Pol. J. Environ. Stud. Vol. 21, No. 2 (2012), 387-393

Original Research

Ultrasonic Stimulation of Co-Immobilized *Saccharomyces cerevisiae* Cells and β-Galactosidase Enzyme for Enhanced Ethanol Production from Whey Ultrafiltration Permeate

Marta Kisielewska*

Department of Environmental Protection Engineering, University of Warmia and Mazury in Olsztyn, Prawocheńskiego 1, 10-957 Olsztyn, Poland

Received: 27 May 2011
Accepted: 18 October 2011

Abstract

Low energy ultrasound irradiation (20 kHz, 1.0 $W \cdot L^{-1}$) was applied to enhance bioethanol production from whey ultrafiltration permeate by co-immobilized *Saccharomyces cerevisiae* cells and β-galactosidese enzyme. Sugar utilization and ethanol formation were investigated as a function of hydraulic retention time (HRT) between 12 and 36 h. Maximum ethanol production under HRT of 36 h was 26.30 $g \cdot L^{-1}$ with ultrasound exposure, and 23.60 $g \cdot L^{-1}$ without. Maximum ethanol yield was 0.532 $g \cdot g^{-1}$ lactose in the fermentation process with ultrasound irradiation, and 0.511 $g \cdot g^{-1}$ without. For the continuously operating bioreactors, the maximum rates of sugar utilization were 98.9 and 92.4% for the yeast with and without ultrasound exposure, respectively. These results highlight the positive effect of low-intensity ultrasounds in bioethanol fermentation from whey permeates, raising new perspectives for its disposal.

Keywords: renewable energy, continuous ethanol fermentation, permeate, ultrasound irradiation, *Saccharomyces cerevisiae*

Introduction

Ultrafiltration (UF) whey permeates are the liquids remaining after industrial separation/concentration of milk fat and proteins using the membrane UF process. Due to the low concentration of solid constituents (about 4% w/v), UF permeates have commonly been considered waste products. The disposal of UF permeates is a major problem for the dairy industry. Permeate streams have a high COD (chemical oxygen demand) value (about 60,000 $mg \cdot L^{-1}$), which represents an important environmental problem [1]. Moreover, the chemical and biological instability of permeates result in difficulties, plus high transportation and storage costs. Because of this there is an increasing concern as to how the whey and whey UF permeates can be efficiently and cost-effectively treated without adversely affecting the environment.

Lactose concentration in whey UF permeates is about 50,000 $mg \cdot L^{-1}$, so more than 90% of COD is due to lactose [1]. Moreover, valuable compounds (protein, lactose, vitamins) can be found in their composition. Since whey and whey permeates contain significant quantities of lactose, the way to use these waste products could be as a substrate for ethanol fermentation. In recent years, fermentative pro-

*e-mail: jedrzejewska@uwm.edu.pl

duction of ethanol from renewable resources, such as wastewater from dairy industries, has received attention due to the increasing petroleum shortage [1-3]. Moreover, biofuels derived from wastes and by-products show low environmental effects (greenhouse emissions, use of soil pollutants: pesticides, herbicides, land demand, etc.) [1].

Many reports show the potential applications of yeast strains in ethanol production from permeate streams, but most of them focus on *Kluyveromyces* sp. due to its ability to directly ferment lactose [2-4]. These yeasts generally suffer from low conversion yields (0.4 kg ethanol·kg^{-1} lactose) and are very sensitive to product (ethanol) inhibition at concentrations as low as 20 g·L^{-1} [1, 5]. An alternative is to use indirect fermentation yeasts, such as *Saccharomyces cerevisiae*, which show much higher alcohol tolerance (100-120 g·L^{-1}) and considerably better ethanol fermentation performance (0.520 kg ethanol·kg^{-1} glucose), but has the disadvantage that an enzymatic hydrolysis step is required prior to lactose fermentation [1, 6]. The fact that the yeasts are incapable of directly fermenting lactose can be solved by genetic manipulation of yeasts or facilitate the process with a simultaneous lactose hydrolysis, for example by co-immobilization of yeast cells with the enzyme [6-9].

Higher ethanol production could be achieved by applying different stimulation processes to improve biological activity of yeasts. Many researchers have found that ultrasonic stimulation has the function of promoting the activity of enzyme, cell growth, and cell membrane permeability, improving gas-liquid mass transfer [10-14]. However, the application of ultrasonic irradiation at improper intensity or period has a destructive impact on cells by disrupting the cell membranes and deactivating biological molecules such as enzymes or DNA [12].

The objective of this study was to evaluate the effects of low intensity ultrasound (20 kHZ, 1 W·L^{-1}) for ethanol production from whey UF permeate by the yeast strain *S. cerevisiae* B4 co-immobilized with β-galactosidase enzyme.

