Some factors affecting efficiency of the ultrasound-aided enzymatic hydrolysis of cotton cellulose

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Abstract

The efficiency of the enzymatic ~~catalysed~~ hydrolysis of cellulose with low frequency ultrasound (horn-type reactor) was investigated and characterized by the concentration of reducing sugars liberated. Small squares of bleached cotton fabric were used for comparing the efficiency of different agitation methods (i.e. magnetic stirring, horizontal and vertical mechanical agitation) and ultrasound. At the same enzyme dosage and substrate level, sonication at 40, 60 and 80 % amplitudes (I_{diss} : 16.2, 32.2 and 43.4 W/cm², respectively) intensified the hydrolysis over the most efficient mechanical agitation (i.e. magnetic stirring) alone by 15 %, 24 % and 54 %, respectively. For mapping the ultrasonicated field, fabric layers positioned perpendicularly to the ultrasonic probe at different distances were hydrolysed. The optimal operating conditions were reached at 60 % amplitude and 9 mm ~~nearest position (9 mm)~~. The yield depended mainly on such important factors such as amplitude, the presence of a reflector, distance from horn and form of substrate.

Keywords: Cotton cellulose, Enzymatic hydrolysis, Ultrasound, Mapping of ultrasonic field, Conventional agitation

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1. Introduction

Agitation has a crucial role in heterogeneous enzyme-aided processes due to the slow diffusion controlled transfer of enzyme macromolecules and reaction products in the liquid phase, through the diffusion boundary layer near the solid surface (Yachmenev, Blanchard, & Lambert, 2004). Several conventional agitating systems, including vertical and horizontal agitation at different agitation levels, have been applied and characterized in various biocatalyzed processes by using cellulase, endoglucanase, cutinase, pectinase and protease etc. enzymes. Results clearly demonstrated the positive effect of agitation on the efficiency of enzyme reactions that was explained mainly by the increased mass transfer in the agitated environment (Cavaco-Paulo, & Almeida, 1996; Cavaco-Paulo, 1998; Silva, Araújo, Casal, Gübitz, & Cavaco-Paulo, 2007; O'Neill, Araújo, Casal, Guebitz, & Cavaco-Paulo, 2007; Zhuoliang, Hatfield, & Berson, 2012). Recently, power ultrasound has also been proved to be successful in increasing the rate of mass transfer of enzyme macromolecules to and from the solid surface (Vajnhandl & Le Marechal, 2005; Mason, 2007; Kwiatkowska, Bennett, Akunna, Walker, & Bremner, 2011; Yachmenev, Blanchard, & Lambert, 2004; Szabo, Csiszar, Toth, Szakacs, & Koczka, 2015). The main effect of sonication in liquids is the growth and sudden collapse of bubbles near the solid surface which induces microjet streaming. This is known as asymmetric cavitation. Due to the intensive microstreaming generated near the solid surface, the rate of the enzyme-aided bio-processes can be enhanced significantly (Suslick, 1990), notwithstanding a slight decrease in enzyme activity occurring especially at higher amplitudes and longer durations (Szabo & Csiszar, 2013).

One of the most important enzyme catalyzed processes is the cellulase-aided hydrolysis of cellulosic materials (Rollings, 1985). While it is already widely used in various industrial areas, its applications are continuously increasing in textile finishing (Hebeish, et al., 2009; Ibrahim, El-Badry, Eid, & Hassan, 2011; Yu, et al., 2013). Numerous papers have demonstrated the positive effects of conventional agitation systems on the cellulase action. Despite a meagre decrease in

enzyme activity, enzymatic hydrolysis of cotton cellulose is accelerated by high level agitation (Cavaco-Paulo & Almeida, 1996). The fibre ends and microfibrils, generated by mechanical action, act as active sites for the enzyme attack (Cavaco-Paulo, 1998). The positive effects of ultrasound in enzyme-assisted heterogeneous reactions have also been discussed in many publications (Subhedar & Gogate, 2013; Hao, Wang, Fang, & Liu, 2013). Several types of ultrasonic devices under various conditions were applied; in some cases they were compared in different enzyme-aided processes, while in some certain studies the mapping of the ultrasonic field was also carried out. One of the most controllable laboratory systems is the horn type reactor, capable of creating a standing wave field and well defined zones with the anti-nodal and nodal points. Depending on the distance between the ultrasonic horn tip and the bottom of the reaction vessel, the efficiency of the chemical, physical and homogeneous enzyme catalysed reactions varied significantly. It can be concluded that the closer the horn to the bottom, the higher the efficiency in the horn type ultrasonic system (Gogate, Tatake, Kanthale, Pandit, 2002; Moholkar & Warmoeskerken, 2003; Hagenson & Doraiswamy, 1998; Silva, Ferreira, Little, & Cavaco-Paulo, 2010; Csoka, Katekhaye, & Gogate, 2011).

