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Ultrasound-assisted fermentation enhances bioethanol productivity

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ABSTRACT

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 measureable 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.

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

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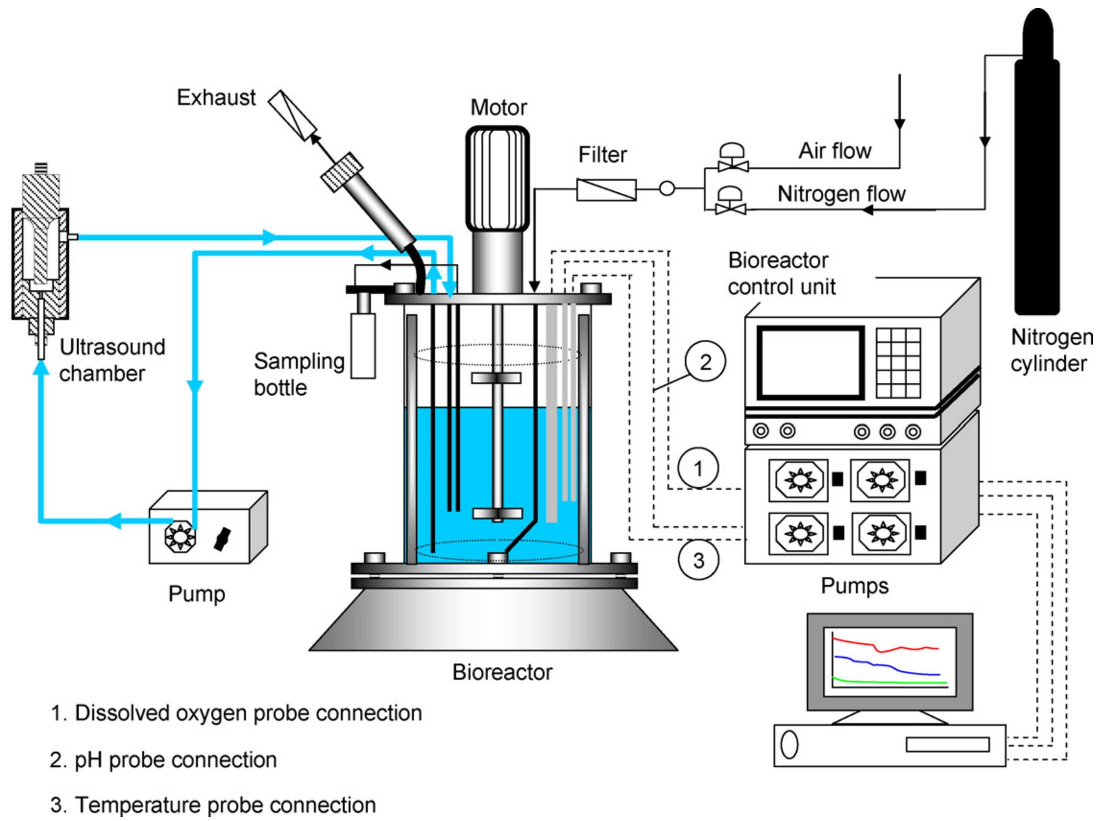

Fig. 1. Ultrasound assisted batch fermentation system.

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 Ca^{2+} [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; $(NH_4)_2SO_4$ 6.25; $MgSO_4{\cdot}7H_2O$ 2; KH_2PO_4 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 $\sim 4 \times 10^7$ 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

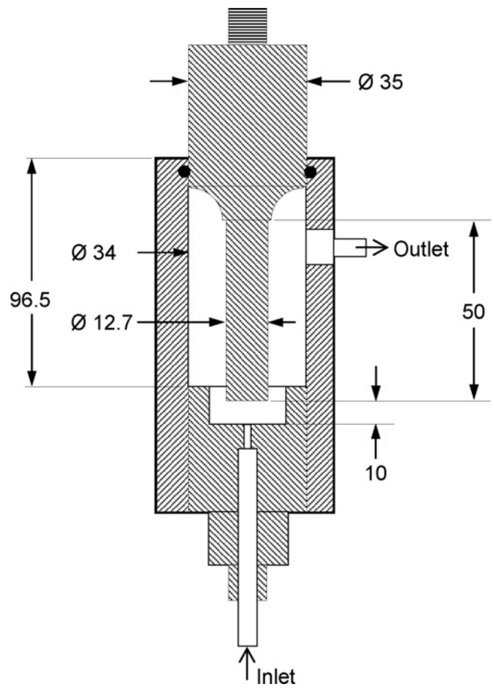

Fig. 2. The ultrasonic flow cell. Dimensions in mm.

sterile hydrophobic membrane filters (0.2 μm; either Sartorious, Gottingen, Germany, or Millipore, Bedford, MA, USA). The assembled bioreactor filled with the earlier specified liquid medium was autoclaved (121 °C, 20 min) with the pH and the dissolved oxygen electrodes installed. The pH electrode (Ingold gel-filled electrode, model no. 465-35-SC-P-K9/270/9848; Mettler-Toledo, www.mt.com) had been calibrated using pH 7.0 and pH 4.0 buffers prior to autoclaving.

