at 30°C) was 1.7 (Supplementary data Figure S1) and the glucose conversion after six hours was 69% in ultraturrax and 47% in incubator.

Effect of yeast shelf-life on the kinetics of glucose fermentation

To evaluate the effect of yeast shelf-life on fermentation kinetics, glucose (20 wt.%) fermentation was performed with fresh (up to one month) as well as old (six months) yeast. The fermentation was performed using ultraturrax at 5,000 rpm and at 10,000 rpm. The rate constant (k) values calculated from each experiment are summarized in Table 2. Using yeast with long shelf-life reduced the fermentation rate. Long shelf-life, nearly six months, was found to decelerate the fermentation rate by almost 3 times and 5.6 times compared to fresh yeast when the stirring speeds were 5,000 rpm and 10,000 rpm, respectively. Thus, yeast shelf-life is an important parameter that affects the fermentation rate. Although dry yeast can be stored at room temperature and performs well for the duration of the package shelf-life, it will always lose some of its viability and activity over time. However, at colder temperatures these losses are less compared to warmer temperatures.

Effect of additives on the glucose fermentation rate

Additives are known to alter the rate of fermentation of sugars. Several additives have been reported to improve the fermentation characteristics of yeast strains through protection against the inhibitory effects of the substrate and the product. These include unsaturated lipids, soy flour, *Aspergillus oryzae* proteolipids, skim milk powder, and chitin. Raman et al. [24] accelerated the rate of fermentation of molasses 1.5 times by adding silicate (zeolite) to the fermentation broth.

In the current study, to investigate whether the addition of activated carbon alters the kinetics of fermentation of glucose (20 wt.%), 2 g of activated carbon was added to the broth. The effect of additive was studied using ultraturrax. The reaction rate was found to decelerate 1.5 times with the addition of activated carbon to the broth ($k_{without\ additive}/k_{with\ additive}=3.80 \pm 0.40 \times 10^{-5}\ s^{-1}/2.60 \pm 0.80 \times 10^{-5}\ s^{-1}$). There was a drastic decrease in pH (from 6.8 to 2) caused by the addition of the activated carbon. To attribute this deceleration in rate to the decrease in pH, the pH of the broth after the addition of activated carbon was raised to 6.8. At a pH of 6.8, in the presence of the additive, the reaction rate was almost the same ($k=3.63 \pm 0.35 \times 10^{-5}\ s^{-1}$) as the reaction rate without any additives ($k=3.80 \pm 0.40 \times 10^{-5}\ s^{-1}$). Thus, the presence of activated carbon had no affect on the rate of glucose fermentation if the pH remained unaltered, in other words activated carbon did not improve the fermentation rate.

Inherently, due to the presence of surface oxygen-containing functional groups such as -COOH, which are formed as a result of the activation process involved in the production of the carbon material, the activated carbon is acidic. The pH of the neutral glucose solution (pH=6.7) decreased to 2.2 solely due to the addition of activated carbon which verifies the inherent acidity of activated carbon. Therefore, the decrease in pH was not due to the presence of the yeast.

Effect of pH on the kinetics of glucose fermentation

One of the main factors that have a significant effect on the performance of yeast is the pH. The pH of the fermentation broth has

been varied (2, 4, and 6.8) using diluted HCl and NaOH. The kinetics of the fermentation reaction at each pH value was monitored. The reaction rate constants at a pH of 2, 4, and 6.8 were calculated as $2.50 \pm 0.74 \times 10^{-5}$ s^{-1}, $3.60 \pm 0.60 \times 10^{-5}$ s^{-1}, and $3.80 \pm 0.40 \times 10^{-5}$ s^{-1}, respectively, where highly acidic pH (=2) decelerated the rate of glucose fermentation by two compared to the rate at a pH of either 4 or 6.8. A pH between 4 and nearly neutral pH (=6.8) had no significant effect on the fermentation rate. Thus, we report a pH range of 4-6.8 as the optimum range for glucose fermentation of the yeast *Saccharomyces cerevisiae*. Lee et al. [25] have reported an optimum pH value of 6.0 for the fermentation of glucose. Chiang et al. [26] reported a pH range of 4-6 as an optimum range for the fermentation of D-xylose using commercial Baker's yeast. An optimum pH range was reported to be 4-5.5 for yeasts other than *Saccharomyces cerevisiae* as well [27].

