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The effect of low-frequency ultrasound on the activity and efficiency of a commercial cellulase enzyme

Orsolya Erzsébet Szabó, Emília Csiszár*

Department of Physical Chemistry and Materials Science, Budapest University of Technology and Economics, P.O. Box 91, H-1521 Budapest, Hungary

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ABSTRACT

A commercial acidic cellulase enzyme complex was chosen in order to gain detailed information about the effect of low-frequency ultrasound (horn at 40 kHz) on the enzyme activity. The performance of the enzyme under sonication was also evaluated in a cellulose–cellulase model reaction. The filter paper activity of the enzyme and the yield of the enzyme catalysed hydrolysis were determined as a function of the parameters of the sonicated environment (treatment time, amplitude, with and without a reflector) and compared with the data measured in a non-sonicated bath. Depending on the parameters of the sonication, the enzyme is susceptible to ultrasound and its activity can significantly decrease. Despite the serious reduction of the enzyme activity, the outcome of the enzyme catalysed hydrolysis was always positive, implying that the advantageous effects of sonication impressed on the heterogeneous enzyme reaction always overcome the undesirable enzyme modifying effect of ultrasound.

1. Introduction

In the last decades ultrasound has been widely used for improving the efficiency of various chemical, physical and biotechnological processes. The effect of ultrasound can be based on a direct interaction with molecular species as well as on the cavitation phenomenon. In the field of biotechnology, low-frequency power ultrasound has recently gained much attention in the intensification of enzyme-aided processes. Ultrasound has a direct effect on the enzyme molecules and enhances the mass transfer in the heterogeneous processes by the local turbulences created by acoustic cavitation (Gogate & Kabadi, 2009; Kwiatkowska, Bennett, Akunna, Walker, & Bremner, 2011; Mason, 2007; Rokhina, Lens, & Virkutyte, 2009; Suslick, 1990).

Cavitation is the formation, growth and collapse of vapour or gas bubbles that occur with ultrasound. In a heterogeneous reaction, collapse of the bubbles near the solid surface results in high velocity micro-jets, which accelerate the transport processes and significantly improve the mass transfer (Moholkar, Nierstrasz, & Warmoeskerken, 2003). Subsequently, power ultrasound can considerably increase the reaction rate in an enzyme-aided heterogeneous system. Furthermore, during acoustic cavitation extremely high local temperatures and high pressure are created, which can have a remarkable effect on the enzyme macromolecule and its activity.

The overall effects of the power ultrasound on the homogeneous and heterogeneous enzyme-aided reactions are well-published in scientific literature. Although most of the papers confirm that the overall effect of the ultrasound is very positive (Basto, Tzanov, & Cavaco-Paulo, 2007; Moholkar et al., 2003; Yachmenev, Blanchard, & Lambert, 2004), the enzymes used in the different bioprocesses can be sensitive to ultrasound irradiation (Macleod & Dunn, 1967; Wood, Aldrich, & Ingram, 1997), and in particular cases, they can sustain damage to a certain extent. Furthermore, the enzyme-modifying effect of the ultrasound can contribute to the alteration of the enzyme activity, which can have far-reaching consequences. Changes in activity suffered by the enzyme in the ultrasonicated bath can be influenced by the parameters of the sonication and the characteristics of the enzyme. Thus, the enzyme macromolecule–ultrasound interaction has a significant effect on the efficiency of the bioprocess.

Despite the fact that ultrasound is able to alter the enzyme activity, surprisingly, only a very few publications have focused on the behaviour of enzymes exposed to power ultrasound. Interestingly, most of the papers published in this field evaluate the efficiency of the ultrasound-aided processes, but draw the conclusions on the enzyme activity modifying effect of the ultrasound, without measuring the enzyme activity itself.

Guiseppi-Elie et al. investigated a commercial glucose oxidase enzyme, which is a broadly utilized enzyme in the field of biosensors and the next generation of biofuel cell systems (Guiseppi-Elie,

* Corresponding author. Tel.: +36 1 463 1423; fax: +36 1 463 3474.
E-mail address: ecsiszar@mail.bme.hu (E. Csiszár).

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Choi, & Deckeler, 2009). The results proved that the ultrasonicated (at 23 kHz and at ice bath temperature for 10, 30 and 60 min) glucose oxidase enzyme showed a different composition with reduced α-helix and β-sheet fractions upon extended sonication compared with the pristine enzyme. Together with the changes of the secondary structure, the enzymatic activity showed a small corresponding decrease.