The concentration of dissolved oxygen (DO) in the broth was measured online using a polarographic electrode (model In Pro 6800 sensor 12/25 mm; Mettler-Toledo, www.mt.com). The DO electrode had been calibrated at 30 °C in the sterilized culture medium. For the calibration, the liquid medium was first bubbled with nitrogen until the dissolved oxygen reading failed to decline further. The DO readout was then adjusted to read 0%. Nitrogen flow was then replaced with a preset air flow of 2.67 vvm, with the impeller rotating at 500 rpm. Once the measured concentration of dissolved oxygen had stabilized, it was adjusted to an air saturation value of 100%.

A 20 kHz, 600 W maximum power, Misonix Sonicator® 3000 (Misonix, Inc., Farmingdale, NY, USA, www.misonix.com) ultrasound generator was used in combination with a standard tapped sonic horn (Misonix, Inc., part no. 200 with 12.7 mm tip diameter, 127 mm length), or sonotrode, installed in an external 800B Misonix Flocell® with a 3.175 mm diameter inlet orifice (Fig. 2). The horn had a replaceable flat tip made of titanium alloy (Misonix, Inc., part no. 406). The flow cell, with the sonic horn in place, was autoclaved (121 °C, 20 min), cooled to room temperature, and connected to the bioreactor aseptically using sterile silicone tubing as shown in Fig. 1. The broth from the bioreactor was recirculated continuously through the sonic chamber using a peristaltic pump (Masterflex model no. 7554-60; Cole Parmer Instrument Co., Chicago, IL, USA). The recirculation flow rate was fixed at 0.2 L min^{-1}. The recirculation commenced after the fermenter had been inoculated and briefly mixed. All fermentations were carried out with recirculation of the broth through the sonic chamber, but ultrasound was not applied to the control fermentation.

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 2.67 vvm, 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:

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

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. A 1 mL sample of the broth was diluted with 24 mL of the sterile medium prior to measurement. This way the spectrophotometric 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:

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

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:

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

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

2.4.3. Ethanol concentration

Ethanol concentration in the broth supernatant was determined using gas chromatography (model GC 6000 Vega Series 2; Carlo Erba Instruments, Milan, Italy) fitted with a flame ionization detector and chromato-integrator (model D-2500; Hitachi, Tokyo, Japan). The carrier gas was nitrogen at a flow rate of 40 mL min^{-1}. The column temperature was 200 °C. Standard ethanol solutions were prepared in the concentration range of 2–8 g L^{-1} by diluting absolute ethanol with deionized water. The sample volume injected was 2 μL. The sample had been prefiltered through a 0.45 μm membrane filter. The ethanol concentration of the culture supernatant sample was calculated by measuring the relative area under the ethanol peak and comparing it with the standard curve prepared using the standard solutions.

2.4.4. Cell viability

Cell viability was determined using the methylene blue staining method [42]. A 10 μL aliquot of serially diluted freshly sampled yeast broth was mixed with of 10 μL of a methylene blue solution and incubated for 5 min [42]. The cell suspension was then counted on a hemacytometer at 400× 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 unit counts on petri dishes.

2.4.5. Activity of β-galactosidase

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

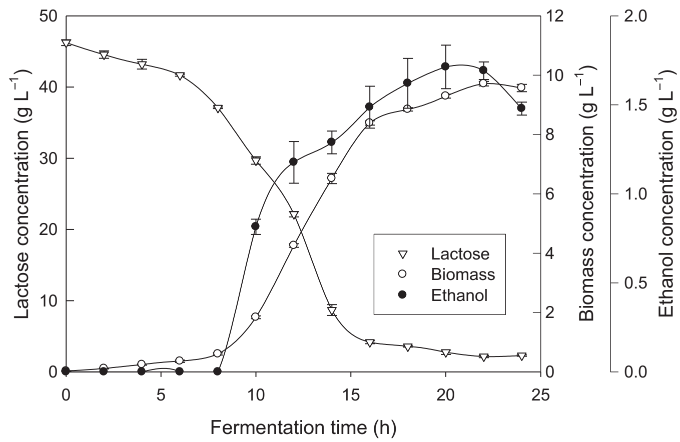

Fig. 3. A typical control fermentation profile.

The intracellular β-galactosidase activity was measured according to the method described by Wang and Sakakibara [13]. A 35 mL sample of the broth was centrifuged (3300 × *g*, 10-min) to recover the cells. The cells were washed (2 × 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 × *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 β-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 1
Comparison of fermentation kinetics.

Kinetic parameter	Sonication regimens (duty cycle)[a]			
	Control (no sonication)	10%	20%	40%
Maximum specific growth rate, μ (h^{-1})	0.203 ± 0.011	0.206 ± 0.027	0.217 ± 0.007	0.179 ± 0.017
Average specific lactose uptake rate, q_s (g g^{-1} h^{-1})	0.206 ± 0.003	0.151 ± 0.010	0.172 ± 0.006	0.208 ± 0.009
Maximum biomass yield on lactose, $Y_{x/s}$ (g g^{-1})	0.220 ± 0.003	0.300 ± 0.020	0.292 ± 0.010	0.218 ± 0.010
Maximum biomass concentration, X_{max} (g L^{-1})	9.712 ± 0.076	13.755 ± 0.850	13.813 ± 0.443	8.388 ± 0.315
Maximum biomass productivity, P_x (g L^{-1} h^{-1})	0.441 ± 0.003	0.625 ± 0.039	0.693 ± 0.022	0.381 ± 0.014
Final ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.034 ± 0.001	0.096 ± 0.009	0.109 ± 0.014	0.052 ± 0.002
Final ethanol concentration (g L^{-1})	1.479 ± 0.036	4.421 ± 0.042	5.199 ± 0.677	2.003 ± 0.086
Final ethanol productivity, P_E (g L^{-1} h^{-1})	0.062 ± 0.002	0.184 ± 0.017	0.217 ± 0.028	0.083 ± 0.004
Average biomass specific ethanol production rate, q_p (g g^{-1} h^{-1})	(6.35 ± 0.16) × 10^{-3}	(13.39 ± 1.47) × 10^{-3}	(15.68 ± 2.10) × 10^{-3}	(9.95 ± 0.57) × 10^{-3}