The distance between the fabric and the sonicator plate (Yachmenev, Blanchard, & Lambert, 2004) or the horn tip (Basto, Tzanov, & Cavaco-Paulo, 2007) was defined in the ultrasound-assisted reactions of textiles. Despite the high number of papers published in the field of ultrasound-textile interaction, little information is available on the comparative analysis of conventional and ultrasonic systems and on the mapping of the ultrasonic field especially in an enzyme-aided reaction. Furthermore, in most cases the enzyme catalysed reaction is evaluated indirectly by testing some of the fabric properties (weight loss, hydrophilicity, surface roughness, tensile properties, etc.), instead of rather than the direct measurement of the reaction produced products, namely the reducing sugars released by the hydrolysis of cellulosic materials. Since the precision of the latter method is much superior to those obtainable in testing the properties of

textiles, more accurate conclusions can be drawn both on the reaction chamber agitated either conventionally or by low frequency ultrasound and on the efficiency of enzymatic catalysis occurring in the different environments.

~~The effect of low frequency ultrasound on the activity and efficiency of a commercial cellulase enzyme was evaluated in our previous work using cotton powder as a substrate (Szabo & Csiszar, 2013). In order to extend our research to different agitating systems conventionally used in the textile research, the enzyme catalysed hydrolysis of bleached cotton cellulose with the aid of magnetic stirring, horizontal shaking and vertical agitation was further studied. Their efficiency was compared to that of the reactions occurred under sonication. In the following experiments the ultrasonicated field was mapped by positioning the fabric perpendicularly to the ultrasonic probe at different distances. The factors studied were the form of cotton swatches, duration of hydrolysis, amplitude of ultrasound, presence/absence of a rigid reflector in the sonicated vessel and distance of fabric to the horn tip. The enzyme catalysed heterogeneous reaction was followed by measuring the reducing sugars liberated from the cellulose macromolecule. Summary of the experimental design can be seen in Table 1.~~

This paper is a continuation of an earlier study which mainly investigated the effect of low frequency ultrasound on the activity of a commercial cellulase enzyme (Szabo & Csiszar, 2013). The objective of the present research was to evaluate the effect of low-frequency ultrasound on the enzyme catalysed hydrolysis of cotton cellulose and to define some of the factors that affect the efficiency of the reaction. Square fabric samples with free movement in the enzyme solution, and circular fabric layers positioned perpendicularly to the ultrasonic probe at different distances, were used in the experiments. The enzyme catalysed heterogeneous reactions were followed by measuring the reducing sugars liberated from the cellulose macromolecule. The factors studied were the form of the cotton swatches, duration of hydrolysis, amplitude of ultrasound, presence/absence of a rigid reflector in the sonicated vessel and the distance of the layered fabric

to the horn tip. In addition, we compared the efficiency of ultrasound to that of different agitating systems, like magnetic stirring, horizontal shaking and vertical agitation, which are conventionally used in the heterogeneous enzyme-aided reactions of fibrous materials. The results were also compared to the data received from the hydrolysis of cotton powder (Szabo & Csiszar, 2013). Summary of the experimental design of present study can be seen in Table 1.

2. Experimental

2.1. Materials

Celluclast 1.5 L, a liquid acidic cellulase (83 FPU/ml) mixture, with very low- β -glucosidase and high 1,4- β -endoglucanase activities (Csiszar, Urbanszki & Szakacs, 2001), produced by *Trichoderma reesei* and purchased from Sigma-Aldrich, was selected ~~used in~~ for the experiments. The optima for the enzyme activities ~~were~~ was a temperature of 50 °C and a pH of 5. In the cellulase enzyme complex, endo- and exo-glucanases act in synergy on the solid surface of the cellulose and these heterogeneous actions can be improved significantly by cavitation, created in the ultrasonicated environment. These actions result in soluble oligosaccharides and cellobiose with reducing ends and their concentration can be measured by the applied DNS (dinitrosalicylic-acid) method (see in Chapter 2.4). The low β -glucosidase activity can only slightly affect the final end products.

