

ENHANCING ENZYMATIC HYDROLYSIS OF CELLULOSE BY ULTRASONIC PRETREATMENT

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ABSTRACT: *Slurries of rice-straw cellulose (obtained by delignification and removal of hemicellulose from the powdered raw material) were subjected to ultrasonic waves at different intensities for various times (constant temperature). Susceptibility of the samples to cellulase-hydrolysis increased initially with pretreatment time, reaching a maximum or a constant level thereafter. Maximum glucose yield (~21%) was obtained under the following conditions: enzyme/substrate ratio ≈ 0.1 ; amplitude 18 μm (peak to peak), and exposure time 30 minutes. This yield is 30X that of the starting raw material, 10X that of the untreated rice-straw cellulose and 12-13X that of the value reported by others regarding pretreatment of wheat-straw cellulose by phenol. Changing the enzyme/substrate ratio to 0.4 raises glucose yield to 42%, under the same conditions. Removal of temperature control during pretreatment (50-80°C) increases the yield up to ~30% without increasing the enzyme/substrate ratio.*

KEY WORDS: *Cellulose, Hydrolysis, Cellulase, Enzyme, Ultrasonic, Physico-mechanical, Pretreatment, Rice-straw.*

Cellulose, the most abundant organic substance, (10^{11} tons/yr), is one of the major components of wood and other woody materials (~50% wt) [1,2]. Essentially it is a poly (β -anhydroglucose) and its hydrolysis yields glucose whose conversion to alcohol fuel has attracted the attention of chemists and other specialists for nearly a century [2-5]. One of the main

obstacles towards this end has been and still is the inaccessibility of cellulose to the hydrolyzing reactants [6-14]. Attempts made to overcome this problem fall in one of the following three categories: a) chemical treatments [15-19]; b) physico-mechanical treatments [20-29]; and c) physico-chemical treatments [30-34]. Despite all these efforts the problem seems far from

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being solved in a desirable technoeconomic fashion. In this article the ultrasonic pretreatment of rice-straw cellulose as a means of enhancing its enzymatic hydrolysis is reported.

EXPERIMENTAL

Materials

Chemicals used were of analytical grade obtained from commercial suppliers (Merck AG, Germany; Fluka, Switzerland; Sigma, Cincinnati, U.S.A; Hopkins & Williams, England). Glucose test kit was purchased from Pars Azmoon, Tehran. Rice-straw samples were obtained from Talesh region in northern Iran.

Equipments

Grinding mill (2840 rpm) equipped with 0.2 mesh screen (model SK-1; Retsch, GmbH, Hannover, Germany); probe type sonicator (exponential geometry, 150 W output, tip made of titanium, tip diam. 3mm, model MK2-3/75 made by MSE, France). The intensity of the ultrasonic waves shown on the equipment were in terms of the average distance between consecutive maximum-minimum peaks (peak to peak) in μm , and are reported as such (intensity is proportional to the square of the amplitude). UV-visible spectrophotometer (model TCC-240A; Shimadzu, Japan); autoclave (Ritter, U.S.A); optical microscope (model AH2; Olympus, Japan), bench top centrifuge (0-5000 rpm), Janetzki, DDR/model T32c; Milipore filter holder and 0.45 μm filters (Milipore Bedford, MA, U.S.).

Methods

a) Pretreatment

Rice-straw was milled (0.2 mesh) and the resulting powder was delignified with 10% potassium hydroxide [35] and its remaining hemicellulose was removed via autoclaving [36]. The resulting cellulose slurry was filtered, washed to neutrality and subsequently dried in a vacuum oven to a constant weight. 0.1 g dry cellulose samples were each taken in 10 mL acetate buffer in a special vial immersed in an ice bath and exposed to ultrasonic waves for various times at different intensities. Vial temperature never exceeded 30°C. Exposed samples were then transferred

to test tubes and diluted to 15 mL with acetate buffer.

b) Hydrolysis

To each sample treated above, was added 2.647 mL of cellulase solution (5U/mg solid from *Penicillium*, 4 mg enz./mL buffer) and gently mixed. Sample tubes were sealed and shaken in a 37°C water bath shaker for 2 hours [37] and subsequently placed in the ice bath to stop further enzyme action. Aliquotes of the supernatant sample solutions which contain glucose, soluble oligosaccharides, cellobiose, xylose, ... were centrifuged in a bench top centrifuge at 2500 rpm and the clear supernatants were further filtered through 0.45 μm milipore filters (to remove any suspended particle).

c) Glucose assay

Glucose assay was carried out by GOD-PAP (based on glucose oxidation via glucose oxidase) method. 10 μL of the above mentioned filtered solution was mixed with 1 mL of the glucose assay solution placed in a 37°C water bath for 15 minutes and its absorbance was measured at 500 nm using a UV-vis spectrophotometer [38].