[a] Except for the control culture, the sonication power intensity was always 11.8 W cm^{-2}.

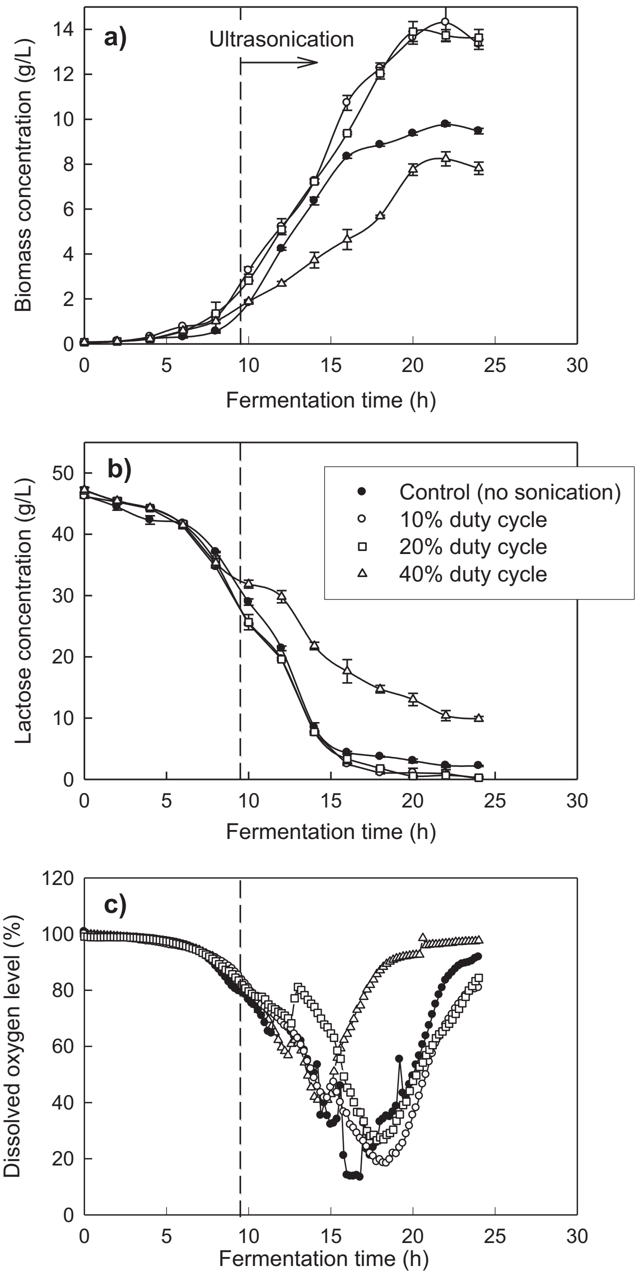

Fig. 4. Effects of sonication on: (a) biomass concentration; (b) lactose concentration; and (c) dissolved oxygen concentration. Except for the nonsonicated control, the sonication intensity was 11.8 W cm^{-2}.

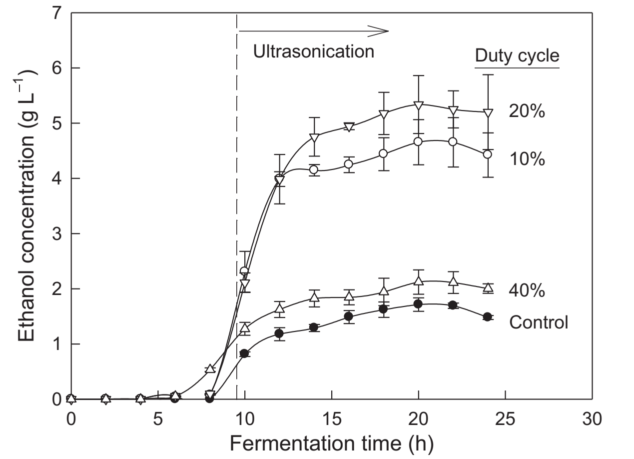

Fig. 5. Ethanol concentration profiles. The sonication intensity was 11.8 W cm^{-2} except for the nonsonicated control culture.

of biomass growth, lactose consumption and the dissolved oxygen concentration are shown in Fig. 4 in comparison to controls. All the profiles were comparable prior to the beginning of sonication. Sonication at duty cycles of 10% and 20% substantially improved the biomass growth rate and final concentration relative to control, but increasing the duty cycle to 40% adversely affected the growth rate and the final biomass concentration (Fig. 4a). The reduced biomass growth and final concentration at the highest duty cycle were clearly reflected in a slower rate of lactose consumption and a higher concentration of the residual lactose for this fermentation (Fig. 4b). Lactose consumption of the fermentations conducted at duty cycles of 10 and 20% was comparable to that of the control (Fig. 4b).