Reusability of yeast using an ultraturrax

To further study whether the yeast can be reused even after exposure to ultraturrax, the yeast was separated from the fermentation broth soon after the complete conversion of glucose (12 h) into ethanol. The fermentation broth was centrifuged and the supernatant was analyzed for ethanol estimation (using ^{1}H NMR). To remove the traces of ethanol, residual yeast was washed repeatedly with distilled water. The regenerated yeast was used for another cycle of fermentation of glucose (20 wt.%). The reaction rate using the regenerated yeast was found to be 8 times lower than that of the fresh yeast ($k_{\text{fresh yeast}}/k_{\text{regenerated yeast}} = 3.80 \pm 0.40 \times 10^{-5}$ $s^{-1}/0.47 \pm 0.06 \times 10^{-5}$ s^{-1}). The lower kinetics may also be due to the loss of some yeast during recycling. However, in industrial bioethanol production, yeast is not regenerated and reused. For every batch of ethanol production, fresh yeast is used. In this study, the reusability was tested to verify that ultraturrax had not damaged the yeast cells. With regenerated yeast, even after 12 h of ultraturrax exposure, the yield of ethanol was 17% of the theoretical yield. Even though it is possible to reuse the yeast even after ultraturrax exposure, the reaction rate constant with the regenerated yeast ($k=0.47 \pm 0.06 \times 10^{-5}$ s^{-1}) is clearly lower than the process performed in an incubator with fresh yeast ($k=1.90 \pm 0.71 \times 10^{-5}$ s^{-1}). Therefore, the reuse of yeast is not recommended in ultraturrax based fermentation process.

Stirring speed (rpm)	Yeast	Reaction rate constant, k ($\times 10^{-5}$ s^{-1})	k_{fresh}/k_{old}
5,000	Old	0.61 ± 0.10	2.95
	Fresh	1.80 ± 0.30	
10,000	Old	0.68 ± 0.06	5.60
	Fresh	3.80 ± 0.40	

Note: Fresh yeast indicates short shelf-life (up to one month) whereas old yeast indicates long shelf-life (six months). k values are mean ± standard deviation, replicate no. *n*=4.

Table 2: Performance of yeast as a function of shelf-life.

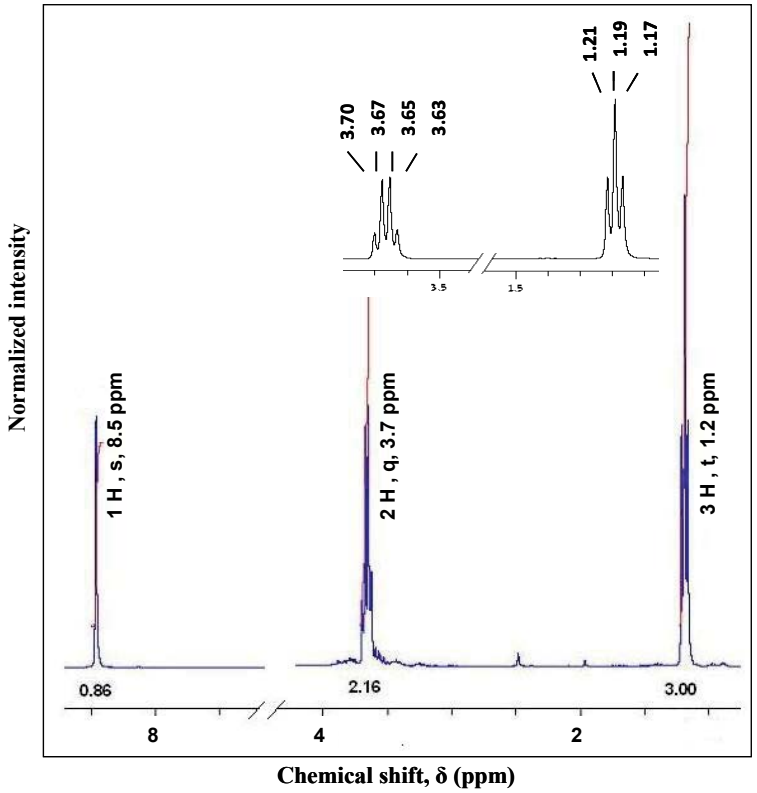

Figure 2: ^{1}H NMR spectrum of an aliquot from glucose (20 wt.%) fermentation broth after 12 h of ultraturrax exposure at 10,000 rpm, 30°C. Inset shows the ethanol peaks, a 3H (t) at 1.2 ppm and a 2H (q) at 3.7 ppm. The singlet peak at 8.5 ppm is the internal standard, HCOONa.