Bleached cotton fabric obtained from Testfabrics Inc., USA, which is regarded as a pure (100 %) cellulose substrate was used for the enzymatic hydrolysis. The average viscometric degree of polymerization (DP: 2200 ± 100) and crystallinity of cellulose in bleached cotton fabric have been discussed in detail elsewhere (Csiszár & Fekete, 2011).

Table 1 Cellulase catalysed hydrolysis of the bleached cotton cellulose. Summary of the experimental design.^a

Way of agitation	Form of the cotton fabric	Varied parameters	Measured values
Horizontal (shaker)	fabric pieces in '□' form i.e. cut-fabric	- time of treatment (min): 0-90	- concentration of the
Vertical (labomat)			reducing sugars liberated
Stirring (magnetic)			
Sonication ^b		- time of treatment (min): 0-90	- concentration of the
		- amplitude of sonication (%): 40, 60 and 80	reducing sugars liberated
		- presence/absence of a reflector	
	4 layers of fabric	- presence/absence of a reflector	
	i.e. framed-fabric	- distance of the fabric layers from the tip of horn (mm): 9; 18 and 27	

^a Parameters of the enzyme treatment: enzyme dosage: 44 FPU/g cotton; substrate level: 7.5 g/l; at 50 °C in acetate buffer (pH 5).

^b Together with magnetic stirring. Control: treatment with magnetic stirring only.

2.2. Conventional agitation methods

In the first experiments, the efficiency of three conventional agitation methods, a magnetic stirrer, a commercial thermostated shaker (Medingen SWB 20, Labortechnik-Medingen, Germany) and a Mathis Labomat (BFA12, Mathis AG, Switzerland) device - usually used in the research of wet textile processes - ~~was~~ were compared to that of sonication. The samples moved ~~were moving~~ horizontally (120 moves/min) in a shaker, while the bulk liquid remained almost stagnant. Turbulent streaming was generated in the jacket-thermostated glass reactor with cylindrical geometry (the same as used for the ultrasound experiments and shown in Fig. 1a) by a magnetic stirrer at 250 rpm. In the Mathis Labomat device, the samples were rotating vertically in closed stainless steel jars.

2.3. Ultrasonic treatment

The experimental set-up consisted of a horn-type reactor (Sonics&Materials, Model: Vibra-Cell VCX 500) with a driving frequency of 20 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 cylindrical glass vessel (inner diameter: 54 mm, height: 120 mm). The distance between the tip and the bottom of the vessel was 37 mm ($\lambda/2$). A stainless steel disc (d=52 mm) was used as a reflector and placed on the bottom of the glass vessel. The reaction mixture was also stirred with a teflon-coated magnetic bar rotating at 250 rpm. The glass reactor was thermostated to 50 ± 2 °C by jacket cooling. Sonication was performed at different amplitudes (40, 60, 80 %) for 90 minute. The intensity of the ultrasound (I_{diss}) in the reaction mixture at 40, 60 and 80 % amplitude was 16.2, 32.2 and 43.4 W/cm², respectively (Szabo & Csiszar, 2013).

The fabric sample was cut into 1×1 cm squares (*cut-fabric*) that were used as substrates for comparing the efficiency of the different agitation methods (i.e. the three conventional agitating methods introduced in 2.2.) and for the ultrasonic irradiation (Fig. 1a) in the first experimental

series of experiments. During sonication, the sound waves were scattered randomly by the cut-fabric pieces, thus, the ultrasonic radiation of the cut-fabric is stochastic.

In the second batch of experiments, the sample consisted of four layers of the bleached fabric cut in the form of circular discs 52 mm in diameter. The four-layer sample packet of this size was fixed in a specially constructed detachable stainless steel frame of 52 mm in diameter (*framed-fabric*), positioned perpendicularly to the probe (Fig. 1b). The frame could be raised or lowered to adjust the distance between the tip of the horn and the fabric layers. The framed-fabric packet was placed at a distance of 9, 18 and 28 mm ($1/8\lambda$, $1/4\lambda$ and $3/8\lambda$, respectively) away from the tip of horn and used for scanning the sonicated field. The perpendicular positioning of the sample to the probe accelerates the transfer of biocatalyst towards and throughout the pores of fabric (Basto, Tzanov, & Cavaco-Paulo, 2007).