Özbek and co-workers investigated the stability of six different enzymes under sonication at low frequency (Özbek & Ülgen, 2000). They demonstrated that operational parameters: such as processing time, time of exposure, acoustic power, wave duty cycle, viscosity of the enzyme solution has a significant effect on the enzyme stability. Furthermore, the stability or inactivation of the enzymes can vary over a wide range depending on the enzyme properties.

A detailed study has been carried out by Souza et al. who evaluated the activity of a commercial amylase enzyme after sonication in an ultrasonic bath at 40 kHz (Souza et al., 2013). Amylases act in the hydrolysis of starch and they are applied in different areas of the food, pulp and paper, textile and bioethanol industry. The enzyme activity was measured in a wide range of temperature (from 30 to 100 °C) and at pH 4.5 with and without sonication. Results clearly proved that at lower temperatures (that is far from the optimum temperature of the enzyme) the sonication can promote enzyme reaction. But, at a higher temperature (that is closer to the enzyme's optimal conditions) sonication depresses the enzyme activity.

Since enzymes are usually used near their optimal conditions, where they exhibit maximum activity, thereby achieving their maximum reaction rate, it is therefore essential to know what the influence of the sonicated environment has on the effectiveness of the enzyme operating under ideal conditions. We therefore believe that there is a need for more research in this area in order to better understand the correlation of the 'sonication–enzyme action'. This would enable us to develop more efficient processes in the field of sono-biotechnology.

Therefore, in this study a commercial acidic cellulase enzyme complex was used in order to characterize the effect of sonication on the enzyme activity and efficiency. Cellulase refers to a group of enzymes which, acting together, hydrolyse cellulose. Cellulases are widely used in different areas of industry, agriculture, as well as in research and development. Two types of experiments were conducted. *First*, the diluted solutions of the enzyme were irradiated with cavitating power ultrasound and subsequently the enzyme activity was measured. The filter paper activity (FPA) of the enzyme was determined as a function of the parameters (treatment time, amplitude, with and without a reflector) of sonication (horn at 40 kHz). In order to ensure the optimal conditions, under which this enzyme exhibits maximum activity, the temperature and pH were maintained at 50 °C and pH 5, respectively. The activity of the enzyme in the sonicated field was compared with that of the enzyme acting in a bath mixed with a magnetic stirrer. *Second*, sonication was applied in an enzyme catalysed reaction (hydrolysis of cellulose powder by cellulase enzyme), in order to get information on the effect of sonication of the efficiency of the enzyme. By varying the above mentioned parameters of the sonication system, the reducing sugar liberation was measured continuously and the results were compared with the enzyme activity data.

2. Experimental

2.1. Cellulase enzyme and cellulose substrate

Celluclast 1.5L, a cellulase mixture produced by *Trichoderma reesei* from Sigma–Aldrich, was used in the experiments. The enzyme exhibits maximal activity near 50 °C and at pH 4.5–5.0. In this research, the filter paper activity of the enzyme was chosen exclusively for characterizing the effect of sonication on the enzyme activity. However, in a previous study (Csiszár, Urbánszki, & Szakács, 2001), beside the FPA, the 1,4-β-endoglucanase and β-glucosidase activities were also determined for a more complete characterization of the enzyme mixture (28,000 EGU/ml, 11 IU/ml, respectively).

Bleached cotton fabric obtained from Testfabrics Inc., USA, which can be seen as a pure (100%) cellulose substrate was used for the cellulose–cellulase model reaction. The fabric was ground in a ball-mill (Csiszár & Fekete, 2011), and the fraction studied in this work was composed of particles with diameters enclosed between 800 and 1000 μm, collected by sieving. All chemicals used were of analytical grade and were purchased from Sigma–Aldrich Co. LLC. and Reanal Private Ltd.

