Ultrasound-assisted fermentation enhances bioethanol productivity

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A B S T R A C T

Production of ethanol from lactose by fermentation with the yeast Kluyveromyces marxianus (ATCC 46537) under various sonication regimens is reported. Batch fermentations were carried out at low-intensity sonication (11.8 W cm$^{-2}$ sonication intensity at the sonotrode tip) using 10%, 20% and 40% duty cycles. (A duty cycle of 10%, for example, was equivalent to sonication for 1 s followed by a rest period (no sonication) of 10 s.) Fermentations were carried out in a 7.5 L (3 L working volume) stirred bioreactor. The sonotrode was mounted in an external chamber and the fermentation broth was continuously recirculated between the bioreactor and the sonication chamber. The flow rate through the sonication loop was 0.2 L min$^{-1}$.

All duty cycles tested improved ethanol production relative to control (no sonication). A 20% duty cycle appeared to be optimal. With this cycle, a final ethanol concentration of 5.20 ± 0.68 g L$^{-1}$ was obtained, or nearly 3.5-fold that of the control fermentation. Sonication at 10% and 20% cycles appeared to stimulate yeast growth compared to the control fermentation, but 40% duty cycle had a measurable adverse impact on cell growth. Sonication at 10% and 20% cycles enhanced both the extracellular and the intracellular levels of β-galactosidase enzyme. Although at the highest duty cycle sonication reduced cell growth, cell viability remained at ≥70% during most of the fermentation. Sonication at a controlled temperature can be used to substantially enhance productivity of bioethanol fermentations.

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1. Introduction

This study is concerned with the ultrasound-induced enhancement of the production of bioethanol from lactose using the yeast Kluyveromyces marxianus.

Ultrasound, or sound of frequency ≥20 kHz, is generally associated with damage to cells and is widely used in laboratory protocols for breaking cell walls to release intracellular products [1]. Enzymes and other fragile macromolecules are known to be susceptible to damage by ultrasound [2]. Nevertheless, suitably applied ultrasound has the potential for enhancing the productivity of bioprocesses involving live cells and bioactive enzymes [3–10].

Effects of sonication for productivity enhancement have been previously reported for certain bacteria [3,5,6,11–16], filamentous fungi [7,8,17] and plant cells [18]. Bakers’ yeast (Saccharomyces cerevisiae) appears to have been the only yeast that has been assessed to some level in ultrasound irradiated fermentations [19–22].

Prior work on sonicated fermentations for producing bioethanol is pertinent to this study and is therefore reviewed here briefly. Nearly all such work focused on the yeast S. cerevisiae. Ultrasound intensity that is otherwise nonlethal to S. cerevisiae, appears to affect the integrity of the cell vacuole and rearrange the intracellular contents [23]. The relatively low power diagnostic ultrasound of the frequency range 1–10 MHz is generally considered less damaging to cells than the power ultrasound (frequency range of 20–100 kHz); nevertheless, 2.2 MHz ultrasound applied continuously at an electrical power input of 14 W to a broth volume of 64 mL killed 25% of the S. cerevisiae cells exposed for 60 min [23]. Continuous sonication at 1 MHz and 10.5 W cm$^{-2}$ has inhibited S. cerevisiae fermentation, but intermittent sonication at the same intensity was less damaging [19].

In production of wine, beer and sake from soluble sugars using immobilized cells of S. cerevisiae, extremely low intensity sonication at 0.3 mW cm$^{-2}$ and 43 kHz stimulated the fermentation to reduce the fermentation time to 50–64% [20]. Ultrasound (20 kHz) used at intensities of 0.2, 0.4 and 0.8 W cm$^{-2}$ was claimed to accelerate the growth of S. cerevisiae in a medium that contained only dissolved nutrients [22], but the data did not clearly support this claim. Marginal improvements to S. cerevisiae growth were observed on controlled exposure to power ultrasound by Lanchun et al. [21].