Experimental Procedures

Microorganisms

The distillery industrial yeast *Saccharomyces cerevisiae* B-4 obtained from the Institute of Agricultural and Food Biotechnology in Warsaw, Poland, was used for assessing the ultrasound exposure effect on ethanol production. The yeast cultures were cultivated on YPG slants (2% glucose, 2% peptone, 1% yeast extract) supplemented with 2% agar at pH 5.0 and 30ºC for 24 h. The active cultures for inoculation were prepared by growing the yeast in a 1 L baffled shake-flask containing sterile water and 100 mL YPG medium at 30ºC for 24 h on orbital shaker table at 200 rpm to a concentration of approximately 10^8 cells mL^{-1}. The cultures in baffled shaken flasks of 100 mL were used to prepare the inocula. After 24 h of incubation at 30ºC, the precultures were centrifuged at 3,800 rpm for 10 min and the cells were resuspended in sterile water to obtain 10^6 cells mL^{-1}. Enzyme β-D-galactosidase (optimum temperature 30ºC, optimum acidity pH 4.5-5.0, activity 8.7 AU mg^{-1} d.m. of the preparation), from *Aspergillus oryzae* manufactured by the SIGMA company (USA), was used for the co-immobilization process. β-galactosidase activity was determined by measuring the release of *o*-nitrophenol from ONPG (*o*-nitrophenyl β-D-galactopyranoside) at 420 nm. The reaction was carried out in a total volume of 2.0 mL containing 1.7 mL of 0.1 M sodium acetate buffer, pH 4.5, 0.1 mL suitably diluted enzyme, and 0.2 mL of 20 mM ONPG at 37ºC for 15 min. The reaction was stopped by adding 2.0 mL 2.0 N sodium carbonate solution. One unit of β-galactosidase activity is defined as the amount of enzyme that liberates 1.0 μmol of *o*-nitrophenol per min in the conditions defined above.

The yeast culture was co-immobilized in the 2% (w/v) sodium alginate by dropping aliquots of 3% free cell inoculum and enzyme solution, each containing a final concentration of 9 AU ml^{-1} into 150 cm^3 0.09 mol·L^{-1} solution of $CaCl_2$ with 10% glucose. The alginate beads containing yeast cells and β-galactosidase enzyme were washed with sterile water and were stored as a fermentation medium in physiological solution at 8ºC, pH 4.7.

Fermentation Medium and Experimental System

Ultrafiltrated (UF) whey permeate (non-deproteinized, non diluted and non-sterilized) with 50 g·L^{-1} lactose concentration from the Dairy Plant in Nowy Dwór Gdański was used as a fermentation substrate.

Continuous fermentation was carried out in a laboratory-scale plant consisting of two anaerobic reactors with a working volume of 5 L each (Fig. 1). These two reactors were used to enable parallel test series with and without ultrasound irradiation. The fermentation medium was pumped continuously to the bottom part of the reaction tank by means of peristaltic pumps. The necessary mixing was achieved through the upward wastewater flow and the magnetic stirrer operated at regular intervals. The reactors were water-jacketed and operated at a constant temperature of 30±1ºC.

The reactors were inoculated with 40% (v/v) of solid beads containing the co-immobilized cells which corresponded to 39.4 g cells DW·L^{-1} of working bioreactor volume. After adding the alginate bead inoculum in the bioreactors, before starting continuous feeding, a batch fermen-

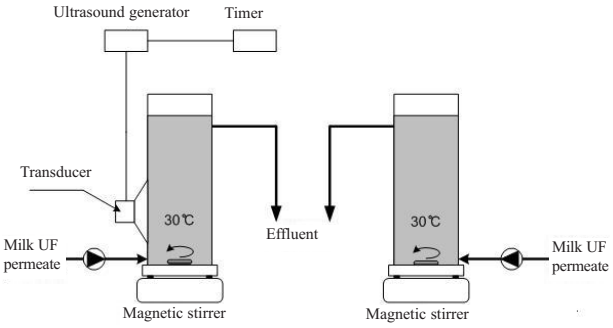

Fig. 1. A scheme of the ultrasound assisted and non-assisted continuous fermentation system.

tation was conducted for 24 h under gentle agitation (100 rpm). Then the reactors were operated at different hydraulic retention times (HRT) of 12, 24, and 36 h. At each HRT the reactor was operated until it reached a steady state (constant ethanol and lactose concentrations in the effluent), thus 30 days of each HRT (12, 24, and 36 h). The fresh inoculum was added to the reactors before each HRT was initiated and the aged one was removed.