Fig. 1. Schematic representation of the ultrasound assisted enzymatic hydrolysis of cotton fabric. Thermostated reaction vessel with a horn-type reactor and a magnetic stirrer. a/ Treatment of squares of cotton fabric (cut-fabric); b/ Treatment of fabric layers placed perpendicularly and fixed in a round frame (framed-fabric) at a distance of 9; 19 and 28 mm from the tip of horn.

2.4. Enzymatic hydrolysis

Bleached cotton was hydrolyzed for 90 min in a 125 ml solution of Celluclast 1.5 L enzyme diluted with a 0.05 M acetate buffer (pH 5) to 250 fold. The concentration of the fabric

was 7.5 g/l and the enzyme dosage was 44 FPU/g fabric. The fabric (either cut-fabric or framed-fabric, see in Figures 1a and b, respectively) was added to the thermostated diluted enzyme solution. The concentration of liberated reducing sugars in the samples taken during the hydrolysis was estimated by the DNS (3,5-dinitrosalicylic-acid) method with spectrophotometric detection according to Miller (1959). The principle of the method is that the 3,5-dinitrosalicylic acid is reduced by sugars to 3-amino-5-nitrosalicylic acid, while the aldehyde groups appear to be oxidized to carboxyl groups. Absorbance of the coloured reaction products can be measured at 540 nm. The method is non-specific and measures any reducing compounds released from cellulose by the enzymatic hydrolysis (Ghose, 1987). Concentration of the reducing sugars was determined as follows: 0.5 ml of enzyme sample solution was diluted with 1 ml of acetate buffer at pH 5, and the enzyme reaction was terminated by the addition of a 3 ml DNS solution, followed by boiling for 5 min. After cooling, the absorbance was read at 540 nm (Szabo, Csiszar, Koczka, Toth, & Klébert, 2016). The liberated reducing sugars in the glucose equivalent were estimated as mentioned above, according to Miller (1959). Each reported value is the average of at least three parallel measurements. ~~All experiments were carried out in triplicates.~~

3. Results and discussion

3.1. Comparing the efficiency of conventional agitation methods

For evaluating the contribution of various agitating systems to the enzymatic hydrolysis of cotton cellulose, the concentration of reducing sugars liberated during the hydrolysis for each of the selected conventional agitating systems (i.e. a shaker, a magnetic stirrer and the Labomat equipment) was determined. Small squares of the bleached cotton were subjected to hydrolysis involving a 90-min cellulase treatment. The reaction products i.e. the reducing sugars were analysed at 15 min intervals. Results in Fig. 2 reveal that there was less than 0.1 g/l reducing sugars in all the solutions in the first 30 min. After 45 min of hydrolysis, however, the sugar

concentration in the solution agitated with a magnetic stirrer was slightly higher than those released with the shaker and the Labomat. When the treatments were terminated (90 min), the final concentrations were 0.23 g/l, 0.28g/l and 0.33 g/l in the reactions agitated with Labomat, a shaker and a magnetic stirrer, respectively. Overall, cut-fabric was more susceptible to enzyme catalysed degradation in the solution agitated with a magnetic stirrer than that agitated either in a shaker or in the Labomat. Therefore, the enzyme treatment agitated with a magnetic stirrer was chosen to be the control for the ultrasonically irradiated system in the subsequent experiments.

Furthermore, magnetic stirring was also used in the ultrasound-aided experiments, in order to avoid sedimentation of the solid particles by continuous mixing the two phases (enzyme solution and fabric sample). Notwithstanding that the local ultrasonic activity decreases with agitation and with increasing flow rate in the reactor (Gogate, Tatake, Kanthale, Pandit, 2002), additional agitation is usually used in the heterogeneous reactions that contributes to the overall performance of hydrolysis.

Fig. 2. The effect of duration and type of agitating methods (vertical – Labomat; horizontal - shaker; magnetic stirrer) on the concentration of reducing sugars liberated during the cellulase catalysed hydrolysis of squares of bleached cotton (cut-fabric) at 50 °C and pH 5.