Some bioethanol fermentations require pretreatment of the substrate. In pretreatment of starch, sonication in the absence of enzymes and microorganisms has been repeatedly shown to enhance the yield of fermentable sugars [24–26] and thereby increase the ethanol yield in a subsequent nonsonicated fermentation. This effect is of course a purely physical consequence...
Fig. 1. Ultrasound assisted batch fermentation system.

of the sonication-induced rupture of the starch granules and does not involve any biological activity. Similar phenomena have been observed in bacterial fermentations for producing ethanol. For example, a 20% enhancement in ethanol yield was reported by intermittent sonication of a paper pulp slurry being enzymatically hydrolyzed and fermented in a combined saccharification–fermentation process that used the bacterium *Klebsiella oxytoca* [14]. Productivity enhancements have been claimed by sonication in some other *S. cerevisiae* fermentations [27]. Power ultrasound has been claimed to enhance the permeability of *S. cerevisiae* cell to proteases [28] and Ca²⁺ [29].

The present study used the well known yeast *K. marxianus* as a model system to investigate the sonication regimens that may be used to enhance cell growth and ethanol production from lactose, a completely soluble substrate. *K. marxianus* has been formerly referred to as *Kluyveromyces fragilis* [30–32]. *K. marxianus* has been widely used to produce ethanol from lactose-containing media [31–40], but in conventional nonsonicated fermentations.

2. Materials and methods

2.1. Microorganism, maintenance and preparation

*K. marxianus* ATCC 46537 was obtained from the American Type Culture Collection, USA (www.atcc.org). The yeast was supplied as a freeze-dried powder in a glass vial. The cells were rehydrated in sterile YM broth, incubated at 30 °C for 24 h and then inoculated on agar slants. After a further incubation period (30 °C, 24 h), the slants were stored at 4 °C. The maintenance agar medium was made using deionized water and had the following composition [31] (g L⁻¹): lactose 50; yeast extract 2; (NH₄)₂SO₄ 6.25; MgSO₄·7H₂O 2; KH₂PO₄ 4; and agar 15. The medium was sterilized by autoclaving (121 °C, 15 min). The slants were kept at 4 °C and subcultured every 2 months. This stock culture was used for inoculum preparation throughout this study.

Agar plates were prepared from slants in the usual way. Seed cultures were prepared by inoculating a single colony from an agar plate into 80 mL of a sterile medium contained in a 250 mL shake flask. The medium was as described above, but without the agar, and had been sterilized as mentioned above. The culture was incubated (30 °C) in an orbital shaking incubator (180 rpm) for 24 h. This culture (50 mL) was used to inoculate 150 mL of the earlier specified sterile medium contained in a 1000 mL shake flask. The flask was incubated as specified above. After the specified incubation period, the inoculum had a spectrophotometric absorbance of 0.7 at 620 nm (Ultraspec 2000, model 80-2106-00 spectrophotometer; Pharmacia Biotech Inc., Piscataway, NJ, USA) and contained ~4 × 10⁷ cells mL⁻¹. All subsequent fermentations were inoculated using the above inoculum at a level of 5% by volume.

2.2. Bioreactor fermentations and ultrasound equipment

A 7.5 L stirred bioreactor (BIOFLO 110 New Brunswick Scientific, East Brunswick, NJ, USA, www.nbsc.com) was used (Fig. 1). The working volume was 3 L. The internal diameter of the jacketed glass bioreactor vessel was 0.18 m. The vessel was fully baffled with 4 vertical baffles spaced equidistance around the periphery. The baffle width was 19 mm. A central shaft supported two 6-bladed Rushton disc turbine agitators. The agitators were identical with a diameter of 59.6 mm and were spaced 0.15 m apart on the shaft. The lower agitator was located 59.6 mm above the bottom of the vessel. A single hole sparger was used for aeration. The sparger hole diameter was 4.3 mm and it was located directly below the lower agitator, about 30 mm above the base of the vessel.

All fermentations were run as aseptic aerobic batch cultures. The air inlet and exhaust ports on the bioreactor were installed with
The sterile bioreactor was inoculated with 150 mL (5% by vol) of the earlier specified inoculum. The final volume of the broth in the fermenter after inoculation was 3150 mL. The fermentation temperature was controlled at 30.0 ± 0.2 °C. The agitation speed and aeration rate were maintained at 500 rpm and 2.67vvm, respectively. The pH and the dissolved oxygen concentration were monitored, but not controlled. Sterile (121 °C, 15 min) antifoam emulsion (catalog no. A 6426-100G, 10 g/100 mL of water; Sigma-Aldrich, St. Louis, MO, USA) was added to the fermenter in response to a foam sensor to automatically suppress severe foaming. Each batch fermentation was run for 24 h. Samples were taken periodically. The optical density and the cell viability were measured immediately after sampling, as specified later in this paper. For the other measurements, the samples were centrifuged at 2000 × g for 10 min (model 0008931 centrifuge; Eppendorf AG, Germany, www.eppendorf.com) immediately after collection and the supernatant was stored at 4 °C for further analysis. The storage period did not exceed 3 days.