Quantification of ethanol from glucose fermentation using ^{1}H NMR spectroscopy

An aliquot from glucose fermentation broth (20 wt.%) after 12 h of ultraturrax exposure (after completion of the reaction as depicted in Figure 1) was collected and analyzed by ^{1}H NMR spectroscopy (Figure 2). A 3H (t) at 1.2 ppm and a 2H (q) at 3.7 ppm are typical of ethanol. The intense singlet peak at 8.5 ppm is typical of HCOONa which was used as an internal standard. The detailed methodology for ethanol quantification is shown in supplementary data section 1.1.

With fresh yeast, complete fermentation of glucose (20 g) yielded 8.2 g ethanol. Theoretically, 20 g glucose should yield 10.2 g ethanol. The observed ethanol yield, after the complete conversion of glucose using an ultraturrax for 12 h, is 80.4% of the theoretical yield. The other 19.6% of the product may be glycerol, an inevitable secondary metabolite, and other byproducts. Moreover, not all glucose in the fermentation broth was consumed for the fermentation by the microorganisms; some of the glucose was also used for the growth and sustenance of the yeast.

Evaluation of kinetics of ethanol production from different carbohydrate feedstock

Sucrose is a disaccharide of glucose and fructose. Relative to glucose, sucrose as an extract from sugar cane is more readily available. Molasses is a by-product of the cane sugar manufacturing process. Typical molasses comprises sucrose, glucose, and fructose. Industrial-grade ethanol, a key product in the conversion of sugars and starches into energy and chemical feedstocks, is produced in India exclusively through the fermentation of sugarcane molasses using yeasts [24]. In addition to glucose fermentation, the effect of mechanical stirring on sucrose and molasses fermentation was also evaluated. Aliquots from fermentation broths (maintained in ultraturrax at 10,000 rpm and in incubator at 30°C) were collected at regular time intervals and ^{13}C NMR

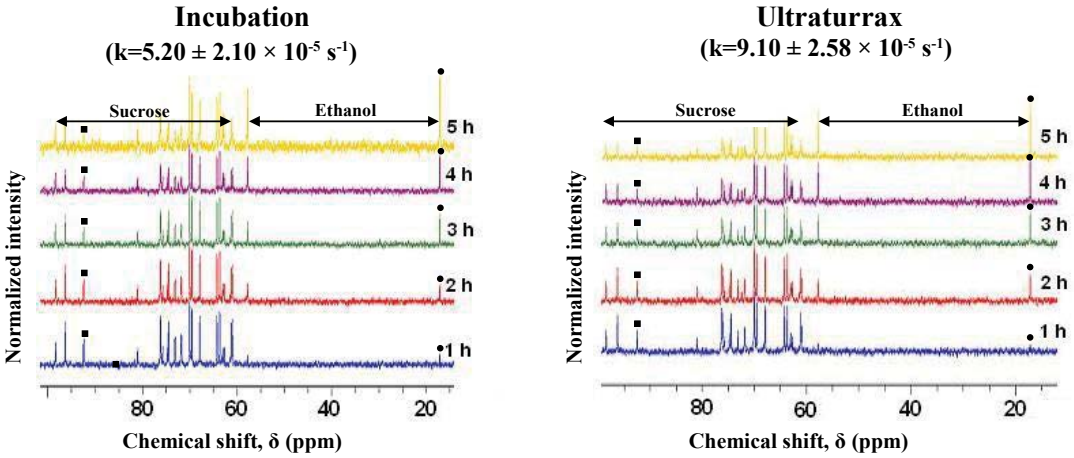

Figure 3: ^{13}C NMR spectra of aliquots from sucrose (20 wt.%) fermentation broth maintained in incubator and ultraturrax at 30°C. The peaks between 60 and 100 ppm belong to sucrose and the peaks at 17 and 57 ppm belong to ethanol. The ratio of the intensity of peaks of ethanol (at 17 ppm, ●) to that of sucrose (at 92 ppm, ■) was used to monitor the reaction kinetics. Reaction rate constant, k, values are mean ± standard deviation, replicate no. *n*=3.