2.2. Sonication system

Fig. 1 shows the schematic illustration of the experimental setup. The ultrasonic experiments were carried out using an ultrasonic horn type reactor (Sonics & Materials, Model: Vibra-Cell VC505), with a driving frequency of 40 kHz, and a power of 500 W supplied by a piezoelectric transducer and with a 13 mm diameter replaceable tip. The horn was fitted by a 10 mm-thick rubber cork to a double walled, cylindrical glass cell with 54 mm inner diameter and 120 mm height, to achieve 1 wavelength distance ($x = 3.7$ cm) between the tip and the bottom of the vessel. In some of the experiments a stainless steel disc ($d = 52$ mm) that acted as a rigid reflector was placed at the bottom of the vessel and was used to intensify the reflectance of the ultrasound waves and to create a standing wave field (Moholkar et al., 2003). The reaction mixture was also stirred with a Teflon-coated magnetic bar rotating at 400 rpm. The system was thermostated to 50 ± 2 °C by jacket cooling, and the actual temperature and power input values were recorded at 10 s intervals. The temperature and power input sensor was connected to a computer so that the sonicated enzyme reaction was monitored by the instrument's software.

The power delivered (P_{deliv}) to the system was measured, and the actual power dissipated (P_{diss}) for each experimental configuration (i.e. at 40, 60 and 80% amplitude/49.6 μm, 74.4 μm and 99.2 μm, respectively; with continuous ultrasonication) was determined by calorimetrically (Hagenson & Doraiswamy, 1998). The intensities of the ultrasound system (I_{diss}) in the reaction mixture were calculated: the P_{diss} was divided by the area of the probe tip (1.27 cm^2).

2.3. Sonication of the enzyme solution and filter paper activity assay

For monitoring the change in enzyme activity, 250-fold dilutions of cellulase enzyme in 0.05 M acetate buffer (pH 5) were sonicated at 40, 60 and 80% amplitude, without and with a reflector, at 50 °C in a period of 0–65 min. The total volume of the reaction mixture was 125 ml. After 5, 20, 35, 50 and 65 min, 0.5 ml of the enzyme solution was taken for the filter paper activity measurement. FPA was determined as described by Ghose (Ghose, 1987). Briefly, a 1×6 cm strip (50 mg) of Whatman No. 1 filter paper was added to a total volume of 1.5 ml enzyme solution and 0.05 M acetate buffer (pH 4.8). The samples were incubated at 50 °C for 1 h. The reaction was terminated by the addition of 3 ml dinitrosalicylic acid (DNS) solution, followed by boiling for 5 min. After cooling, 20 ml distilled water was added and the absorbance was read at 540 nm. The liberated reducing sugars (glucose equivalent) were estimated according to Miller (Miller, 1959). The reducing sugar content of the fermentation media was estimated indirectly from the enzyme blanks of the FPA measurement. Filter paper unit (FPU) was calculated

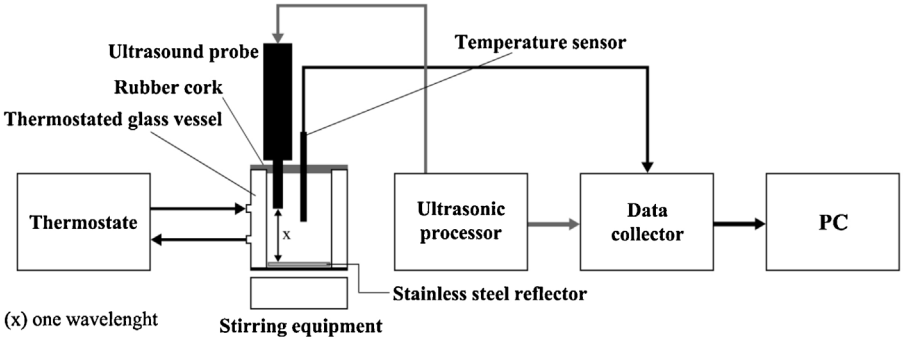

Fig. 1. Configuration of the apparatus used in the sonicated system experiment.

as recommended by Ghose (Ghose, 1987). Control treatment was carried out simultaneously, but without sonication. Each reported FPU value is the result of at least three parallel measurements.

2.4. Hydrolysis of cellulose

In the cellulose–cellulase model reaction 250 fold dilutions of cellulase enzyme in 0.05 M acetate buffer (pH 5) were also used. The amount of the ground bleached cotton substrate in the enzyme solution (125 ml) was 5 g/l and the enzyme concentration was 66 FPU/g substrate. Reactions with sonication were carried out at three different ultrasonic amplitudes, with and without a reflector, as described above. All experiments were conducted at 50 °C and under isothermal conditions. Control treatment was carried out simultaneously, but without sonication. The effect of sonication on the efficiency of the enzymatic hydrolysis was followed by measuring the concentration of the reducing sugars liberated from the cotton cellulose during the hydrolysis, in a period of 0–65 min.