2.3. Sonobioreactor fermentations

For ultrasound-assisted fermentations, the ultrasound power level could be varied by adjusting the amplitude setting of the sonotrode and the cumulative average ultrasound dose could be varied by adjusting the duty cycle. The amplitude was set at position 2 to correspond to a power input $P$ of 15 W, or a sonication intensity $I$ of 11.8 W cm$^{-2}$. The sonication intensity was calculated using the following equation:

$$ I = \frac{P}{A} \quad \text{(1)} $$

where $A$ (cm$^2$) was the area of the sonotrode tip. The $A$ value was 1.27 cm$^2$.

The cumulative sonic energy imparted to the fluid depended on the duty cycle of sonication. The duty cycle determined the proportion of the time that the sonication was “on”. A duty cycle of 10% was equivalent to sonication for 1 s followed by a rest period (no sonication) of 10 s. A sonication duty cycle of 100% meant uninterrupted sonication. The time units of seconds were used in setting the duty cycle. Duty cycles of 10%, 20% (1 s sonication, 5 s rest period) and 40% (2 s sonication, 5 s rest period) were used.

2.4. Analyses

2.4.1. Biomass concentration

Biomass concentration was determined by measuring the optical density of the fermentation broth at 620 nm ($A_{620}$) with a spectrophotometer (Ultraspec 2000, model 80-2106-00; Pharmacia Biotech Inc., Piscataway, NJ, USA) against a blank of sterile medium prior to measurement. This way the spectroscopic absorbance was always $<0.7$. A calibration curve was used to convert the optical density data to the dry biomass concentration. The equation of the calibration curve was the following:

$$ \text{Dry biomass concentration (g/L)} = \frac{A_{620}}{6.95 \times 10^{-2}} \quad \text{(2)} $$

2.4.2. Lactose concentration

Lactose concentration was estimated using a modified dinitrosalicylic acid (DNS) method based on Miller [41]. Thus, a 1% (w/v) solution of DNS reagent was prepared by dissolving 10 g DNS and 2 g of phenol in 1000 mL of a solution of sodium hydroxide (10 g L$^{-1}$) and sodium sulfite (0.5 g L$^{-1}$). The broth supernatant sample containing lactose was appropriately diluted with deionized water. The diluted sample (3 mL) was mixed with 3 mL of DNS reagent and heated for 15 min on a boiling water bath. One milliliter of Rochelle
salt solution (potassium–sodium tartrate, 400 g L\(^{-1}\)) was added and the resulting mixture was cooled to ambient temperature in a cold water bath. The absorbance of the cooled solution was measured at 575 nm (Ultraspec 2000, model 80-2106-00 spectrophotometer; Pharmacia Biotech Inc., Piscataway, NJ, USA) against a blank that had been prepared using deionized water instead of the sample. The absorbance was converted to lactose concentration using a standard curve. The standard curve had been prepared using lactose solutions of known concentrations. The equation of the standard curve was the following:

\[
A_{575} = \frac{5.2 \times 10^{-3}}{w}
\]

where \(A_{575}\) was the spectrophotometric absorbance at 575 nm. The above equation applied to an absorbance range of 0–0.7.

2.4.5. Activity of colony forming unit counts on petri dishes.

In prior unpublished work, this method had been rigorously validated as the ratio of the unstained cell count and the total count.

2.4.4. Cell viability

Cell viability was determined using the methylene blue staining method [42]. A 10 \(\mu\)L aliquot of serially diluted freshly sampled yeast broth was mixed with 10 \(\mu\)L of a methylene blue solution and incubated for 5 min [42]. The cell suspension was then counted on a hemacytometer at 400 \(\times\) magnification. The viability was calculated as the ratio of the unstained cell count and the total count. In prior unpublished work, this method had been rigorously validated for \(K.\) marxianus using the highly reliable but cumbersome colony forming units on petri dishes.