RESULTS AND DISCUSSION

Disintegration of cellulosic particles

The first obvious effect of ultrasonic treatment was the gradual expansion of the cellulosic mass, Fig. 1. In all cases the expansion is initially an increasing

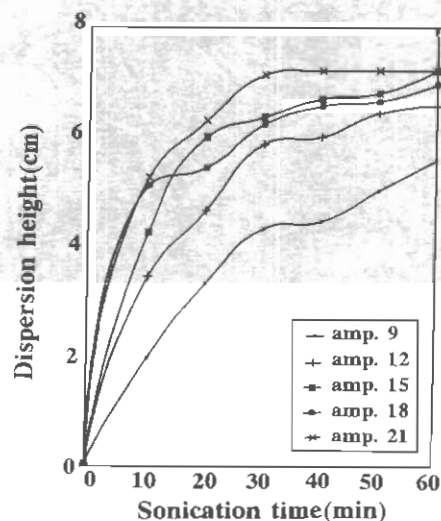


Fig. 1: Height of particle pack as a function of sonication time at different amplitudes (intensities).

function of the exposure time, usually reaching a rather constant level in about 30 minutes. The apparent changes in the physical shape and structure of the resultant particles (as a consequence of delignification and subsequent ultrasonic treatment) may be assessed by microscopic examination Figs. (2-5). Obviously, a tremendous disruption and dispersion of cellulosic particles takes place which is expected to lead to an increased substrate surface area for the heterogeneous enzymatic hydrolysis.



Fig. 2: Microscopic view of initial raw rice-straw particles, 100X magnification.



Fig. 3: Microscopic view of delignified nonsonicated rice-straw cellulose particles, 50X.

Glucose yield

Fig. 6 depicts the amount of glucose produced as a function of ultrasonic intensity and exposure time. As can be seen, the yield has an increasing trend up to 30 minutes when it usually reaches a maximum,



Fig. 4: Microscopic view of rice-straw cellulose particles after 10 minutes sonication at the amplitude of 15 μ m and prior to hydrolysis, 100X.



Fig. 5: Microscopic view of sonicated cellulose particles (60 minutes at 15 μ m), after 2 hours enzymatic hydrolysis.

showing the highest yield at 18 μ m (peak to peak) intensity. The same yield is achieved at the amplitudes of 12 μ m and 15 μ m in about 60 minutes. The reasons for the increase in the yield are rather obvious and straightforward, specially in light of the increased specific surface area of the substrate. In addition to physical disintegration of the particles as a consequence of cavitation phenomenon, there are possibilities of disruption of some intermolecular interactions, and probable reduction in cellulose crystallinity [39-41].

The decrease in the yields during longer periods may presumably be due to one or any combination of the following factors: formation and collection of large bubbles around the probe thereby reducing its

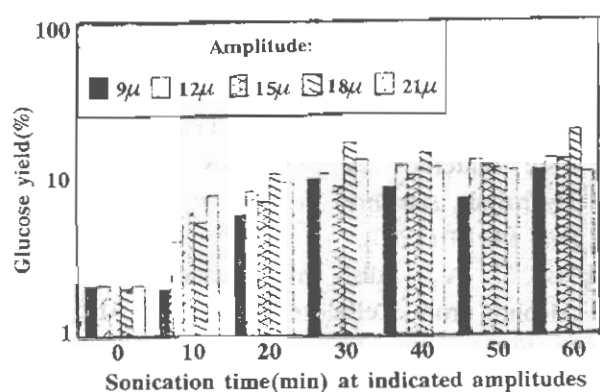


Fig. 6: Glucose yield as a function of pretreatment (sonication) time at different amplitudes. Enzymatic hydrolysis, 2 hours at 37°C, enzyme/substrate 0.1; $\lambda=500\text{nm}$.

energy transmission[40], gelation phenomenon and the increase in viscosity with their consequent reducing effects on cavitation [41]; occurrence of side reactions (e.g. oxidation and repolymerization, at 21 μm amplitude the sample slurry turns darker at longer exposure times); retarding effects on the enzyme by metal ions released from the probe [40] and inhibitory effects of glucose and cellobiose on the enzyme [9]. No attempts were made to elucidate any of these effects.