Concentration of the reducing sugars was determined as described at FPA assay: 0.5 ml of enzyme sample solution was added to 1 ml acetate buffer solution (0.05 M, pH 4.8). After incubation the enzyme reaction was terminated by the addition of 3 ml DNS solution, followed by boiling for 5 min. After cooling, the absorbance was read at 540 nm. The liberated reducing sugars (in glucose equivalent) were estimated according to Miller (Miller, 1959). Each reported value is the result of at least three parallel measurements.

3. Results and discussion

3.1. Characterization of the ultrasound system in the experimental apparatus

For characterizing the intensity of the ultrasonic energy inside the reaction chamber, the temperature change of the distilled water caused by sonication was observed for a period of 0–10 min. The power delivered to the system and the actual temperature values were measured and recorded at 10 s intervals. The recorded temperature values, as a function of time for each amplitude applied are shown in Fig. 2. Although, Fig. 2 demonstrates the results recorded in the absence of the reflector, almost the same curves were obtained from the experiments carried out with the reflector. For both systems (i.e. without or with a reflector), the P_{deliv} measured by the power input sensor and the P_{diss} and I_{diss} calculated are presented in Table 1.

It is obvious that the applied sonication has a significant effect on the temperature of the liquid in the reaction chamber (Fig. 2). Depending on the amplitude, the temperature of the distilled water increased from room temperature to 50, 70 and 85 °C at 40, 60 and 80% amplitudes, respectively, within 10 min of continuous exposure to ultrasound and in the absence of the reflector. Furthermore, at higher amplitudes the power delivered to the system and the rate of the energy dissipation was also higher (Table 1). There are considerable differences between the P_{deliv} and P_{diss} values, indicating that 32, 35 and 40% of the energy delivered to the horn at 40, 60 and 80% amplitudes, respectively, were lost in the energy transfer. The intensities of the applied configurations (without the reflector) calculated from the dissipated energy are 16.2, 32.2 and 43.4 W/cm^2. Based on the data in Table 1, it can also be concluded that the reflector has only a negligible effect on the power characteristics of the apparatus, thus there is only a slight difference between the systems operated with or without a reflector at 40, 60 and 80% amplitudes.

3.2. Effect of sonication on the activity of cellulase enzyme

For analysis and evaluation of various enzymes associated with cellulose–cellulase system, the FPA assay is usually and widely applied (Hagenson & Doraiswamy, 1998). This is the best method to measure the ability of a cellulase enzyme complex to hydrolyse cellulose. The enzyme assay is carried out on filter paper as a solid substrate and under favourable hydrolysis conditions. The reducing sugars released from the filter paper strip by the cellulase enzyme tested, are measured.

The original Celluclast 1.5L enzyme has about an FPA of 83 FPU/ml, which remained practically unchanged in the control (non-sonicated, only magnetically stirred) process during the period of 65 min. To assess the effect of sonication on the enzyme activity, the cellulase enzyme solution was sonicated continuously

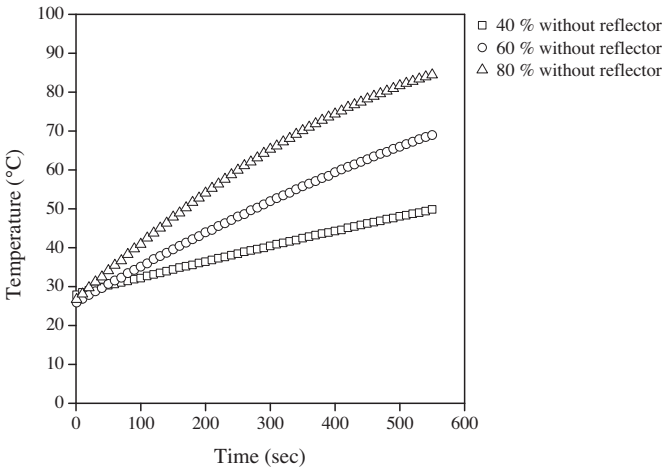

Fig. 2. Determination of the ultrasonic energy dissipated in the reactor at different amplitudes and without the reflector.

Table 1
Power characteristics of the applied ultrasound system for each experimental configuration.