3.2. *Ultrasound-aided enzymatic hydrolysis of squares of bleached cotton fabric*

For evaluating the contribution of sonication to the hydrolytic potential of a cellulase enzyme toward a pure cellulose substrate, the cut-fabric was hydrolysed by varying the parameters of sonication (i.e. amplitude, duration and presence/absence of reflector). Results in Fig. 3a show that the cellulase enzyme was more effective in hydrolysis of cellulose in the sonicated systems than when sonication was absent (i.e. in the control system, agitated only with a magnetic stirrer). It is also clear that the yield of reaction depended largely on the amplitude of sonication. The highest concentration of reducing sugars was measured at 80 % amplitude. When the treatment was terminated (90 min), the concentration was above 0.5 g/l (Table 2). At 40 and 60 % amplitude, the final concentrations were close to 0.4 g/l (0.38 and 0.40 g/l, respectively). It should also be noted that in the control treatment the final concentration was only 0.33 g/l. Thus, sonication at different amplitudes (40, 60 and 80 %) enhances the yield of the enzymatic hydrolysis of cellulose over mechanical agitation (i.e. magnetic stirring) alone by 15 %, 24 % and 54 %, respectively.

For the bleached cotton powder (Szabo & Csiszar, 2013), a significantly higher increase in the concentration of reducing sugars (50, 250 and 275 % at 40, 60 and 80 % amplitude, respectively) was achieved after 60 min duration with the aid of sonication. In the latter experiment, however, the enzyme-dosage was also higher (66 versus 44 FPU/g substrate). Nevertheless, the surface of the substrate available for the enzyme attack can be significantly be larger for cotton powder than for squares of bleached cotton. Furthermore, the average degree of polymerization and the crystallinity of cotton powder are lower than those of the fabric (DP: 1880 ± 50 vs. 2200 ± 100 , respectively; Csiszar & Fekete, 2011). They both could also contribute to the higher reducing sugar liberation. ~~from the cotton powder.~~

Fig. 3. The effect of duration and amplitude of ultrasound (US) on the concentration of reducing sugar liberated during the cellulase catalysed hydrolysis of squares of bleached cotton (cut-fabric) at 50 °C and pH 5 without (A) and with (B) a reflector (R). Control treatment: agitated with a magnetic stirrer only.

Results in Figure 3b show the concentration of reducing sugars by applying a stainless steel reflector. It is obvious that all the experiments showed a continuous increase in concentration up to 90 min. ~~Comparing~~ When compared to the control treatment, it can be stated that only a slight increase was observed in the sonicated systems with a reflector and the amount of degradation products of hydrolysis ~~was~~ were not dependent upon the amplitude of sonication.

Table 2 compares the final reducing sugar concentrations liberated from the cut-fabric after 90 min of enzymatic hydrolysis of cellulose, at various amplitudes, with and without reflector. ~~Notwithstanding that we detected~~ In spite of detecting an increase in each of the experiments upon sonication, a significantly noticeable effect could only be detected at 80 % amplitude without a reflector.

At 40 and 60 % amplitude, the final concentrations were close to those measured without the reflector (about 0.4 g/l), thus, applying a reflector during sonication does not significantly alter

the efficiency of the enzyme catalysed hydrolysis. However, at 80 % amplitude a slight decrease in concentration was observed in the presence of a reflector compared to the data received without the reflector (0.45 g/l versus 0.51 g/l, respectively). Therefore, in this latter experiment the effect of reflector was negative and the final concentration of reducing sugars decreased by about 11 % from 0.51 to 0.45 g/l. These results ~~were in good agreement~~ conformed with the data obtained from the hydrolysis of powder cotton (Szabo & Csiszar, 2013). In the cited paper it was also shown that sonication at 80 % amplitude for 60 min with a reflector decreased the filter paper activity of a cellulase enzyme by 26.5 % ~~that~~ and this can be the reason for the less effective hydrolysis at 80 % amplitude in the presence of a reflector. The data, however, contradict ~~are contradictory with~~ the statement that a stainless steel disc completely reflects ~~fully~~ the sound waves, therefore it can usually ~~can~~ enhance the positive effect of ultrasound on the heterogeneous reactions (Moholkar and Warmoeskerken, 2003).