Ultrasonic Equipment

Ultrasonic irradiation of the reactor with yeasts was done using a special ring with transducer (Intersonic S.C. Poland) that was attached at the bottom of the reactor. The range of the frequency generator was adjustable between 20-25 kHz, with maximum power of 50 W. The experiments were carried out using stable sonication power of 1 $W \cdot L^{-1}$ and frequency of 20 kHz.

Analytical Methods

Lactose and ethanol concentrations in distillate were determined according to Standard Methods PN-EN [15]. The samples were analyzed in triplicates and results were reproducible within 3% deviation. The ethanol yield was determined as:

$$Y_{P/S} \text{ (g EtOH} \cdot \text{g}^{-1} \text{ lactose utilized)} = P_f - P_i \,/\, S_i - S_f$$

...where P_f is the final ethanol concentration ($g \cdot L^{-1}$), P_i is the initial ethanol concentration ($g \cdot L^{-1}$), S_i is the initial lactose concentration ($g \cdot L^{-1}$), and S_f is the final lactose concentration ($g \cdot L^{-1}$).

The percentage lactose consumption was determined as:

$$\textit{lactose consumption } (\%) = 100 \cdot [1 - (S_f \,/\, S_i)]$$

Statistical Analysis

All fermentation steps connected with different HRTs were carried out in triplicate. Significant differences between the effects obtained in the two reactors with and without ultrasound exposure were analyzed using an ANOVA F-test (Statistica 7.1 software, Statsoft Inc.). A 5% probability level was applied for all the tests. If $p<0.05$ from an ANOVA F-test, the differences between the effects were considered to be significantly different from one another.

Results and Discussion

Sonication Parameters

In the experiment, the frequency of applied ultrasounds was 20 kHz and the power input was 1.0 $W \cdot L^{-1}$. The initial experiments were done to find the best irradiation period. The experiments revealed that continuous low energy ultrasound irradiation during 12, 24, and 36 h did not enhance ethanol productivity by co-immobilized *S. cerevisiae*. Moreover, the ethanol yield coefficients were lower than those obtained in experiments without ultrasound irradiation. The subsequent experiments were carried out using time intervals with and without ultrasonic irradiation in order to obtain the positive influence of ultrasound on biological activity of *S. cerevisiae*. Sonication cycles of 4 min sonication followed by a 6 h rest period, 3 min sonication followed by a 6 h rest period, 2 min sonication followed by a 6 h rest period, 1 min sonication followed by a 6 h rest period were tested at HRT of 24 h. The adverse effects of sonication on ethanol concentration and ethanol yield at 4 min, 3 min, and 2 min sonication cycles were noted. At the cycles of 4 min sonication every 6 h, 3 min sonication every 6 h, and 2 min sonication every 6 h, the final ethanol concentrations were 9.8, 12.3, and 18.1 $g \cdot L^{-1}$, respectively, and ethanol yields were as low as 0.306, 0.332, and 0.460 $g \cdot g^{-1}$, respectively. In the control fermentation, ethanol concentration was as high as 21.79 $g \cdot L^{-1}$, while ethanol yield was 0.487 $g \cdot g^{-1}$. During the shortest sonication step, the obtained results were better than in the control fermentation. The cycle of 1 min sonication every 6 h was chosen for further investigation.

This was similar to results obtained by Marques et al. [16]. They investigated the effect of ultrasound pulses on enzymatic activity of *S. cerevisiae*. Their results showed that the ultrasound pulse at low frequency (20-25 kHz) for a short sonication period of 1 and 2 min increased cell permeability, and the viability rate of yeasts was over 95%. However, in the 4 min sonication, the rate decreased to 46%.

The use of ultrasounds to stimulate biological activity and ethanol production by *S. cerevisiae* is reported by Schläfer et al. [13]. After testing several different frequencies and power levels, they carried out the experiments at 25 kHz, 0.3 and 12 $W \cdot L^{-1}$. At an ultrasound intensity of 12 $W \cdot L^{-1}$ there was no recognizable difference in the biological activity of yeasts with and without ultrasound. The authors stated that some pauses are needed between ultrasound exposures to obtain positive effects on biological activity of yeast *S. cerevisiae*. Moreover, an increase in biological activity appeared after irradiation and high activity of ultrasound-activated cultures stopped for some hours after irradiation. The authors stated that discontinuous ultrasonic irradiation of *S. cerevisiae* was more beneficial for activating fermentation than continuous exposure, because only a few steps in intracellular metabolisms are supported by ultrasound and others are not or may even be inhibited.