Amplitude (%)*	Without a reflector			With a reflector		
	P_{deliv} (W)	P_{diss} (W)	I_{diss} (W/cm^2)**	P_{deliv} (W)	P_{diss} (W)	I_{diss} (W/cm^2)**
40	30.3	20.6	16.2	29.5	19.9	15.7
60	62.6	40.9	32.2	62.2	40.5	31.9
80	91.5	55.1	43.4	96.0	54.4	42.8

* Amplitudes: 49.6 μm, 74.4 μm and 99.2 μm, respectively.
** Area of the probe tip: 1.27 cm^2.

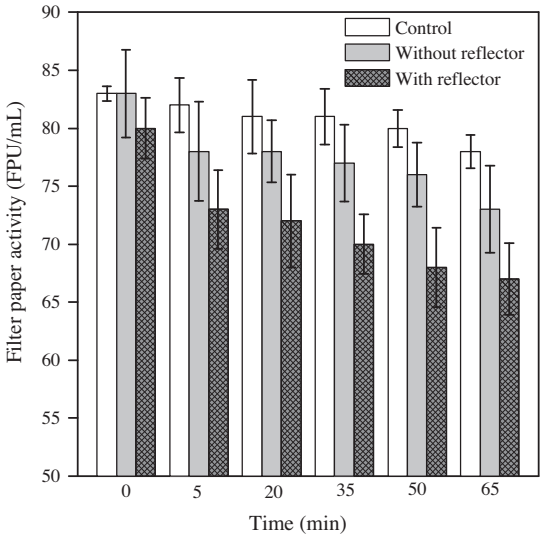

Fig. 3. Filter paper activity of Celluclast 1.5L enzyme sonicated at 40% amplitude, with and without the reflector as a function of time.

at different amplitudes of 40, 60 and 80% (Figs. 3–5, respectively), without and with a reflector. As the figures show, sonication can modify the ability of the cellulase enzyme to hydrolyse cellulose. The influence of the sonicated environment on the activity of the enzyme operating under ideal conditions (pH ~5, 50 °C) is significant. The FPA of the enzyme sonicated with 40% amplitude (without reflector, Fig. 3) decreased to 73 FPU/ml when the time of treatment increased to 65 min, which represents a 12% reduction. By increasing the amplitude, the loss in enzyme activity became more significant: the enzyme activity decreased to 67 FPU/ml (80% of the original, Fig. 4) and 63 FPU/ml (75% of the original, Fig. 5) when the amplitude increased to 60% and 80%, respectively.

The application of a reflector to intensify efficiency of the sonication has a more pronounced effect on the enzyme activity. As the results in Figs. 3–5 show, the hydrolytic potential of commercial cellulase enzyme can be deteriorated significantly. The losses in FPA increased as the time of treatment and the amplitude increased. At a certain amplitude, the FPA values measured without the reflector were always higher than those measured in the presence of the reflector. At 80% amplitude (Fig. 5), the final loss in enzyme activity is 25.0% in the absence of the reflector, while it is 27.4% in the presence of the reflector.

Results clearly prove that even a short sonication has a significant effect on the enzyme activity. This statement is in accordance with the results published in a previous study (Özbek & Ülgen, 2000), where the activity of a β-galactosidase enzyme, which is also a hydrolytic enzyme, like cellulase tested in this study, decreased by about 20–40% after a very short (2 × 30 s) sonication (at 20 kHz, 30 and 40 W acoustic power, duty cycle of 10 and 90%, respectively). In that previous study, the β-galactosidase enzyme was proven to be the second most stable of the six enzymes investigated there.

Sensitivity of the enzyme macromolecules to sonication is different. The alteration in enzyme activity that occurred during sonication depended largely on both the characteristics of the enzyme and the sonication system (Macleod & Dunn, 1967). It has not been established whether either the dissociation of the enzymes into subunits or the thermal denaturation of the enzymes by the shear forces or high localised temperatures, respectively, arising from the collapse of cavitation bubbles, are responsible for the damage of the enzyme occurring in the sonicated environment (O'Donnell, Tiwari, Bourke, & Cullen, 2011). But we can conclude