Demonstrating the enhancement

To clearly demonstrate the enhanced enzymatic hydrolysis of the sonicated cellulosic substrates, a comparison was made between the initial raw material, (I); nonsonicated cellulose obtained therefrom, (II); and sonicated samples, (III); Table 1.

As can be seen, at all amplitudes the yields for the sonicated samples are much higher, reaching 21% yield at 18 μm .

Table 1: Comparison of maximum glucose yields for the raw rice-straw(I), nonsonicated rice-straw cellulose (II) and sonicated rice-straw cellulose (III)

Sonication amplitude (μ Peak-Peak)	Yield(III) (%)	Yield(III)/yield(I)	Yield(III)/yield(II)
9	11	15.7	5.2
12	13.4	19.1	6.4
15	13.6	19.4	6.5
18	21	30	10.0

Yield(I) = 0.7%, Yield(II) = 2.1%, Yield(III) = 21%

Effect of enzyme/substrate ratio

The preceding results were obtained with an enzyme/substrate ratio of about 0.1. Repeating the experiments under maximum yield conditions (18 μm amplitude for 60 minutes) while increasing enzyme/substrate ratio, leads to increase in yield, Fig. 7. At the ratio of 0.4 the yield becomes 42%.

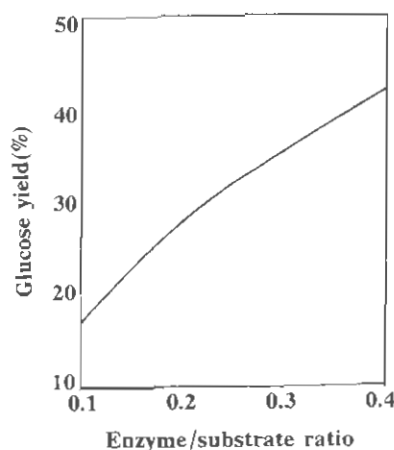


Fig. 7: Glucose yield as a function of enzyme/substrate ratio. Ultrasonic pretreatment at 18 μm for 30 minutes. Enzymatic hydrolysis; 2 hours at 37°C.

Temperature effect

In all experiments described so far the temperature was deliberately kept constant during sonication, whereas under no control, the temperature would increase automatically. Since the raise in temperature leads to increased reaction rates, an experiment was run at 18 μm amplitude for 30 minutes with no temperature control (50-80°C). The resultant cellulose when hydrolyzed enzymatically showed a glucose yield 1.4X that of temperature controlled.

Comparison with others

Wheat straw has been chemically pretreated with phenol and the resultant cellulose hydrolyzed enzymatically (under constant enzyme/substrate ratio of 0.1) [42]. Our yields are 12.6 and 13.6X greater than this one showing its superiority.

CONCLUSIONS

1. As expected ultrasonic treatment leads to disintegration of cellulosic particles, increasing their

apparent surface area for enzymatic attack, thereby enhancing their hydrolysis (an increase by a factor of ≈ 30 compared to the raw rice-straw).

2. Glucose yield is proportional to sonication amplitude and the exposure time (up to ~ 30 minutes).

3. Best yield is obtained at $18 \mu\text{m}$ amplitude in 30 minutes.

4. Ultrasonic pretreatment is far superior to chemical treatments.

5. Allowing the spontaneous temperature rise during sonication, improves the yield.

6. The yield is also proportional to the enzyme/substrate ratio (in the range 0.1-0.4 studied).

7. These results clearly indicate that the increase in specific surface area of the cellulosic substrate is the key determining factor in its enzymatic hydrolysis.