2.4.5. Activity of \(\beta\)-galactosidase

Activity of the extracellular \(\beta\)-galactosidase was measured in the cell-free culture supernatant as specified in the Sigma enzymatic assay for \(\beta\)-galactosidase [43]. The activity was determined using the synthetic substrate o-nitrophenyl-\(\beta\)-D-galactopyranoside, ONPG (catalog no. N1127-25G; Sigma-Aldrich, St. Louis, MO, USA). One unit of \(\beta\)-galactosidase activity was defined as the amount of the enzyme that liberated 1.0 mmol of o-nitrophenol from 5 mM ONPG per minute at pH 3.5 and 25 °C.

\[
\text{Lactose concentration (} \mu\text{g/mL}) = \frac{A_{575}}{5.2 \times 10^{-3}} 
\]

The intracellular \(\beta\)-galactosidase activity was measured according to the method described by Wang and Sakakibara [13]. A 35 mL sample of the broth was centrifuged (3300 \(\times\) g, 10-min) to recover the cells. The cells were washed (2 \(\times\) 35 mL) with 0.1 M phosphate buffer, pH 6.5. The washed cells were resuspended in 35 mL of deionized water using a vortex mixer. The suspension was cooled in an ice-water bath at 4 °C and sonicated at 550 W, 20 kHz, for 30 s (Misonix Sonicator\® 3000, Misonix, Inc., Farmingdale, NY, USA). The sonicated suspension was centrifuged (12000 \(\times\) g, 30-min; Hitachi CR-22GII refrigerated centrifuge, Hitachi Koki Co., Ltd., Tokyo, Japan) at 4 °C. The supernatant was collected and analyzed in accordance with the procedure given above for the determination of the extracellular \(\beta\)-galactosidase activity.

3. Results and discussion

3.1. Baseline determination (nonsonicated batch fermentation)

The results of duplicate nonsonicated batch fermentations are shown in Fig. 3 as baseline data for comparison with the sonicated fermentations. The fermentation was essentially complete by 24 h (Fig. 3). The biomass growth, the ethanol production and lactose consumption profiles are consistent with expectations for an aerated fermentation. The error bars in Fig. 3 demonstrate a good reproducibility of the fermentations. The baseline fermentation kinetic parameters determined from Fig. 3 are compared later (Table 1) with those of the sonicated fermentations.

3.2. Effects of ultrasound

Sonication at 11.8 W cm\(^{-2}\) and the specified duty cycle commenced 9.5 h after inoculation of a batch fermentation. The profiles

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Sonication regimen (duty cycle)*</th>
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<tbody>
<tr>
<td></td>
<td>Control (no sonication)</td>
</tr>
<tr>
<td></td>
<td>10%</td>
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<tr>
<td></td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>40%</td>
</tr>
<tr>
<td>Maximum specific growth rate, (\mu) (h(^{-1}))</td>
<td>0.203 ± 0.011</td>
</tr>
<tr>
<td>Average specific lactose uptake rate, (q_1) (g g(^{-1}) h(^{-1}))</td>
<td>0.206 ± 0.027</td>
</tr>
<tr>
<td>Maximum biomass yield on lactose, (Y_{x}) (g g(^{-1}))</td>
<td>0.217 ± 0.007</td>
</tr>
<tr>
<td>Maximum biomass concentration, (X_{x}) (g L(^{-1}))</td>
<td>0.179 ± 0.017</td>
</tr>
<tr>
<td>Maximum biomass productivity, (P_x) (g L(^{-1}) h(^{-1}))</td>
<td>0.208 ± 0.009</td>
</tr>
<tr>
<td>Final ethanol yield on substrate, (Y_{e}) (g g(^{-1}))</td>
<td>0.218 ± 0.010</td>
</tr>
<tr>
<td>Final ethanol concentration (g L(^{-1}))</td>
<td>0.218 ± 0.010</td>
</tr>
<tr>
<td>Final ethanol productivity, (P_e) (g L(^{-1}) h(^{-1}))</td>
<td>0.381 ± 0.014</td>
</tr>
<tr>
<td>Average biomass specific ethanol production rate, (q_p) (g g(^{-1}) h(^{-1}))</td>
<td>0.381 ± 0.014</td>
</tr>
</tbody>
</table>

* Except for the control culture, the sonication power intensity was always 11.8 W cm\(^{-2}\).