The adverse effect of sonication at 40% duty cycle was reflected also in the dissolved oxygen concentration profiles (Fig. 4c). Thus, at the 40% duty cycle, because of a reduced rate of consumption of lactose, the decline in the dissolved oxygen concentration during exponential growth was less than for the other fermentations and the oxygen concentration recovered earlier (Fig. 4c) suggesting an earlier end to exponential growth even though plenty of lactose remained in the broth. Clearly, even at a relatively high intensity of 11.8 W cm^{-2}, ultrasound stimulated growth of *K. marxianus* on a soluble 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

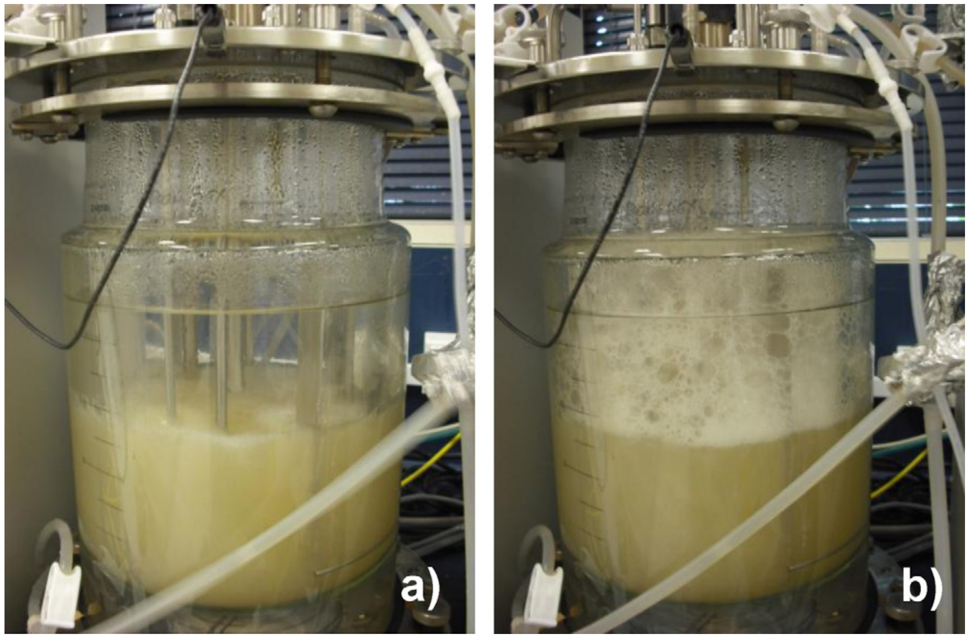

Fig. 6. Foaming behavior of the fermentation: (a) just before sonication commenced 9.5 h after inoculation and (b) the same fermentation 10 min after sonication commenced at a power intensity of 11.8 W cm^{-2} and a duty cycle of 20%.

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 sonciation 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, μ:

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

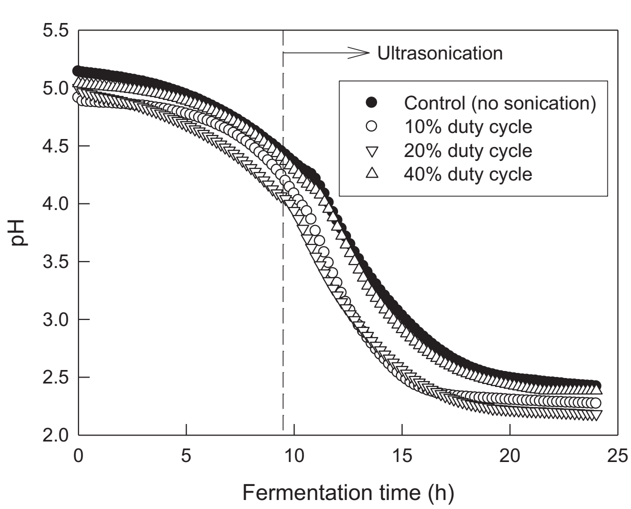

Fig. 7. The pH profiles. The sonication intensity was 11.8 W cm^{-2} except for the nonsonicated control culture.

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:

$$q_s = -\frac{\Delta S}{\Delta X t} \tag{5}$$

where ΔS is the substrate consumed by time t (= 22 h) and ΔX is the increase in biomass concentration by time t.

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

$$Y_{x/s} = -\frac{\Delta X}{\Delta S} \tag{6}$$

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

Maximum biomass productivity, P_x:

$$P_x = \frac{X_{max} - X_0}{t} \tag{7}$$

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, $Y_{p/s}$:

$$Y_{p/s} = -\frac{\Delta P}{\Delta S} \tag{8}$$

where ΔP is the change in ethanol concentration during the fermentation.