Table 2 Comparison of the hydrolytic capacities of sonicated systems. The final reducing sugar concentrations after 90 min of cellulase enzymatic hydrolysis of cut-fabric and framed-fabric, at 50 °C and pH 5, at an enzyme dosage of 44 FPU/g fabric and at 40, 60 and 80 % amplitude.^a

Sample	Amplitude (%)	Distance (mm) ^b	Reducing sugar concentration (g/l) ^c		Increase by sonication (%) ^{c,d}	
			-R	+R	-R	+R
Cut-fabric	40		0.38	0.39	15	8
	60		0.40	0.41	21	24
	80		0.51	0.45	54	36
Framed-fabric	40	9	0.25	0.32	9	39
		19	0.24	0.23	4	0
		28	0.11	0.17	-52	-26

60	9	0.62	0.57	170	148
	19	0.28	0.31	22	35
	28	0.31	0.31	35	35
80	9	0.35	0.36	52	56
	19	0.31	0.31	35	35
	28	0.30	0.32	30	39

^a Control treatments: agitated with a magnetic stirrer. Concentration of reducing sugars for the cut-fabric and framed- fabric: 0.33 g/l and 0.23 g/l, respectively.

^b Framed-fabric: positioned perpendicularly to the probe at a distance of 9, 19 and 28 mm, without a reflector.

^c Data measured without (-R) and with (+R) a reflector.

^d Calculation is based on the concentration measured in the respective control treatment.

3.3. Testing the ultrasonic field in a cylindrical glass cell by positioning the fabric

For evaluating the efficiency of ultrasonicated field in the cellulose-cellulase reaction, four layers of cotton fabric samples fixed in a frame were positioned perpendicularly to the probe at a distance of 9, 19 and 28 mm from the tip (Figure 1b). Sonication was performed at different amplitudes in the period of 0-90 min. Results in Figs. 4, 5 and 6 show the concentrations of the reducing sugars liberated from the fabrics at 40, 60 and 80 % amplitudes, respectively, without (A) and with (B) a reflector.

Fig. 4. The effect of the duration of sonication at 40 % amplitude on the concentration of reducing sugars liberated during the cellulase catalysed hydrolysis of framed-fabric positioned perpendicularly to the probe at a distance of 9, 19 and 28 mm from the tip, at 50 °C and pH 5 without (A) and with (B) a reflector (+ R). Control treatment: agitated with a magnetic stirrer only.

For each of the experiments, the highest reducing sugar concentration was measured at a distance of 9 mm, which indicates that cavitation most likely reached its maxima in this closest position to the probe, and the effect of the reflector is negligible. Furthermore, the figures clearly show that the most pronounced effect of sonication on the enzyme-aided hydrolysis of bleached cotton fabric was measured at 60 % amplitude at a distance of 9 mm (Figs. 5A and B). When the hydrolysis was terminated, the concentration of reducing sugars was about 2.7 times higher (0.62 g/l) than in the control solution (0.23 g/l). A similar value was measured with a reflector (0.57 g/l). Far Further from the probe (at 18 and 27 mm), however, the hydrolysis reaction is less notably accelerated by sonication (Table 2).

Fig. 5. The effect of the duration of sonication at 60 % amplitude on the concentration of reducing sugars liberated during the cellulase catalysed hydrolysis of framed-fabric positioned perpendicularly to the probe at a distance of 9, 19 and 28 mm from the tip, at 50 °C and pH 5 without (A) and with (B) a reflector (+ R). Control treatment: agitated with a magnetic stirrer only.

The influence of sonication at 80 % amplitude is evident and the hydrolysis pattern with and without the reflector were almost identical (Figs. 6A and B). Although a distinct improvement was observed, the final concentration values are quite low and they are in the range of 0.30-0.36 g/l. The cavitation activity is the lowest at 40 % amplitude. Except for the results received from the position at 9 mm with the reflector (Fig. 4A), the acoustic forces at 40 % amplitude and 18 and 27 mm were too weak to accelerate the hydrolysis of the fabric layers (Figs. 4A and B).

It can be summarized that the final yield in the system with framed-fabric depends on such important factors as amplitude, presence of a reflector and the distance from the top of the tip, and what is measured is the resultant of these effects. Hagenson and Doraiswamy (1998) proved the wave pattern to be dissipated and the mass transfer elevated near the probe tip, decreasing with increasing distance from the tip of the probe. At low powers (i.e. 8 W), in a radial direction the maximum intensity was measured at the centre of the transducer and the distribution of the intensity was fairly homogeneous. At high input power (i.e. 200 W), however, the active region

was equal only to the diameter of the horn, and the intensity decreased from the axis and became negligible at the remaining region toward the reactor wall. The power delivered to our system at 40, 60 and 80 % amplitude was about 30, 62 and over 90 W, respectively (Szabo & Csiszar, 2013). As the final yields in Table 2 reveal, our system reaches the optimal operating circumstances conditions at 60 % amplitude at the nearest position (9 mm), where the mass transfer is high in the center of the reactor and comparable over the cross section. Furthermore at 60 % amplitude, the decrease in enzyme activity caused by sonication is moderate (Szabo & Csiszar, 2013).