Liu et al. [12] investigated the changes of biological activity of aerobic activated sludge after ultrasonic irradiation. The activity of microorganisms rose sharply after ultrasonic exposure of 0.3 W cm^2, 35 kHz for 10 min, and reached a peak level in 8 h after exposure (100% higher than that of the initial level immediately after exposure). Then it dropped rapidly in the next 8 h. In 24 h after ultrasonic irradiation, the enhancement effect induced by ultrasound almost disappeared, and cell activity returned to the normal state as control cells without ultrasound stress. The authors stated that enhancement might be due to defense

response of microorganisms evoked by mechanical stress. These reactions are usually observed when cells are challenged by biotic or abiotic stress.

Pitt and Ross [17] used ultrasonic irradiation to increase the growth rate of bacterial cells attached to a polyethylene surface. It was found that low frequency ultrasound (70 kHz) of low intensity (<2 $W \cdot cm^{-2}$) increased the growth rate of the cells compared to growth without ultrasonic waves. They stated that ultrasounds can increase the rate of transport of oxygen and nutrients to the cells and the rate of transport of waste products away from the cells, thus enhancing their growth.

Xie et al. [18] studied the enhancement effect of low-intensity ultrasound (35 kHz) on anaerobic sludge activity. The experiments showed that optimal ultrasonic intensity and the irradiation period were 0.2 $W \cdot cm^{2}$ and 10 min, respectively.

To summarize optimal ultrasonic intensity and the irradiation period are varied in each biological process enhanced by ultrasound, and should be found experimentally.

Effect of HRT on Ethanol Fermentation

In order to estimate optimal fermentation time in this study, parameters such as ethanol concentration, ethanol volumetric productivity, ethanol yield, and lactose consumption were investigated.

The maximum values of ethanol concentration and lactose consumption were achieved when the HRT was 36 h. Under the HRT of 36 h in ultrasound-assisted fermentation, the average ethanol concentration of 26.30 $g \cdot L^{-1}$, ethanol yield of 0.532 $g \cdot g^{-1}$ lactose, and lactose consumption of 98.9% were obtained (Figs. 2-4). Using *S. cerevisiae* without ultrasound exposure gave the results as 23.60 $g \cdot L^{-1}$, 0.511 $g \cdot g^{-1}$, and 92.4 %, respectively, and the differences were statistically significant ($p<0.05$). Shortening the HRT to 24 h allowed remaining high ethanol yield of 0.520 $g \cdot g^{-1}$ with sonicated *S. cerevisiae*, but in the control fermentation step it was as low as 0.487 $g \cdot g^{-1}$ ($p<0.05$). When the HRT was 12 h the ethanol yields were in the range of -0.318-0.365 $g \cdot g^{-1}$, depending on using ultrasound devices (Fig. 4). From the economic viewpoint, shortening the fermentation time (HRT) could reduce costs of industrial ethanol production. The study showed that there is no need to extend the HRT over 36 h or more, because most of the lactose was converted into ethanol during 24 h (98.9 % in the ultrasound-assisted fermentation). Nikolić et al. [19] stated that optimal fermentation time for free and immobilized *S. cerevisiae* was 38 h. Ozmihci and Kargi [2] studied ethanol production from cheese whey powder (CWP) solution containing 50 g $sugar \cdot L^{-1}$ at six different HRTs varying between 17.6 and 64.4 h by *Kluyveromyces marxianus* strains. Percent sugar utilization, effluent ethanol concentration, and ethanol yield increased with increasing HRT from 17.6 to 50 h. Further increasing HRT to 64.4 h resulted in a decrease of the analyzed parameters. Moreover, the time for fermentation decreased at higher initial substrate concentrations [2, 7, 19]. According to Guimarães et al. [7] the fermentations with 50-150 g $lactose \cdot L^{-1}$ reached completion in about the same time of 27 h, but the maximum ethanol concentration increased linearly with increasing initial lactose concentration from 6.5 g $ethanol \cdot L^{-1}$ with 20 g $lactose \cdot L^{-1}$ to 57 $g \cdot L^{-1}$ with 200 $g \cdot L^{-1}$. They also stated that

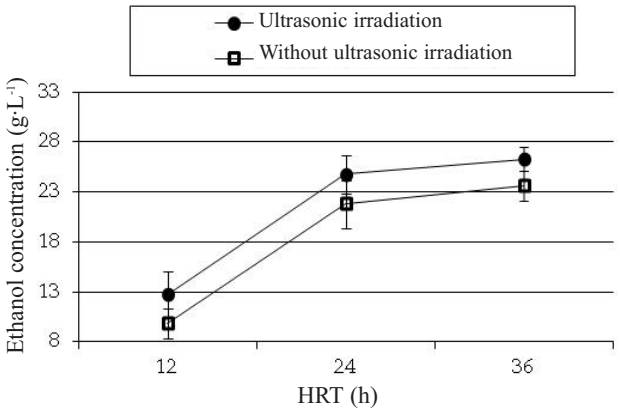

Fig. 2. Effects of hydraulic retention time (HRT) and ultrasound irradiation on the ethanol concentration in effluent distillate with standard deviations.