Fig. 3. A typical control fermentation profile.
Sonication intensity was 11.8 W cm$^{-2}$ and (c) dissolved oxygen concentration. Except for the nonsonicated control, the Effects of sonication on: (a) biomass concentration; (b) lactose concentration; (c) dissolved oxygen concentration are shown in Fig. 4 in comparison to controls. All of biomass growth, lactose consumption and the dissolved oxygen concentration were clearly reflected in a slower rate of lactose consumption and biomass growth and final concentration at the highest duty cycle rate and the final biomass concentration (Fig. 4 a). The reduced but increasing the duty cycle to 40% adversely affected the growth the biomass growth rate and final concentration relative to control, the biomass of 11.8 W cm$^{-2}$, ultrasound stimulated growth of K. marxianus on a soluable substrate so long as the duty cycle was appropriately selected. Each sonication event had to be followed by a recovery period of no sonication to prevent adverse impact on the yeast. No other work has been reported on sonication of K. marxianus, but continuous sonication of S. cerevisiae with diagnostic ultrasound (1 MHz) at a lower intensity (10.5 W cm$^{-2}$) than used by us, has proved to be inhibitory [19] while intermittent sonication was less damaging.

The effects of pulsed sonication on ethanol production are shown in Fig. 5 in comparison to the control fermentation. All duty cycles tested improved ethanol production relative to control, but the duty cycles of 10% and 20% were clearly the most effective. With the best duty cycle of 20%, the final ethanol concentration of 5.20 ± 0.68 g L$^{-1}$ was nearly 3.5-fold that of the control fermentation. For this sonication regimen, the ethanol yield on lactose was 0.109 g g$^{-1}$ compared to a yield of 0.034 g g$^{-1}$ for the control culture. The ethanol productivity of the culture sonicated at a duty cycle of 20% was 3.5-fold greater than for the control.

Ultrasonication is known to improve interfacial mass transfer. Mass transfer enhancements have been attained at power intensities as low as 2.2 W cm$^{-2}$ [3]. Therefore, a plausible improved gas–liquid mass transfer of oxygen as a consequence of sonication [44] may potentially explain the observed increase in the concentration of the biomass (Fig. 4a) relative to control; however, it does not explain the increased concentration of ethanol (Fig. 5) that is normally produced optimally under conditions of a low dissolved oxygen concentration [32]. In the present study, the dissolved oxygen concentration did not drop to much less than 20% of air saturation as shown in Fig. 4c.

Improved production of ethanol (Fig. 5) must therefore have a different explanation. One of the products of the fermentation is carbon dioxide. Elevated concentrations of dissolved carbon dioxide are known to inhibit S. cerevisiae [45,46] and have a similar effect on K. marxianus [32]. Improved gas–liquid mass transfer may have contributed to improved removal of the highly soluble carbon dioxide from the broth to enhance the ethanol productivity relative to control. Rapid desorption of carbon dioxide from a fermentation broth commonly produces foaming, as it does in a glass of beer. The fermentation broth was indeed observed to foam within the period of no sonication to prevent adverse impact on the yeast. No other work has been reported on sonication of K. marxianus, but continuous sonication of S. cerevisiae with diagnostic ultrasound (1 MHz) at a lower intensity (10.5 W cm$^{-2}$) than used by us, has proved to be inhibitory [19] while intermittent sonication was less damaging.

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Fig. 6. Foaming behavior of the fermentation: (a) just before sonication commenced 9.5 h after inoculation and (b) the same fermentation 10 min after sonication commenced at a power intensity of 11.8 W cm$^{-2}$ and a duty cycle of 20%.

minutes of commencing sonication as shown Fig. 6. At the recycle rate used, nearly 63% of the broth in the bioreactor had passed through the sonication chamber at least once by 10 min when the picture (Fig. 6b) was taken. Foaming may also be attributed to release of intracellular proteins, but up to a sonication duty cycle of 20% biomass growth was in fact better than in the control culture (Fig. 4a), suggesting little or no cell lysis. No distinct pH changes attributable to a possible change in the concentration of dissolved carbon dioxide could be observed. The pH values for the different sonication regimens were generally within ±0.2 pH units of the measured value (Fig. 7).