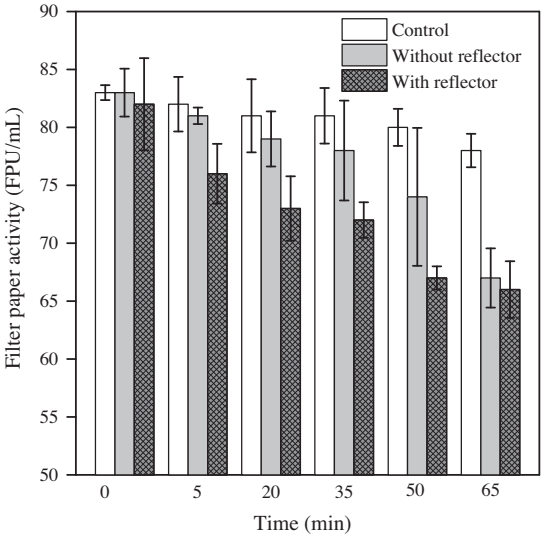

Fig. 4. Filter paper activity of Celluclast 1.5L enzyme sonicated at 60% amplitude, with and without the reflector as a function of time.

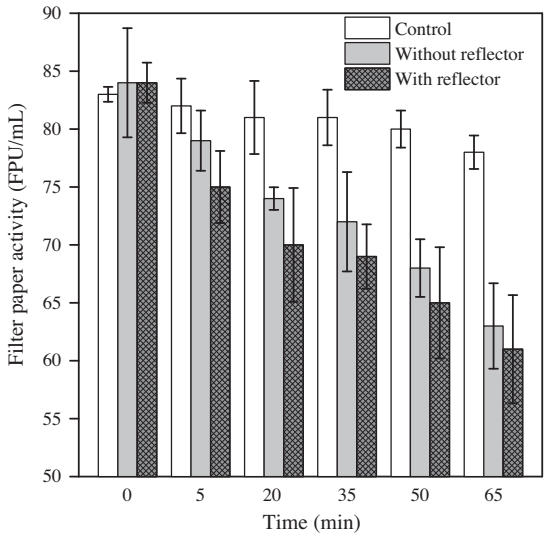

Fig. 5. Filter paper activity of Celluclast 1.5L enzyme sonicated at 80% amplitude, with and without the reflector as a function of time.

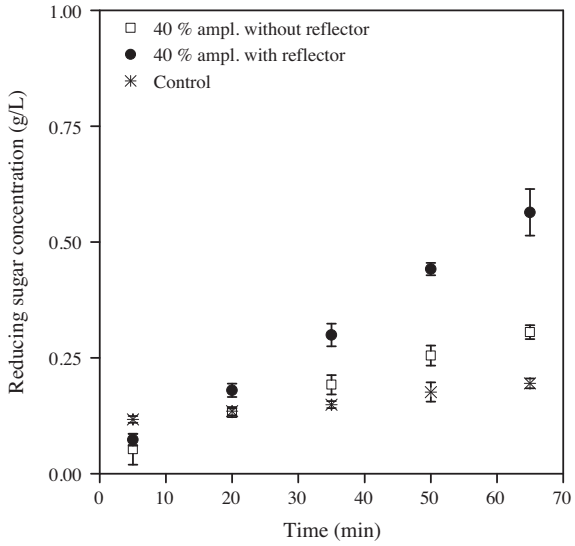

Fig. 6. Reducing sugar liberation at 40% amplitude, with and without the reflector as a function of time. Concentration of the solid substrate: 5 g/l.

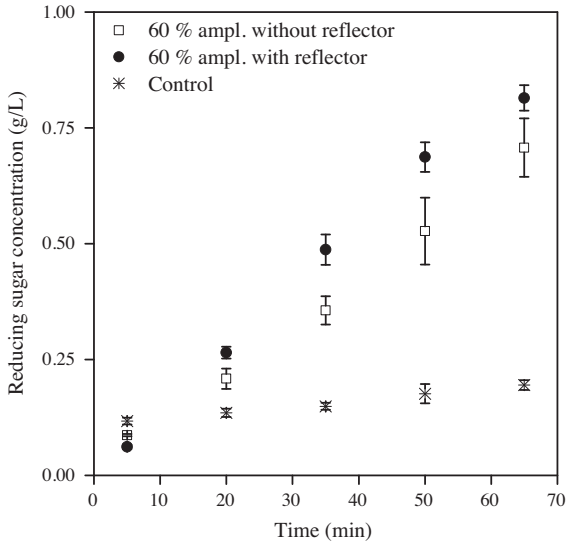

Fig. 7. Reducing sugar liberation at 60% amplitude, with and without the reflector as a function of time. Concentration of the solid substrate: 5 g/l.

from the results that sonication has a "sonochemical" effect on the enzyme molecule, which is manifested unambiguously in the diminished enzyme activity. Consequently, optimisation of the parameters of the sonicated system is required specifically for each of the enzymes used.