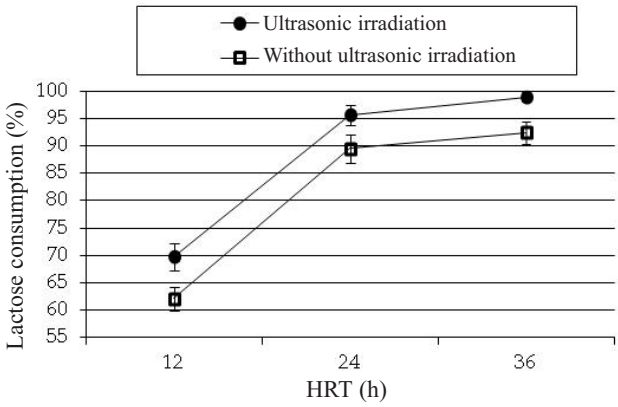

Fig. 3. Effects of hydraulic retention time (HRT) and ultrasound irradiation on lactose consumption by co-immobilized *Saccharomyces cerevisiae* cells and β-galactosidese enzyme with the initial lactose concentration of 50 $g \cdot L^{-1}$ and with standard deviations.

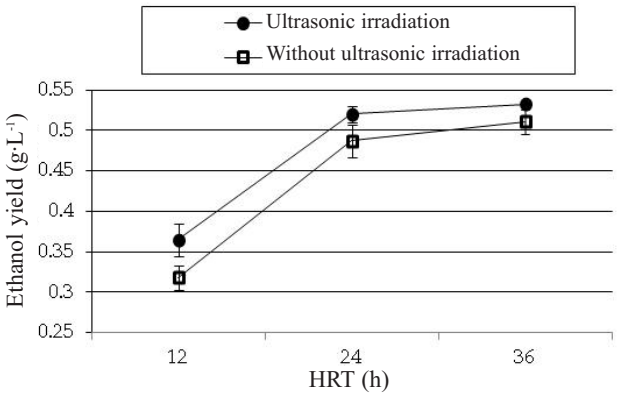

Fig. 4. Effects of hydraulic retention time (HRT) and ultrasound irradiation on ethanol yield with standard deviations.

Table 1. Effects of hydraulic retention time (HRT) on ethanol volumetric productivity.

HRT	Ethanol volumetric productivity in the ultrasound-assisted fermentation system, $g \cdot L^{-1} \cdot h^{-1}$	Ethanol volumetric productivity in the control fermentation system, $g \cdot L^{-1} \cdot h^{-1}$
12 (h)	1.060 (0.15)[a]	0.822 (0.13)
24 (h)	1.035 (0.16)	0.908 (0.13)
36 (h)	0.730 (0.09)	0.655 (0.08)

[a]standard deviations are presented in brackets

increasing lactose concentration led to incomplete fermentation and impaired fermentation due to nutrient limitations.

Interestingly, the volumetric productivities of ethanol decreased at longer HRT (Table 1). Maximum productivities of ethanol were observed under HRT of 12 h: 1.060 $g \cdot L^{-1} \cdot h^{-1}$, when the culture has been sonicated and under HRT of 24 h: 0.908 $g \cdot L^{-1} \cdot h^{-1}$ in the fermentation process without ultrasound irradiation. The differences were significant. The volumetric ethanol productivity obtained in this work was higher than that reported for batch or fed-batch fermentations with recombinant *S. cerevisiae* strains: 0.3 $g \cdot L^{-1} \cdot h^{-1}$ [9], 0.46 $g \cdot L^{-1} \cdot h^{-1}$ [8], 0.14-0.6 $g \cdot L^{-1} \cdot h^{-1}$ [20], and 1 $g \cdot L^{-1} \cdot h^{-1}$ [21]. Ozmihci and Kargi [22], using *Kluyveromyces marxianus* to ferment concentrated cheese whey powder solution, obtained higher volumetric ethanol productivity over 2 $g \cdot L^{-1} \cdot h^{-1}$, but after 120 h fermentation.