Final ethanol productivity, P_E:

$$P_E = \frac{E_f - E_0}{t_f} \tag{9}$$

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:

$$q_p = \frac{\Delta E}{X_{max} t} \tag{10}$$

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

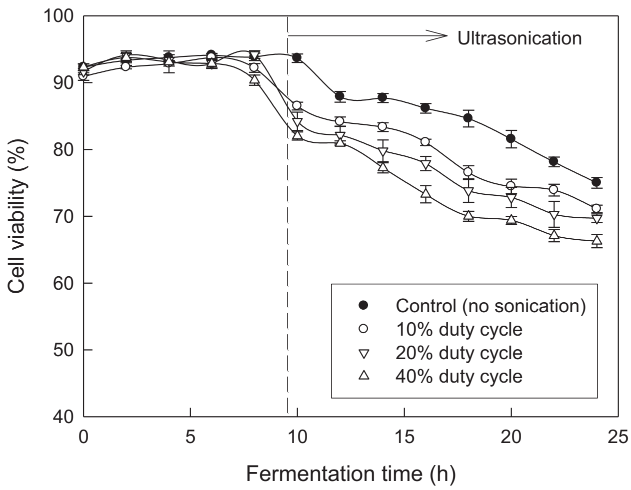

Fig. 8. Cell viability profiles. The sonication intensity was 11.8 W cm^{-2} except for the nonsonicated control culture.

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

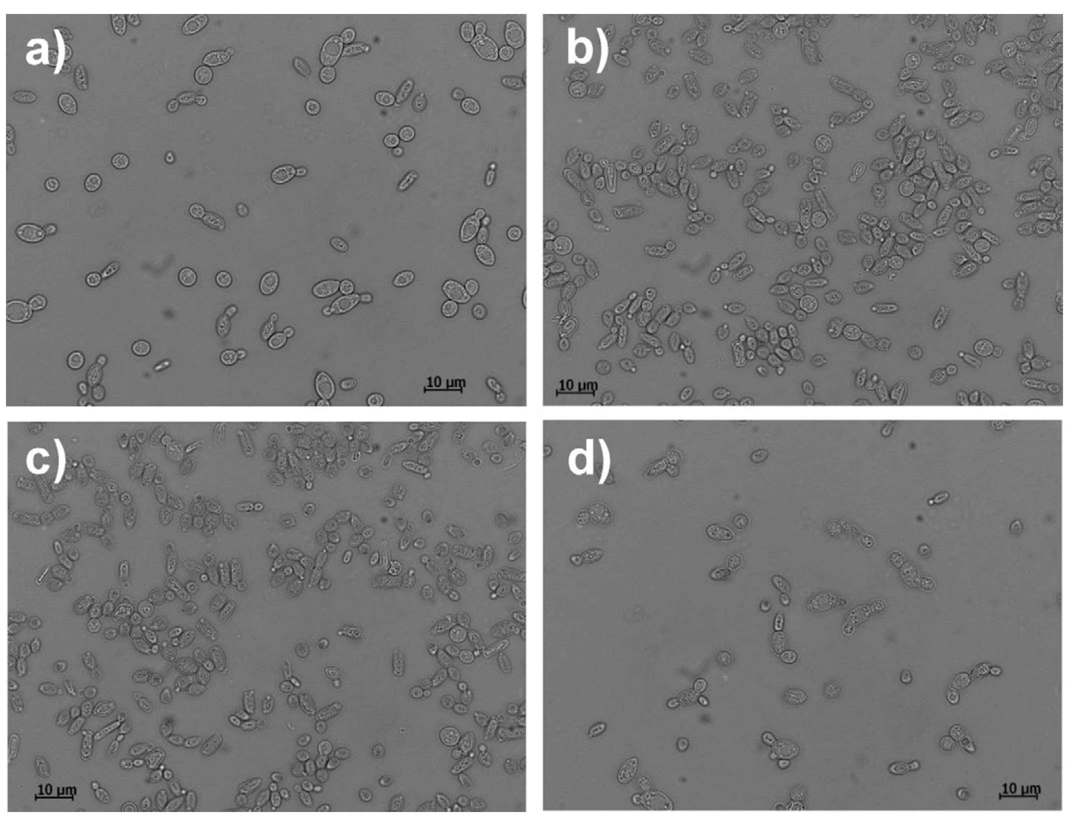

Fig. 9. Yeast cell morphology (1000× magnification) at 22 h of fermentation: (a) control (no sonication), (b) sonication at 10% duty cycle, (c) sonication at 20% duty cycle and (d) sonication at 40% duty cycle. The sonication intensity was always 11.8 W cm^{-2}.