3.3. *Effect of sonication on the efficiency of the cellulose–cellulase model reaction*

In this study, a commercially available and widely used cellulase enzyme was selected for a detailed investigation of the interactions of low frequency ultrasound with the enzyme. Results presented above revealed that this enzyme is susceptible to ultrasound and its activity can significantly decrease, depending on the parameters of the sonication. On the other hand, many papers have shown that the overall effect of the ultrasound on the cellulase-catalysed reactions is very positive, and sonication results in a significant improvement in the enzyme efficiency (Herper & Aliyu, 2000; Yachmenev, Calamari, & Lambert, 2006).

For evaluating the hydrolytic potential of the enzymes toward a pure cellulose substrate in sonicated solution, a simple cellulose–cellulase model reaction was investigated here. The parameters of the sonicated environment were varied to determine the effect of these variables (amplitude, time and reflector) on the yield of the enzyme catalysed reaction expressed in reducing sugar concentration, which was monitored continuously.

Figs. 6–8 show the concentration of the reducing sugars liberated from the solid cellulose substrate during the enzymatic hydrolysis. It is obvious that the efficiency of the cellulase enzyme in the hydrolysis of the ground cellulose is notably higher in sonicated solution than when sonication is absent (control). This means that the addition of ultrasound to the model reaction leads to a significant increase in the degradation of cellulose to oligosaccharides and glucose with reducing end groups, as shown in the figures. Furthermore, the presence of the reflector in the reaction vessel has an additional intensifying effect, especially at 40% amplitude (Fig. 6). At 80% amplitude, however, its effect is negligible.

Fig. 9 compares the final reducing sugar concentrations after 65 min of enzymatic hydrolysis on pure cellulose, at various amplitudes, with and without the reflector. In general, the overall catalytic effect of the applied cellulase enzyme complex can be improved significantly by sonication. The yield of the reaction as expressed in reducing sugar concentration, increases from 0.2 g/l (control) by about 50, 250 and 275% to 0.31, 0.71 and 0.75 g/l at 40, 60 and 80% amplitudes (without a reflector), respectively. For the 40% of amplitude, the presence of the reflector also accelerates the enzyme catalysis notably and results in an 87% increase in the reducing sugar concentration of the solution. At higher amplitudes (60 and 80%) the reflector has less additional effect, and approximately a 15% increase can be measured at both amplitudes.

Comparing the efficiency of the cellulase enzyme in the above model reaction to the enzyme activity values measured in sonicated solution (Figs. 3–5), we can see that despite the serious reduction of the enzyme activity, which occurred by sonication, the outcome of the enzyme catalysed hydrolysis is always positive. It means that the advantageous effects of sonication impressed on the heterogeneous enzyme reaction always overcome the undesirable, enzyme modifying effect of ultrasound. Interestingly, the highest yield was measured at 80% amplitude and in the presence of the reflector, where the enzyme sustained a 27% loss in enzyme activity.

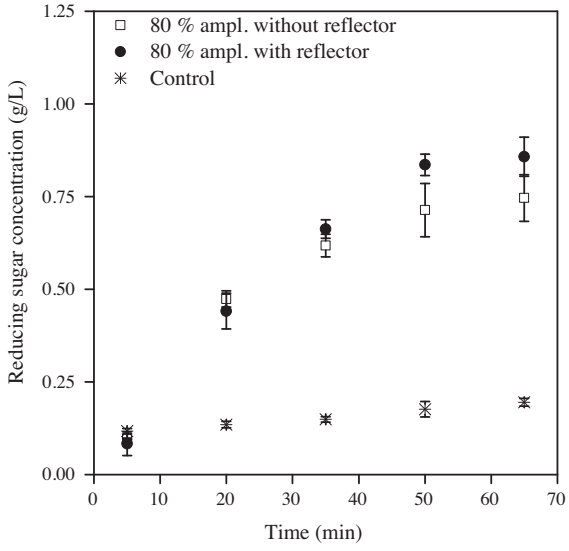

Fig. 8. Reducing sugar liberation at 80% amplitude, with and without the reflector as a function of time. Concentration of the solid substrate: 5 g/l.