Effect of Ultrasound on Ethanol Fermentation

In all HRTs, significantly higher ethanol production in the ultrasound-assisted fermentation process than in the control fermentation process was recorded ($p<0.05$). When the HRT was 12 h, the ethanol concentration without ultrasonic treatment was 9.87 $g \cdot L^{-1}$ and it was significantly lower by 2.85 $g \cdot L^{-1}$ than the production in the process stimulated with low-intensity ultrasounds ($p<0.05$) (Fig. 2). Lactose consumption was only 62.1%, but application of ultrasound increased it to 69.7% ($p<0.05$) (Fig. 3). The best results were obtained with the longest HRT of 36 h. Ethanol concentration increased to 26.30 $g \cdot L^{-1}$ when the culture was sonicated, while in the fermentation process without ultrasound irradiation it was only 23.60 $g \cdot L^{-1}$, and the differences were statistically significant ($p<0.05$) (Fig. 2). Lactose consumption was as high as 98.9% and was significant higher by 6.5% than consumption in the reactor without ultrasonic irradiation ($p<0.05$) (Fig. 3.). High ethanol production and lactose consumption were observed when shortening the HRT to 24 h. *S. cerevisiae* stimulated with low intensity ultrasound produced 24.85 g ethanol$\cdot L^{-1}$, while lactose consumption was 95.6% (Figs. 2-3). In the control fermentation there was 21.79 $g \cdot L^{-1}$ and 89.5%, respectively. The differences between the data obtained in the assisted-fermentation process and in the control process were statistically significant ($p<0.05$). Under the HRT of 36 h, in the fermentation process with ultrasound irradiation the maximum ethanol yield of 0.532 $g \cdot g^{-1}$ lactose was observed, whereas using biocatalyst *S. cerevisiae* without ultrasound exposure gave the result as 0.511 $g \cdot g^{-1}$ (Fig. 4) ($p<0.05$). Shortening the HRT to 24 h allowed a remaining high ethanol yield of 0.520 $g \cdot g^{-1}$ with sonicated *S. cerevisiae*, but in the control fermentation process it was as low as 0.487 $g \cdot g^{-1}$ ($p<0.05$). When the HRT was 12 h the ethanol yield was only 0.365 and 0.318 $g \cdot g^{-1}$, respectively ($p<0.05$).

Only a few experiments have investigated the enhancement of ethanol production by ultrasonic stimulation of *S. cerevisiae*. Schläfer et al. [13] improved biological activity of *S. cerevisiae* by low-energy ultrasound assisted bioreactors operated at a frequency of 25 kHz and a power input of 0.3 $W \cdot L^{-1}$. Ethanol production without ultrasonic treatment varied between 3-12 $g \cdot L^{-1}$, while ultrasonic stimulation increased it to 30 $g \cdot L^{-1}$. The highest ethanol concentrations were obtained with a cycle regime of ultrasound exposure and a pause, because during continuous ultrasound irradiation no stimulation in the ethanol fermentation process was recorded.

Lanchun et al. [23] investigated the influence of low intensity ultrasound on physiological characteristic of *S. cerevisiae*. The results of their study showed that ultrasounds in the frequency of 24 kHz and power efficiency of 2 W with 1 s irradiation time every 15 s and 30 min duration cycle stimulated material transport and improved the cell's metabolism by changing the membrane osmotic. Consequently, transfer of substance was speeded up, enzyme synthesis was driven up and enzyme activity was enhanced.

Sulaiman et al. [14] operated a batch fermentation system at low-intensity sonication (11.8 $W \cdot cm^{-2}$, 20 kHz) using 10%, 20% (1 s sonication, 5 s rest period), and 40% (2 s sonication, 5 s rest period) cycles. All sonication cycles tested improved ethanol production by *K. marxianus*, but increasing the duty cycle to 40% had an adverse impact on yeast. The best sonication regime of a 20% cycle enhanced the biomass yield on lactose by 33%, the final ethanol yield by 3-fold greater, and the final ethanol concentration by 3.5-fold greater compared to control.

The positive results of the ultrasound treatment on the ethanol production by co-immobilized *S. cerevisiae* cells and β-galactosidase enzyme observed in this work seem to be a combination of different processes, including activating the yeast by improving the mass transfer rate of nutrients in the liquid, enhancing the uptake of foreign substances and the release of intracellular products in cells, improving cell growth, and degassing CO_2 [12, 14, 23, 24]. According to Sulaiman et al. [14], elevated concentrations of dissolved CO_2 are known to inhibit *S. cerevisiae*. The authors explained that improving ethanol production by