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REFERENCES

- [1] Turbak, A. F., Durso, D. F., Battista, O. A and Bolker, H. L., Cellulose in "Kirk-Othmer Encyclopedia of Chemical Technology", 2nd Ed., Vol. 5, John Wiley, N.Y., pp. 170-89(1979).
- [2] Sarbolouki, M. N. and Moacanin, J., Chemicals from Biomass-U.S. Prospects for the Turn of the Century, *J. Solar Energ.*, **25**, 303(1980).
- [3] Sjostrom, E., "Wood Chemistry-Fundamentals and Applications", Academic Press, N.Y., pp. 170-200 (1981).
- [4] Parisi, F., Advances in Lignocellulosics Hydrolysis and in the Utilization of the Hydrolysis, *Advances in Biochem. Eng./Biotechnol.*, **38**, 54 (1989).
- [5] Maiorella, B. L., *Ethanol in "Comprehensive Biotechnology"*, Vol. 3, Moo-young, M.(Ed.), Pergamon Press. London, pp. 861-914(1985).
- [6] Vollander, L. and Eriksson, K. E. L., Production of Ethanol from Lignocellulosic Materials-State of the Art, *Advances in Biochem. Eng./Biotechnol.*, **42**, 63(1990).
- [7] Caufield, D. F. and Moore, W. E., Effect of varying Crystallinity of Cellulose on Enzyme Hydrolysis, *Wood Sci.*, **6**, 375(1974).
- [8] Sidiras, D. K., Koullas, D. P., Vgenopoulos, A. G. and Koukios, E. G., Cellulose Crystallinity as Affected by Various Technical processes, *Cellulose Chem Technol.*, **24**, 309(1990).
- [9] Fan, L. T., Lee, Y. H. and Beardmore, D. H., Major Chemical and Physical Features of Cellulosic Materials as Substrates for Enzymatic Hydrolysis, *Advances in Biochem. Eng./Biotechnol.*, **14**, 101(1980).
- [10] Hsu, T. A., Ladish, M. R. and Tsao, G. T., Alcohol from Cellulose, *Chemtech.*, 315., May (1980).
- [11] Fan, L. T., Gharpuray, M. N. and Lee, Y. H., "Cellulose Hydrolysis", Springer-Verlag, N. Y., pp. 5-120(1987).
- [12] Cowling, E. B. and Kirk. T. K., Properties of Cellulose and Lignocellulosic Materials as Substrates for Enzymatic Conversion Processes, *Biotechnol./ Bioeng. Symp.*, No. 6, 95(1976).
- [13] Sinitsyn, A. P., Gusakov, A. V. and Vlasenko, E. Y., Effect of Structural and Physico-Chemical Features of Cellulose Substrates on the Efficiency of Enzymatic Hydrolysis, *Appl. Biochem. Biotechnol.*, **30**, 43(1991).
- [14] Peters, L. E., Walker L. P., Wilson, D. B. and Irwin, D. C., The Impact of Initial Particle Size on the Fragmentation of Cellulose by the Cellulases of *Thermomonospora Fusca*, *Biores. Technol.*, **35**, 313(1991).
- [15] Shah, F. H. and Rehman, Z. U., Ethanol Production from Dilute Acid Hydrolyzate of Cellulose Treated with Hydrogen Peroxide., *Pakistan Sci. Ind. Res.*, Nos. 5-6, 199, Oct-Dec.(1981).
- [16] Oqudipe A and Lu J. Y., Improvement in the Digestibility of Rice-straw by Alkali Treatment, *Biomass*, **20**, 291(1989).
- [17] Kappert, D. R., *Partial Acid Hydrolysis Pretreatment for Enzymatic Hydrolysis of Cellulose*, Ph. D. Thesis, Dartmouth College, N. H. U.S.A.(1981).
- [18] Holtzapfle, M. T., The Pretreatment and Enzymatic Saccharification of poplarwood, *Appl. Biochem. Biotechnol.*, **28/29**, 59(1991).
- [19] Kirk, T. K., Ibach, R. Mozauch, M. D., Conner, A. H. and Highley, T. L., Characteristics of Cotton Cellulose Depolymerization by Brown Rot Fungus, by Acid, or by Chemical Oxidants, *Holz-forschung* **45**, 239(1991).