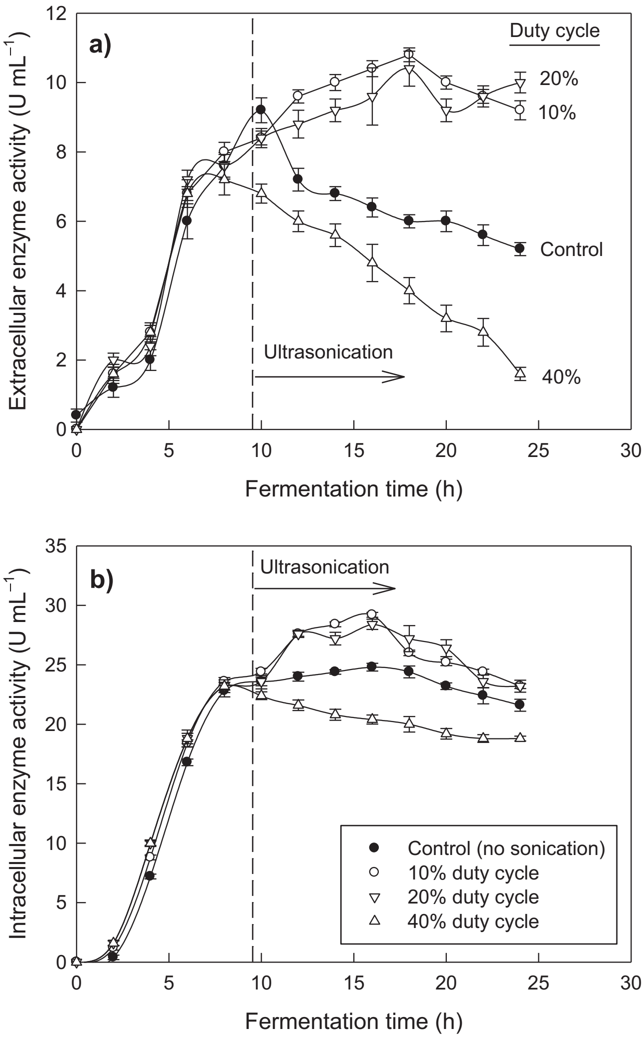

Fig. 10. β-Galactosidase activity profiles during fermentation: (a) extracellular enzyme activity and (b) intracellular enzyme activity. The sonication intensity was 11.8 W cm^{-2} except for the nonsonicated control culture.

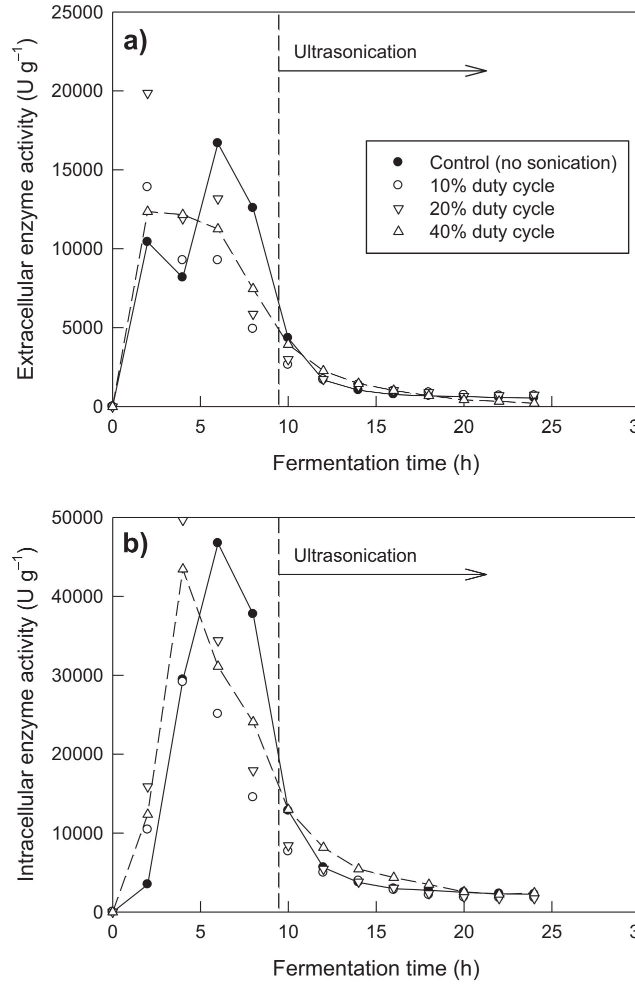

Fig. 11. Biomass specific β-galactosidase activity profiles during fermentation: (a) extracellular enzyme activity and (b) intracellular enzyme activity. The sonication intensity was 12.5 W cm^{-2} except for the nonsonicated control culture. For clarity, lines are plotted only through the data for the control culture (solid lines) and the culture sonicated at the 40% duty cycle (dashed lines).

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:

$$t_R = \frac{V_L}{Q_L} \tag{11}$$

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]:

$$t_\theta = \frac{-\ln\left(1-\theta\right)}{1.06N(D/T)^{2.17}(T/H)^{0.5}} \tag{12}$$

where t_θ is the time required to attain a fractional homogeneity of θ (e.g. a θ-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