yeasts may have contributed to improved removal of the highly soluble CO_2 from the broth in the ultrasound-assisted fermentation system. Stimulating enzyme activity is done by increasing the mass transfer rate of the reagents to the active site [12]. Ultrasound irradiation can cause thermal and mechanical stress to biological materials [24]. High-energy ultrasonic waves break the cells and denaturate enzymes [12, 16]. Low-energy ultrasounds can produce a variety of effects on biological materials, including the inhibition or stimulation of cellular metabolisms, enzyme activity, alteration of cell membranes and other cellular structures [11, 12]. According to Xie et al. [25], cavitation is the primary basis of biological effects of low-intensity ultrasound. Cavitation bubbles produced by low-intensity ultrasound can cause acoustic microstreaming [25]. The microstreaming surrounding the cells will cause shear stress and enhance mass transfer, which may stimulate metabolic activities inside the cells [17, 24, 25]. When ultrasonic intensity is sufficiently low, a stable cavitation occurs and leads to the enhancement of mass transfer and fluid mixing, which produces positive effects on the rate of biological reactions in the exposure systems [12].

The growth activity of yeast cells is hardly changed within the early period of sonication regardless of either damage to cell wall, or complete inactivation of the yeast located in the cavitation zone [26]. A short sonication time up to 5 min of irradiation indicated bactericidal effects, but the cells were able to repair the damage. According to Guerrero et al. [27], yeasts, including *S. cerevisiae*, are highly resistant to ultrasound damage. Moreover, at relatively low intensity of ultrasounds, microorganisms can adapt to irradiation exposure and their biological activity increases [12, 14]. With a relatively short irradiation period, cell damage and membrane permeability induced by ultrasounds appear to be temporary and reversible. Lanchun et al. [23] also stated that sonication cannot influence the fermentation strength of *S. cerevisiae* descendants.

Conclusions

The process of whey UF-permeate fermentation to ethanol in continuous mode by co-immobilized *S. cerevisiae* cells and β-galactosidase enzyme was improved by the application of low-intensity ultrasounds. Optimal ultrasonic intensity and irradiation period vary for each biological process and should be found experimentally. According to this experiment, stimulation of yeast activity could be achieved in the presence of low-intensity ultrasound (1 $W \cdot L^{-1}$, 20 kHz), and a 1 min every 6 h irradiation period is favourable to increase ethanol production efficiency. Moreover, the short exposure of yeast to ultrasound could reduce operational costs compared with continuous irradiation.

For the continuously operating bioreactors, the maximum rates of sugar utilization were 98.9 and 92.4% for the yeast with and without ultrasound exposure ($p<0.05$), respectively. Maximum ethanol yield was 0.532 $g \cdot g^{-1}$ lactose, while without ultrasound exposure 0.511 $g \cdot g^{-1}$ was attained. This study showed that there is no need to extend the HRT over 36 h or more, because most of the lactose was converted into ethanol during 24 h (95.6% in the ultrasound-assisted fermentation).

The results obtained here raise new perspectives for whey permeate ultrasound-assisted fermentation, and hence for whey UF permeate disposal.

Acknowledgements

This study was supported by grant No. N523 049 32/1753 from the Polish Ministry of Science and Higher Education in 2007-08.

References

1. De GLUTZ F. N. Fuel bioethanol production from whey permeate http://biblion.epfl.ch/EPFL/theses/2009/4372/EPFL_TH4372.pdf **2009**.
2. OZMIHCI S., KARGI F. Ethanol production from cheese whey powder solution in a packed column bioreactor at different hydraulic residence times. Biochem. Eng. J. **42**, 180, **2008**.
3. SILVEIRA W. B., PASSOS F. J. V., MANTOVANI H. C., PASSOS F. M. L. Ethanol production from cheese whey permeate by *Kluyveromyces marxianus* UFV-3: A flux analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen levels. Enzyme Microb. Technol. **36**, 930, **2005**.
4. KOURKOUTAS Y., DIMITROPOULOU S., KANELLAKI M., MARCHANT R., NIGAM P., BANAT I. M. High-temperature alcoholic fermentation of whey using *Kluyveromyces marxianus* IMB3 yeast immobilized on delignified cellulosic material. Bioresour. Technol. **82**, 177, **2002**.
5. TAHOUN M. K., EL-NEMR T. M., SHATA O. H. Ethanol from lactose in salted cheese whey by recombinant *Saccharomyces cerevisiae*. Z. Lebensm. Unters. Forsch. **208**, 60, **1999**.
6. COTÉ A., BROWN W. A., CAMERON D., van WALSUM G. P. Hydrolysis of lactose in whey permeate for subsequent fermentation to ethanol. J. Dairy Sci. **87**, 1608, **2004**.
7. GUIMARÃES P. M. R., FRANÇOIS J., PARROU J. L., TEIXEIRA J. A., DOMINGUES L. Adaptive evolution of a lactose-consuming *Saccharomyces cerevisiae* recombinant. Appl. Environ. Microbiol. **74**, 1748, **2008**.
8. GUIMARÃES P. M. R., TEIXEIRA J. A., DOMINGUES L. Fermentation of high concentrations of lactose to ethanol by engineered flocculent *Saccharomyces cerevisiae*. Biotechnol. Lett. **30**, 1953, **2008**.
9. RUBIO-TEXEIRA M., CASTRILLO J. I., ADAM A. C., UGALDE U. O., POLAINA J. Highly efficient assimilation of lactose by a metabolically engineered strain of *Saccharomyces cerevisiae*. Yeast **14**, 827, **1998**.
10. CHISTI Y. Sonobioreactors: using ultrasound for enhanced microbial productivity. Trends Biotechnol. **21**, (2), 89, **2003**.
11. LIU Y., TAKATSUKI H., YOSHIKOSHI A., WANG B., SAKANISHI A. Effects of ultrasound on the growth and vacuolar H^+-ATPase activity of aloe arborescens callus cells. Colloids Surf. B **32**, 105, **2003**.