Fig. 6. The effect of the duration of sonication at 80 % amplitude on the concentration of reducing sugars liberated during the cellulase catalysed hydrolysis of framed-fabric positioned perpendicularly to the probe at a distance of 9, 19 and 28 mm from the tip, at 50 °C and pH 5 without (A) and with (B) a reflector (+ R). Control treatment: agitated with a magnetic stirrer only.

It is also important to consider the final reducing sugar concentrations liberated from either cut-fabric or framed-fabric samples at the same enzyme dosage (44 FPU/g) and substrate level (7.5 g/l) (Figs. 4-6 and Table 2). For the control enzyme treatments, the hydrolysis of the cut-fabric (0.33 g/l) is more efficient than that of the framed-fabric (0.23 g/l), which can be explained especially by the higher accessibility of the small fabric squares, the significantly higher number of protruding fibres on the edge of small fabric squares available easily for the enzymes, and the

free movement of the small fabric squares. These can all ~~can~~ contribute to the higher reducing sugar concentrations for cut-fabric.

In a sonicated environment, contrary to our expectations, the increase in reducing sugar liberation from cut-fabric and framed-fabric was almost in the same range (excluding the best configuration). It was proved that fabrics form a practically transparent boundary for acoustic waves, thus textiles do not have any individual impact on the ultrasound wave field (Moholkar & Warmoeskerken, 2003). On the other hand, the ultrasound intensity is sensitive to the presence of particles in the sonicated field and the waves are much more attenuated with the increase of particle size (Romdhane, Gadri, Contamine, Gourdon, & Casamatta, 1997). Here it has to be noted that for an efficient heterogeneous enzyme catalysed reaction, small particles are required for intensifying the enzyme-substrate interaction. Thus, besides the exact parameters of the sonicated environment, the overall performance of an enzyme reaction in a sonicated environment is influenced by many factors that can have positive and negative effects or both. All of these have ~~has~~ to be considered in a comprehensive investigation.

4. Conclusions

In this study we broadened our evaluation of the effects of low frequency ultrasound generated by a horn type reactor, on the cellulase enzyme catalysed hydrolysis of bleached cotton cellulose to include parameters such as amplitude of sonication, presence/absence of a rigid reflector on the bottom of the glass vessel, duration of hydrolysis, form of the cotton substrates and their free or impeded movement, and the distance of fabric layers positioning perpendicularly to the tip of horn. Results of the present work clearly show that low frequency ultrasound has a much more pronounced effect on the enzyme catalyzed hydrolysis of cotton cellulose than the conventional agitating methods such as magnetic stirring, horizontal and vertical mechanical agitation.

Hydrolysis of the approximately 1x1 cm sized squares of bleached cotton fabric revealed that the yield of the reaction as expressed in reducing sugar concentration, increased from 0.33 g/l (control) by about 15 %, 24 % and 54 % at 40, 60 and 80 % amplitudes (without a reflector), respectively. For amplitudes of 40 % and 60 %, the presence of the reflector slightly accelerated further the enzyme catalysis. At 80 % amplitude with a reflector, however, the yield of hydrolysis decreased which can be attributed to the adverse effect of ultrasound on the cellulase enzyme activity (Szabo & Csiszar, 2013). Mapping of the ultrasonicated reactor by following the hydrolysis of fabric layers positioned perpendicularly at different distances to the ultrasonic probe revealed that the reducing sugar concentrations were maximised ~~maximum~~ at a distance of 9 mm at each amplitude and decreased ~~with~~ when increasing the distance from the radiating surface.

Results clearly proved that besides exact parameters of the sonicated environment, the form of the fabric (including particle size), its positioning and free or impeded movement in the reaction chamber directly influenced the final outcome of the diffusion-limited enzymatic hydrolysis of cellulose.

Acknowledgements

The research was financed by grant from the Hungarian National Science Foundation (grant number OTKA K82044). This work is connected to the scientific program of the "Development of quality-oriented and harmonized R+D+I strategy and functional model at BME" project supported by the New Hungary Development Plan (Project ID: TAMOP-4.2.1/B-09/1/KMR-2010-0002).

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