- [20] Morehead, F. F., Ultrasonic Disintegration of Cellulose Fibers before and after hydrolysis, *Text. Res. J.*, **20**, 549(1950).
- [21] Saeman, J. F. and Millett, M. A., Effect of High Energy Cathode Rays on Cellulose, *Ind. Eng. Chem.*, **44**(12), 2848(1952).
- [22] Sofonova, V. V. and Klenkova, N. I., Change in the Reactivity of Cellulose Fibers under the Influence of Ultrasound, *Zh. Prikl. Khim.*, **42**(11), 2636(1969).
- [23] Rolz, C., Effect of Ultrasound on Enzymatic Saccharification, *Rev. ICAITI*, **2**(1), 6(1986).
- [24] Attala, R. H., Ellis, J. D. and Schroeder, L. R., Some Effects of Elevated Temperatures on the Structure of Cellulose and Its Transformations, *J. Wood Chem. Technol.*, **4**, 465(1984).
- [25] Rivers, D. B. and Emert, G. H., Lignocellulose Pretreatment: A Comparison of Wet and Dry Ball Attrition, *Biotechnol. Lett.*, **9**, 365(1987).
- [26] Yamashiki, T., Matsui, T., Saitoh, M., Okajima, K., Kamide, K. and Savada, T., Characterization of Cellulose Treated by the Steam Explosion Method, *Pts I & II. Br. Polym. J.*, **22**, 73 and 201(1990).
- [27] Ramos, L. P., Breuil, C., Kushner, D. N. and Saddler, J. N., Steam Pretreatment Conditions for Effective Enzymatic by Hydrolysis and Recovery yields of *Eucalyptus vimindalis* Wood Chips, *Holzforchung*, **46**, 149(1992).
- [28] Saddler, J. N., Ramos, L. P. and Breuil, C., Steam Pretreatment of Lignocellulosic Residues, in "Bio-conversion of Forest and Agricultural plant Residues", J. N. Saddler(Ed.), CAB. Internation, pp. 73-92(1993).
- [29] Shimizu, K., Sudo, K., Ono, H., Ishihara, M. and Fujii, T., Total Utilization of Wood Components by Steam Explosiom Pretreatment, in "Advanced Materials, 93, VLA: Economaterials", *Trans. Mat. Res. Soc Jpn.*, **18A**, R Yamamoto, (Ed.), 481 (1994).
- [30] Millett, M. A., Baker A. J. and Satter, L. D., Physical and Chemical Pretreatments for Enhancing Cellulose Saccharification, *Biotechhol. Bioeng. Symp.*, No. 6, 125(1967).
- [31] Mackie, K. L., Brownell, H. H., West, K. L. and Saddler, J. N., Effect of Sulfur Dioxide and Sulfuric Acid on Steam Explosion of Aspenwood, *J. Wood Chem. Technol.*, **5**, 405(1985).
- [32] Clark, T. A and Mackie, K. L., Steam Explosion of Softwood *Pinus Radiata* with Sulfur Dioxide Addition I. Process Optimization, *J. Wood Chem. Technol.*, **7**, 373(1987).
- [33] Schuchardt, U. L. and Ines, J., Hydrolysis of Sugar Cane Bagasse with Hydrochloric Acid, Promoted by Ultrasound, *Chem. Tech. Biotech.*, **39**(2), 115(1987).
- [34] Grethlein, H. E. and Converse, D. A., Common Aspects of Acid Prehydrolysis and Steam Explosion for Pretreating Wood, *Biores. Techn.*, **36**, 77(1991).
- [35] Mandels, M., Mantz, L. and Nystrom, J., Enymatic Hydrolysis of Waste Cellulose, *Biotechnol. Bioeng.*, **XVI**, 1473(1974).
- [36] Zemann, A. J., Kubikova, J., Bobleter, O. and Kam, L., *Utilization of Chemical Free Sugar Cane Bagasse and Sugar Cane Leaves as Plup.*, 207th National ACS Meeting, San Diego, March (1994).
- [37] "Workingtion Enzyme Mannual", Worthingtion Biochem. Corp./Freehold N. J., U.S.A p. 97 (1972).
- [38] Dietzler, D. N. and Smith, C. H., Enzymatic Determination of Glucose, in "Gradwohl's Clininal Laboratoy Methods and Diagnosis" Vol. 1, Sonnewirth A. O. and jarett, L(Eds.), 8th Ed. the C.V. Mosby Co., St. Louis, London, pp. 225-35 (1980). Barham, D. and Tinder P., *Analyst*, **97**, 142(1972).
- [39] Takavar, A. and Saghari, M., "Medical Physics" (in Presian); Nashre-Enghelab, 2nd Ed., Tehran, pp. 153-59(1992).
- [40] Mason, T. J. "Chemistry with ultrasound", Elsevier, Amsterdam, pp. 1-25(1990).
- [41] Mason, T. J. and Lorimer, J. P. "Sono-chemistry-Theory, Applications and Uses of Ultrasound in Chemistry", Ellis Horwood, Chichester, pp. 98-138(1988).
- [42] Zacchi, G., Skoog, K. and Hahan-Hagerdal, D., Economic Evaluation of Enzymatic Hydrolysis of Phenol-Pretreated Wheat Straw, *Biotechnol. Bioeng.*, **32**, 460(1988).