The kinetic parameters for the various fermentations are compared in Table 1. The equations used in calculating the parameters [47] were as follows:

Specific growth rate, $\mu$:

$$\mu = \frac{1}{(t_2 - t_1) \ln \frac{X_2}{X_1}} \quad (4)$$

where $X_1$ is the biomass concentration at time $t_1$ (= 8 h) and $X_2$ is the biomass concentration at time $t_2$ (= 14 h) during exponential growth.

Average specific lactose consumption rate, $q_s$:

$$q_s = -\frac{\Delta S}{\Delta X} \quad (5)$$

where $\Delta S$ is the substrate consumed by time $t$ (= 22 h) and $\Delta X$ is the increase in biomass concentration by time $t$.

Maximum biomass yield on substrate, $Y_{x/s}$:

$$Y_{x/s} = -\frac{\Delta X}{\Delta S} \quad (6)$$

where $Y_{x/s}$ is calculated at the instance of the maximum biomass concentration $X_{\text{max}}$.

Maximum biomass productivity, $P_x$:

$$P_x = \frac{X_{\text{max}} - X_0}{t} \quad (7)$$

where $P_x$ is calculated at the instance $t$ of the maximum biomass concentration in the fermentation. In Eq. (7), $X_0$ is the biomass concentration at the beginning of the fermentation.

Final ethanol yield on substrate, $Y_{p/s}$:

$$Y_{p/s} = -\frac{\Delta P}{\Delta S} \quad (8)$$

where $\Delta P$ is the change in ethanol concentration during the fermentation.

Final ethanol productivity, $P_E$:

$$P_E = \frac{E_f - E_0}{t_f} \quad (9)$$

where $E_0$ is the initial concentration of ethanol, $E_f$ is the final concentration of ethanol and $t_f$ is the duration of the fermentation.

Average specific ethanol production rate, $q_p$:

$$q_p = \frac{\Delta E}{X_{\text{max}} t} \quad (10)$$

where $q_p$ is calculated at the instance $t$ of the maximum biomass concentration. In Eq. (10) $\Delta E$ is the increase in ethanol concentration by time $t$ during the fermentation.

Fig. 7. The pH profiles. The sonication intensity was 11.8 W cm$^{-2}$ except for the nonsonicated control culture.
Under the best sonication regimen of a 20% duty cycle, the sonicated fermentation was substantially superior to the control culture (Table 1). For example, compared to control, the biomass yield on lactose was 33% greater for the sonicated culture; the maximum biomass concentration was 42% greater; the maximum biomass productivity was 57% greater; the final ethanol yield on lactose was 3-fold greater; the final ethanol concentration was 3.5-fold greater; and the final ethanol productivity was 3.5-fold greater (Table 1).

Cell viability profiles for the fermentations are shown in Fig. 8. Prior to the beginning of sonication at 9.5 h, the cell viability in all fermentations exceeded >90%, but in all cases, the viability continuously declined as the fermentations progressed. For the control culture, this decline could be explained by a progressive accumulation of ethanol, a well known inhibitor of yeasts [48,49] including K. marxianus [32]. The beginning of the viability decline (Fig. 8) coincided with the instance of the rapid increase in ethanol concentration around 9.5 h (Fig. 3). The viability decline of the sonicated cultures was also due to accumulation of ethanol (Fig. 5), but sonication appears to have been an additional contributing factor. Thus, at any instance after the sonication began, the viability was progressively reduced with the increasing value of the duty cycle of sonication (Fig. 8). Although sonication enhanced the viability decline, by the end of the fermentation >65% of the yeast cells were still viable in the culture that was sonicated at a duty cycle of 40% (Fig. 8). Ethanol is known to affect the structure of cell membranes [49] and this likely explained the increased susceptibility of cells to ultrasound once the ethanol concentration had increased.

Under certain conditions, ultrasound is known to affect the morphology of cells without causing a loss in viability [16,17,23]. Therefore, the cell morphology was examined photographically at 22 h of various fermentations (Fig. 9). By this time the yeast broth had passed through the sonication chamber 50 times. Compared to nonsonicated culture (Fig. 9a), no morphological changes were discerned in cells sonicated at 10 and 20% duty cycles (Fig. 9b and c). However, the culture that had been sonicated at the 40% duty cycle contained many ghost cells (i.e., cells that had lost most or all of their contents) and cells with clearly broken envelopes (Fig. 9d). This concurred with the lower biomass concentration (Fig. 4a) and cell viability (Fig. 8) in this fermentation, as discussed earlier.