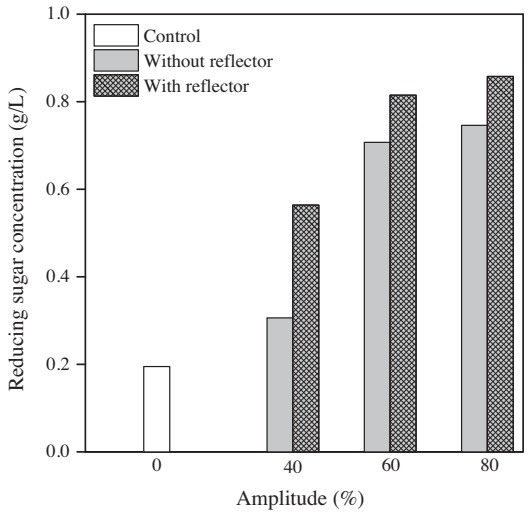

Fig. 9. Comparison of the hydrolytic capacities of the sonicated systems. Final reducing sugar concentrations after 65 min of enzymatic hydrolysis on pure cellulose at different amplitudes, with and without the reflector. Concentration of the solid substrate: 5 g/l.

Enzyme catalysed hydrolysis of a solid cellulosic substrate is a heterogeneous reaction. In the cellulase system of the industrially important cellulolytic fungus *Trichoderma reesei*, three major types of activities are found: endoglucanases (EG), exoglucanases including glucohydrolases and cellobiohydrolases (CBH), and β-glucosidases or cellobiases (Li, Yoshimoto, Tsukuda, Fukunaga, & Nakao, 2004). The enzyme action can be simplified as a two-step process. The first is a heterogeneous reaction between the solid substrate and the enzyme solution. In this rate limiting step, the synergistic interaction between exo- and endo-acting cellulase components leads to soluble oligosaccharides and cellobiose. In the second process they are hydrolysed to glucose in a homogeneous reaction by the action of β-glucosidases (Kubicek, 1992). Since Celluclast 1.5L used in this research is a low-β-glucosidase-containing cellulase enzyme (Csiszár et al., 2001), the heterogeneous reaction is likely to be more pronounced than the homogeneous one.

It is obvious that the applied sonication has a sonochemical/sonomechanical effect on the enzyme sonicated in a homogeneous solution, which is manifested in the alteration of the enzyme activity (Figs. 3–5). But, it does not influence chemically and/or mechanically the pure water hydrolysis of the solid substrate. This latter statement was proved by the results of the tests, where we did not detect any reducing sugars released from the cellulose substrate during the treatment in water only, with sonication but without enzyme. It means that the applied sonication is not able to depolymerise the cellulose macromolecules of the ground bleached cotton under hydrolysis conditions.

Sonication, however, can significantly affect the heterogeneous, enzyme catalysed hydrolytic reactions of cellulose (Figs. 6–9). The very frequent and accidental encounters of thousands of small particles of the ground cellulose substrate with cavitation bubbles induce asymmetric cavitation, which creates the right conditions for the effective interaction between the enzyme solution and the solid substrate, and results in a significant enhancement in the yield of the hydrolytic reactions.

4. Conclusions

In this paper a commercial acidic cellulase enzyme complex was chosen to get sufficiently detailed information about the effect of sonication on the enzyme activity. The performance of the enzyme under sonication was also evaluated in a model reaction, where pure cellulose in ground form was hydrolysed by the enzyme. The filter paper activity of the enzyme and the yield of the enzyme catalysed hydrolysis, expressed in reducing sugar concentration were compared with the data measured in a non-sonicated bath, mixed only with a magnetic stirrer.

Sonicating the enzyme solution at different amplitudes, the cellulase enzyme underwent changes, and the serious reduction of the enzyme activity which occurred was highly dependent on the amplitude. Without a reflector at 40% amplitude, a 12% loss in enzyme activity was measured in a period of 65 min. By increasing the amplitude to 60 and 80%, the decrease in enzyme activity became more significant, and after 65 min the enzyme possessed only 80 and 75% of the original activity, respectively. Application of a reflector to intensify the sonication had a pronounced effect and the loss in enzyme activity was always higher in the presence of the reflector.

In spite of the fact that the applied sonication depressed the activity of the cellulase enzyme, the outcome of the enzyme catalysed hydrolysis in the model reaction was always positive, implying that the advantageous effects of sonication impressed on the heterogeneous cellulose–cellulase reaction always overcome the undesirable, enzyme modifying effect of the ultrasound.

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