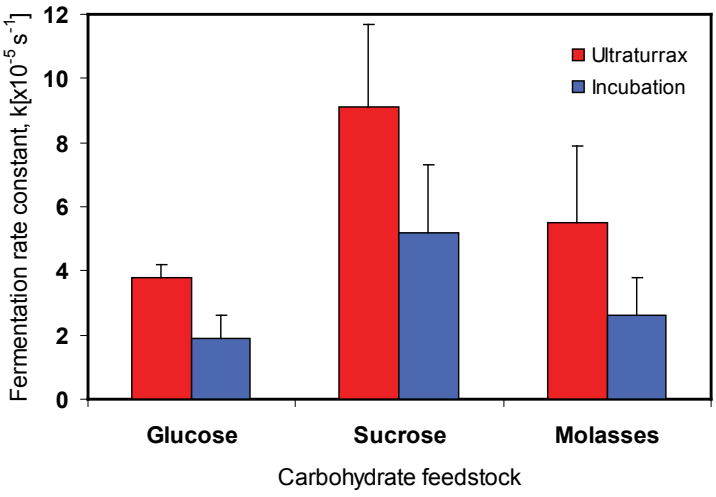

Figure 4: Effect of carbohydrate feedstock (glucose, sucrose, and molasses) on fermentation kinetics (replicate no. *n*=3 for sucrose and molasses and *n*=4 for glucose; error bars indicate standard deviation, SD).

spectra were recorded (Figure 3 and Supplementary data Figure S2). The effect of mechanical stirring on the fermentation rates of sucrose and molasses is summarized in Table 3. The accelerating effect of the mechanical stirring by ultraturrax exposure was also dependent on the feedstock. With either sucrose or glucose, a 1.7 times acceleration in reaction rate was observed whereas the acceleration rate was 2.1 times for molasses, which indicated that the acceleration in fermentation was slightly higher in molasses than in glucose and sucrose.

Moreover, the fermentation rate was found to be a function of the feedstock used. The effect of carbohydrate feedstock, such as glucose, sucrose, and molasses, on fermentation kinetics is shown in Figure 4. As depicted in Figure 4, the rate of sucrose fermentation was nearly two times faster than either glucose or molasses regardless of the fermentation method used. According to our results, it is evident that sucrose is an ideal feedstock for the production of ethanol as it was found to be more feasible to be fermented.

D'Amore et al. [28] reported that, in the initial stages of fermentation, sucrose is rapidly hydrolyzed into glucose and fructose by the action of the periplasmic enzyme invertase, prior to the sugars being transported across the cell membrane. Glucose was taken up preferentially over the other hydrolysis product fructose. Growth of *Saccharomyces cerevisiae* on a medium consisting of a mixture of glucose and fructose also resulted in the preferential uptake of glucose. However, when glucose and fructose were added separately, the uptake profile for each sugar was very similar. Since glucose is a monosaccharide, it is a common misconception that it should provide a higher rate of fermentation as it does not need to be broken down. However, glucose enters the yeast cells by facilitated diffusion which requires carrier proteins. When glucose is being absorbed, there will be a point where the rate reaches its maximum and all the carrier proteins are being used. When sucrose is the substrate, it splits into glucose and fructose. When all the carrier proteins are transporting glucose, different proteins are used for fructose (due to the tertiary structure of the protein), so the composite monosaccharides of sucrose can enter the yeast at a higher rate [28]. Hence, the rate is faster in the case of sucrose fermentation.

Conclusions

A way to expedite ethanol production from carbohydrates was evaluated in this study. The effect of mechanical stirring (ultraturrax) on the kinetics of fermentation was critically analyzed. In conclusion, nearly two times increment in the reaction rate was observed in the fermentation of glucose, sucrose, and molasses by using a high-speed stirrer. Use of an ultraturrax is suggested as a new method for enhancing the catalytic function of *Saccharomyces cerevisiae* leading to ethanol production. Thus, an effective, fast, and green method was developed for bioethanol production using renewable feedstocks. Future studies should investigate the use of hydrolyzates from lignocellulosic biomass for bioethanol production using the proposed method.

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