Transport of lactose into cells of K. marxianus is mediated by lactose permease [32]. Once internalized, the lactose is hydrolyzed by β-galactosidase and the resulting glucose and galactose are metabolized by separate biochemical pathways [32]. As most of the lactose is hydrolyzed intracellularly, most of the β-galactosidase activity resides within the cells. The observed
sonication-dependent changes in growth metabolism and ethanol production may be potentially linked to possible effects of sonication on the enzyme β-galactosidase. Considering this, the activity of the intercellular and extracellular β-galactosidase was measured in the various fermentations (Fig. 10).

Until the beginning of sonication at 9.5 h, the profiles for all fermentations were identical for both the extracellular and the intracellular enzyme activity (Fig. 10). Irrespective of the fermentation, the extracellular enzyme activity was relatively small compared to the intracellular activity at any given instance (Fig. 10), as expected. The extracellular β-galactosidase was a consequence of either cell leakage or an ongoing lysis of a small fraction of the growing cell population. Sonication at 10 and 20% duty cycles appears to have stimulated the production of the enzyme inside the cells relative to control (Fig. 10b), whereas sonication at the 40% duty cycle appears to have suppressed enzyme synthesis. In fact these apparent effects are entirely explained by the differences in the biomass concentrations of the various fermentations (Fig. 4a) and not by any direct effect of sonication on the production or release of the enzyme. This is confirmed in Fig. 11 where the measured extracellular and intracellular activities of β-galactosidase are plotted per unit of dry cell mass present at any given instance during fermentation. From 9.5 h onwards, all the sonicated cultures had nearly the same biomass specific enzyme activity as did the control culture. Therefore, sonication had no effect at all on production or release of β-galactosidase. During exponential growth, i.e. prior to 9.5 h, the biomass always had a much higher enzyme activity than later in the fermentation. This was likely because production of β-galactosidase was up regulated during rapid growth that demands a rapid hydrolysis of lactose to feed the resulting sugars into the energy consuming metabolic pathways.

For the experimental system used, the bioreactor could always be considered to be well mixed. This could be readily demonstrated by comparing the mixing time in the bioreactor with the residence time of the recycle flow in the reactor. Thus, the residence time $t_R$ of the recycle stream was calculated as follows:

$$t_R = \frac{V_L}{Q_L}$$

(11)

where $V_L$ is the working volume (3 L) in the bioreactor and $Q_L$ is the previously specified recycle flow rate. The residence time was always 15 min. The mixing time in the bioreactor was calculated using the following equation [50]:

$$t_\theta = \frac{-\ln (1 - \theta)}{1.06N(D/T)^2(1/T)H^{0.5}}$$

(12)

where $t_\theta$ is the time required to attain a fractional homogeneity of $\theta$ (e.g. a $\theta$-value of 0.99 is equivalent to 99% of the fully mixed state),
N is the rotational speed of the impeller, D is the diameter of the impeller, T is the diameter of the mixing vessel and H is the depth of fluid in the tank. For the earlier specified bioreactor geometry and H = T, the mixing time for attaining a 99% homogeneity was found to be 0.096 min. Thus, the residence time in the bioreactor was nearly 150-fold greater than the time required for mixing.

4. Concluding remarks

Intermittent sonication with power ultrasound (20 kHz) at duty cycles of <20% stimulated biomass production, lactose metabolism and ethanol production in K. marxianus at a relatively high sonication intensity of 11.8 W cm\(^{-2}\). Increasing the duty cycle to 40% had a clear adverse impact on the yeast. Under the best conditions, sonication enhanced the final ethanol concentration by nearly 3.5-fold relative to control. This corresponded to a 3.5-fold enhancement in ethanol productivity, but required 952 W of additional power input per cubic meter of broth through sonication. This additional requirement for energy was certainly within acceptable operational norms for bioreactors and, for high value products, could be easily compensated by the increased productivity. In view of the potential benefits of sonication and its cost effectiveness in some processes, a wider investigation of its applications in biotechnology based processing is warranted.

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References