[1] Y. Chisti, M. Moo-Young, Disruption of microbial cells for intracellular products, Enzyme and Microbial Technology 8 (1986) 194–204.
[2] M.V. Potapovich, A.N. Eryomin, D.I. Metelitza, Ultrasonic and thermal inactivation of catalases from bovine liver, the methylotrophic yeast *Pichia pastoris*, and the fungus *Penicillium piceum*, Applied Biochemistry and Microbiology 41 (2005) 529–537.
[3] R. Bar, Ultrasound enhanced bioprocesses: cholesterol oxidation by *Rhodococcus erythropolis*, Biotechnology and Bioengineering 32 (1988) 655–663.
[4] N.J. Kilby, C.S. Hunter, Repeated harvest of vacuole-located secondary product from in vitro grown plant cells using 1.02 MHz ultrasound, Applied Microbiology and Biotechnology 33 (1990) 448–451.
[5] M. Zabaneh, R. Bar, Ultrasound-enhanced bioprocess. II. Dehydrogenation of hydrocortisone by *Arthrobacter simplex*, Biotechnology and Bioengineering 37 (1991) 998–1003.
[6] J. Chu, B. Li, S. Zhang, Y. Li, On-line ultrasound stimulates the secretion and production of gentamicin by *Micromonospora echinospora*, Process Biochemistry 35 (2000) 569–572.
[7] D. Chuanyun, W. Bochu, D. Chuanren, A. Sakanishi, Low ultrasonic stimulates fermentation of riboflavin producing strain *Ecemothecium ashbyii*, Colloids and Surfaces B: Biointerfaces 30 (2003) 37–41.
[8] D. Chuanyun, W. Bochu, Z. Huan, H. Conglin, D. Chuanren, L. Wangqian, Y. Toyama, A. Sakanishi, Effect of low frequency ultrasonic stimulation on the secretion of riboflavin produced by *Ecemothecium ashbyii*, Colloids and Surfaces B: Biointerfaces 34 (2004) 7–11.
[9] Y. Chisti, Sonobioreactors: using ultrasound for enhanced microbial productivity, Trends in Biotechnology 21 (2003) 89–93.
[10] P.R. Gogate, A.M. Kabadi, A review of applications of cavitation in biochemical engineering/biotechnology, Biochemical Engineering Journal 44 (2009) 60–72.
[11] C.M. Runyan, J.C. Carmen, B.L. Beckstead, J.L. Nelson, R.A. Robinson, W.G. Pitt, Low-frequency ultrasound increases outer permeability of *Pseudomonas aeruginosa*, Journal of General and Applied Microbiology 52 (2006) 295–301.
[12] M. Sakakibara, D. Wang, K. Ikeda, K. Suzuki, Effect of ultrasonic irradiation on production of fermented milk with *Lactobacillus delbrueckii*, Ultrasonics Sonochemistry 1 (1994) S107–S110.
[13] D. Wang, M. Sakakibara, Lactose hydrolysis and β-galactosidase activity in sonicated fermentation with *Lactobacillus* strains, Ultrasonics Sonochemistry 4 (1997) 255–261.
[14] B.E. Wood, H.C. Aldrich, L.O. Ingram, Ultrasound stimulates ethanol production during the simultaneous saccharification and fermentation of mixed waste office paper, Biotechnology Progress 13 (1997) 232–237.
[15] H. Wu, G.J. Hulbert, J.R. Mount, Effects of ultrasound on milk homogenization and fermentation with yogurt starter, Innovative Food Science and Emerging Technologies 1 (2000) 211–218.
[16] E. Joyce, S.S. Phull, J.P. Lorimer, T.J. Mason, The development and evaluation of ultrasound for the treatment of bacterial suspensions. A study of frequency, power and sonication time on cultured Bacillus species, Ultrasonics Sonochemistry 10 (2003) 315–318.
[17] N.S. Herran, J.L.C. Lopez, J.A.S. Perez, Y. Chisti, Effects of ultrasound on culture of *Aspergillus terreus*, Journal of Chemical Technology and Biotechnology 83 (2008) 593–600.
[18] H. Böhm, P. Anthony, M.R. Davey, L.G. Briarty, J.B. Power, K.C. Lowe, E. Benes, M. Gröschl, Viability of plant cell suspensions exposed to homogeneous ultrasonic fields of different energy density and wave type, Ultrasonics 38 (2000) 629–632.
[19] J.M. Anderson, Effects of ultrasonic radiation on growth and fermentation in yeast, *Saccharomyces cerevisiae*, Biochimica et Biophysica Acta 11 (1953) 122–137.
[20] K. Matsuura, M. Hirotsune, Y. Nunokawa, M. Satoh, K. Honda, Acceleration of cell growth and ester formation by ultrasonic wave irradiation, Journal of Fermentation and Bioengineering 77 (1994) 36–40.
[21] S. Lanchun, W. Bochu, L. Zhiming, D. Chuanren, D. Chuanyun, A. Sakanishi, The research into the influence of low intensity ultrasonic on the growth of *S. cerevisiae*, Colloids and Surfaces B: Biointerfaces 30 (2003) 43–49.
[22] C. Jomdecha, P. Prateepasen, The research of low-ultrasonic affects to yeast growth in fermentation process, in: Proceedings of the 12th Asia-Pacific Conference on Nondestructive Testing, 5–10 November, Auckland, New Zealand, 2006.
[23] S. Radel, A.J. McLoughlin, L. Gherardini, O. Doblhoff-Dier, E. Benes, Viability of yeast cells in well controlled propagating and standing ultrasonic plane waves, Ultrasonics 38 (2000) 633–637.
[24] S.K. Khanal, M. Montalbo, J.H. van Leeuwen, G. Srinivasan, D. Grewell, Ultrasound enhanced glucose release from corn in ethanol plants, Biotechnology and Bioengineering 98 (2007) 978–985.
[25] S. Nikolić, L. Mojović, M. Rakin, D. Pejin, J. Pejin, Ultrasound-assisted production of bioethanol by simultaneous saccharification and fermentation of corn meal, Food Chemistry 122 (2010) 216–222.
[26] S. Nitayavardhana, P. Shrestha, M.L. Rasmussen, B.P. Lamsal, J.H. van Leeuwen, S.K. Khanal, Ultrasound improved ethanol fermentation from cassava chips in cassava-based ethanol plants, Bioresource Technology 101 (2010) 2741–2747.
[27] O. Schläfer, M. Sievers, H. Klotzbücher, T.I. Onyeche, Improvement of biological activity by low energy ultrasound assisted bioreactors, Ultrasonics 38 (2000) 711–716.
[28] S. Lanchun, W. Bochu, Z. Lianchai, L. Jie, Y. Yanhong, D. Chuanren, The influence of low intensity ultrasonic on some physiological characteristics of *Saccharomyces cerevisiae*, Colloids and Surfaces B: Biointerfaces 30 (2003) 61–66.
[29] W. Bochu, S. Lanchun, Z. Jing, Y. Yuanyuan, Y. Yanhong, The influence of Ca^{2+} on the proliferation of S. cerevisiae and low ultrasonic on the concentration of Ca^{2+} in the S. cerevisiae cells, Colloids and Surfaces B: Biointerfaces 32 (2003) 35–42.
[30] M.A.F. Belem, B.H. Lee, Production of bioingredients from *Kluyveromyces marxianus* grown on whey: an alternative, Critical Reviews in Food Science and Nutrition 38 (1998) 565–598.
[31] T. Lukondeh, N.J. Ashbolt, P.L. Rogers, Fed-batch fermentation for production of *Kluyveromyces marxianus* FII510700 cultivated on a lactose-based medium, Journal of Industrial Microbiology and Biotechnology 32 (2005) 284–288.
[32] P.M.R. Guimarães, J.A. Teixeira, L. Domingues, Fermentation of lactose to bioethanol by yeasts as part of integrated solutions for the valorisation of cheese whey, Biotechnology Advances 28 (2010) 375–384.
[33] M.A. Mehaia, M. Cheryan, Hollow fibre bioreactor for ethanol production: application to the conversion of lactose by *Kluyveromyces fragilis*, Enzyme and Microbial Technology 6 (1984) 117–120.
[34] I. Marison, Uv. Stockar, A calorimetric investigation of the aerobic cultivation of *Kluyveromyces fragilis* on various substrates, Enzyme and Microbial Technology 9 (1987) 33–43.
[35] M. Ozilgen, D.F. Ollis, D. Ogrydziak, Kinetics of batch fermentations with *Kluyveromyces fragilis*, Enzyme and Microbial Technology 10 (1988) 165–172.
[36] S.E. Barberis, R.F. Segovia, Dissolved oxygen concentration controlled feeding of substrate into *Kluyveromyces fragilis* culture, Biotechnology Techniques 11 (1997) 797–799.
[37] N. Bojorge, B. Valdman, F. Acevedo, J.C. Gentina, A semi-structured model for the growth and β-galactosidase production by fed-batch fermentation of *Kluyveromyces marxianus*, Bioprocess and Biosystems Engineering 21 (1999) 313–318.
[38] L. Krzystek, S. Ledakowicz, Stoichiometric analysis of *Kluyveromyces fragilis* growth on lactose, Journal of Chemical Technology and Biotechnology 75 (2000) 1110–1118.
[39] S. Grba, V. Stehlik-Tomas, D. Stanzer, N. Vaheie, A. Škrlin, Selection of yeast strain *Kluyveromyces marxianus* for alcohol and biomass production on whey, Journal of Chemical Technology and Biotechnology 16 (2002) 13–16.
[40] S. Zafar, M. Owais, M. Saleemuddin, S. Husain, Batch kinetics and modelling of ethanolic fermentation of whey, International Journal of Food Science and Technology 40 (2005) 597–604.
[41] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Analytical Chemistry 31 (1959) 426–428.
[42] K. Painting, B. Kirsop, A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining, World Journal of Microbiology and Biotechnology 6 (1990) 346–347.