12. LIU H., YAN Y., WANG W., YU Y. Low intensity ultrasound stimulates biological activity of aerobic activated sludge. Front. Energy Power Eng. China **1**, 67, **2007**.
13. SCHLÄFER O., SIEVERS M., KLOTZBÜCHER H., ONYECHE T. I. Improvement of biological activity by low energy ultrasound assisted bioreactors. Ultrason. **38**, 711, **2000**.
14. SULAIMAN. A. Z., AJIT A., YUNUS R. M., CHISTI Y. Ultrasound-assisted fermentation enchances bioethanol productivity. Biochem. Eng. J. **54**, 141, **2011**.
15. PN-EN. Standard Methods for the Examination of Water and Wastewater, PN-67/A-86430, PN-A-79528-3:**2007**.
16. MARQUES L. L. M., BUZATO J. B., CELLIGOI M. A. P. C. Effect of raffinose and ultrasound pulses on invertase release by free and immobilized *Saccharomyces cerevisiae* in loofa (*Luffa cylindrica*) sponge. Braz. Arch. Biol. Technol. **49**, 1516, **2006**.
17. PITT W.G., ROSS S.A. Ultrasound increases the rate of bacterial cell growth. Biotechnol. Prog. **19**, 1038, **2003**.
18. XIE B., LIU H., YAN Y. Improvement of the activity of anaerobic sludge by low-intensity ultrasound. J. Environ. Manag. **90**, 260, **2009**.
19. NIKOLIĆ S., MOJOVIĆ L., PEJIN D., RAKIN M., VUKAŠINOVIĆ M. Production of bioethanol from corn meal hydrolyzates by free and immobilized cells of *Saccharomyces cerevisiae* var. *ellipsoideus*. Biomass Bioenergy, **34**, 1449, **2010**.
20. RAMAKRISHNAN S., HARTLEY B. S. Fermentation of lactose by yeast cells secreting recombinant fungal lactase. Appl. Environ. Microbiol. **59**, 4230, **1993**.
21. COMPAGNO C., PORRO D., SMERALDI C., RANZI B. M. Fermentation of whey and starch by transformed *Saccharomyces cerevisiae* cells. Appl. Environ. Microbiol. **43**, 822, **1995**.
22. OZMIHCI S., KARGI F. Kinetics of batch ethanol fermentation of cheese-whey powder (CWP) solution as a function of substrate and yeast concentrations. Bioresour. Technol. **98**, 2978, **2007**.
23. LANCHUN S., BOCHU W., LIANCAI Z., JIE L., YANHONG Y., CHUANREN D. The influence of low-intensity ultrasonic on some physiological characteristics of *Saccharomyces cerevisiae*. Colloids Surf. B **30**, 61, **2003**.
24. LIU Y., YOSHIKOSHI A., WANG B. C., SAKANISHI A. Influence of ultrasonic stimulation on the growth and proliferation of *Oryza sativa* Nipponbare callus cells. Colloids Surf. B **27**, 287, **2003**.
25. XIE B., WANG L., LIU H. Using low intensity ultrasound to improve the efficiency of biological phosphorus removal. Ultrason. Sonochem. **15**, 775, **2008**.
26. TSUKAMOTO I., CONSTANTINOIU E., FURUTA M., NISHIMURA R., MAEDA Y. Inactivation effect of sonication and chlorination on *Saccharomyces cerevisiae*. Calorimetric Analysis. Ultrason. Sonochem. **11**, 167, **2004**.
27. GUERRERO S., TOGNON M., ALZAMORA S. M. Response of *Saccharomyces cerevisiae* to the combined action of ultrasound and low weight chitosan. Food Control **16**, 131, **2005**.