[43] Sigma, Enzymatic assay of β-galactosidase, Sigma-Aldrich Corporation, 1994.
[44] M. Ashokkumar, J. Lee, S. Kentish, F. Grieser, Bubbles in an acoustic field: an overview, Ultrasonics Sonochemistry 14 (2007) 470–475.
[45] J.S. Norton, R.W. Krauss, The inhibition of cell division in *Saccharomyces cerevisiae* (Meyen) by carbon dioxide, Plant and Cell Physiology 13 (1972) 139–149.
[46] R.P. Jones, P.F. Greenfield, Effect of carbon dioxide on yeast growth and fermentation, Enzyme and Microbial Technology 4 (1982) 210–223.
[47] P.M. Doran, Bioprocess Engineering Principles, Academic Press, London, 1995.
[48] M.F. Rosa, I. SaCorreia, Intracellular acidification does not account for inhibition of *Saccharomyces cerevisiae* growth in the presence of ethanol, FEMS Microbiology Letters 135 (1996) 271–274.
[49] P. Lucero, E. Penalver, E. Moreno, R. Lagunas, Internal trehalose protects endocytosis from inhibition by ethanol in *Saccharomyces cerevisiae*, Applied and Environmental Microbiology 66 (2000) 4456–4461.
[50] J.B. Fasano, W.R. Penney, Avoid blending mixups, Chemical Engineering Progress 87 (10) (1991) 56–63.