ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC RESIDUES: A PROMISSING TECHNOLOGY FOR ETHANOL PRODUCTION

Boutros Fouad Sarrouh[1], Silvio Silvério da Silva[1,2]

[1]Escola de Engenharia de Lorena-USP. Departamento de Biotecnologia. Estrada Municipal de Campinho, CEP: 12.602-810, Lorena-SP. Email: bsarrouh@yahoo.es

[2]Fatea - Faculdades Integradas Teresa D'Ávila, Av. Peixoto de Castro, 539 Vila Celeste - Cep:12.606-580, Lorena-SP. Email: silvio@debiq.eel.usp.br

Abstract- The shortage and extremely high price of fossil fuels has become increasingly serious in many developing countries as a result of increased population density and industrial expansion. From this point of view the enzymatic saccharification of cellulosic materials, primarily renewable agricultural residues, presents an important solution to this problem, especially given the extremely large quantity of cellulosic resources being produced each year. It appears likely that the enzymatic saccharification of cellulose to glucose followed by its refinement or conversion to edible sugars (e.g., fructose) or its fermentation into liquid fuels presents a very promising technology. In this paper we present some important concepts of enzymatic conversion of cellulosic biomass to ethanol, including enzyme sources, molecular mechanisms of enzymatic cellulose degradation mechanisms and kinetics, enzyme inhibition models and the economic feasibility of the process.

.

Keywords: cellulose, enzymatic hydrolysis, degradation mechanism, kinetics, ethanol

Área do Conhecimento: Engenharia Química

Introduction

Biomass in the form of agricultural and forest wastes accumulates every year in large quantities both in industrial and developing countries. This results in a deterioration of the environment and a loss of potentially valuable resources. Some of the potential biodegradable agricultural and agro-industrial cellulosic residues like, sugarcane bagasse (KLYOSOV, A. et. al., 1982a):

Natural cellulose is a crystalline polymer generally associated with hemicellulose and lignin in a matrix and is highly resistant to enzymatic attack. Therefore, pretreatment is necessary. Most approaches separate the different types of pretreatment into mechanical, chemical, physical (other than mechanical), biological, or a combination of these methods (FAN, L. T., 1981).

Most pre-treatment processes have energy requirements that depend on the severity of the process. Severe mechanical and/or thermochemical processes have substantial energy requirements and when combined with those for product separation, can make some cellulosics bioconversion processes very inefficient. It appears likely that the enzymatic saccharification of cellulose to glucose followed by its refinement or conversion to edible sugars (e.g., fructose) or its fermentation into liquid fuels presents a very promising technology. With respect to the acid hydrolysis of the cellulosic biomass, one of its major shortcomings is the low total yield of sugars (50-55%) owing to side product formation. The estimated costs of chemical pretreatment of wheat straw vary from $0.4/kg for caustic pretreatment to $11.25/kg for ethylene glycol treatment. In comparison, the costs of mechanical pretreatments vary from $0.01/kg to $2.24/kg (LYND, L. R. 1990, SPANO, L., et. al., 1980). In this paper we present some important concepts of enzymatic conversion of cellulosic biomass to ethanol, including enzyme sources, molecular mechanisms of enzymatic cellulose degradation mechanisms and kinetics, enzyme inhibition models and the economic feasibility of the process.

Enzyme sources

Although many fungi can degrade cellulose, metabolic products consist usually of carbon dioxide and methane. In addition, although numerous fungi degrade soluble cellulose derivatives such as carboxymethyl cellulose, only comparatively few of them can produce high levels of extracellular cellulases capable of extensively degrading insoluble cellulose to soluble sugars in vitro. These fungi include *Trichoderma reesei (= Trichoderma viride), T. koningii, T. lignorum, T. longibrachiatum, Phanerochaete chrysosporium (= Sporotrichum pulverulentum, = Chrysosporum*

lignorum), Geotrichum candidum, Penicillium funiculosum, P. iriensis, Eupenicillium javanicum, Schizophylium commune, Polyporus adustus, Fusarium solani, F. lini, Sclerotium rolfsii, Aspergillus wentii, Asp. terreus, Asp. niger, Asp. foetidus. Thermophilic microorganisms are viewed as a source of thermostable cellulases; however, cellulases from thermophiles may not necessarily be more heat-stable than cellulases from mesophiles (KLYOSOV, A. A., 1982b).

Until recently, most of the applied work in the area of the enzymatic conversion of cellulose to glucose utilized strain *Trichoderma viride* QM 9414 as a source of cellulase. However, the development of hyper producing and catabolite repression resistant strains *T. reesei* Rut C-30 and Rut-P37 as well as strains *T. reesei* VTT-D-80132 and -80133 (VTT Biotechnical Laboratory, Finland), has led to a reevaluation of these processes (KLYOSOV, A. et. al., 1982a). There obviously is a major potential in the area of biotechnological mutagenesis of cellulases for increasing the availability and choice of novel bioengineered cellulases, designed for specific technological purposes.

Molecular mechanisms of enzymatic cellulose degradation

Cellulases are the group of hydrolytic enzymes that are capable of hydrolyzing insoluble cellulose to glucose. They are produced by microorganisms, plants and animals (in the latter case by symbiotic microorganisms) usually as a cellulase system composed of several distinct enzymes. Three types of enzymes are traditionally assigned to the cellulase system: endoglucanases (endo-1,4-ß-glucanases, or 1,4-ß-D-glucan 4-glucanohydrolases, EC 3.2.1.4), cellobiohydrolases (exo-1,4-ß-glucanases, or 1,4-ß-D-glucan cellobiohydrolases, EC 3.2.1.91) and cellobiases (ß-glucosidases, or ß-D-glucoside glucohydrolases, EC 3.2.1.21) (KLYOSOV, A. A., 1982b).

Kinetics and mechanisms of enzymatic cellulose degradation

It was well known that cellulase systems from some biological sources can hydrolyze both *amorphous* and *crystalline* cellulose ("complete", "full-value", or "true" cellulases), whereas other cellulase systems are active only toward *amorphous* cellulose ("non-complete", or "low-value").

The behavior of enzyme kinetics can be deduced from Michaelis-Menten classical equations and applied on the system under study. For the case of beta-glycosidase, which is soluble, it hydrolyzes the soluble substrate of cellobiose to glucose and also the mentions equations can be applied in this case. But for the cellulase that produces cellubiose from cellulose these equations are inadequate. This fact is due to that the cellulase when acting over the insoluble substrates (cellulose) it deviate the reaction kinetic from Michaelis-Menten model due to several factors:

- The enzyme can be adsorbed or no to the substrate, but only the adsorbed enzyme can act over the cellulose.
- It's not clear which substrate concentration should be considered, that of all the substrate or only which is in direct contact with the enzyme

Some characterizations are made by considering the cellulase complex as a whole. In this sense its behavior can be expressed as a function of the enzyme dose in the system as shown in equation 1 (KLOYOSOV, A. A. et. al, 1981, KLOYOSOV, A. A. et. al., 1986a,b):

$$G = K*E_{ads}^{r} \qquad (1)$$

G = produced glucose mg/g cellulose.
K = rate constant
E_{ads} = adsorbed enzyme concentration mg/g cellulose.
r = reaction order

For cellulose: 0.15> r < 0.7. The reaction fractional order, not only characterizes many cellulase effect but also glucose production (KLOYOSOV, A. A. et. al., 1986a,b).

Adsorption of cellulases on cellulose

The adsorption of cellulases on cellulose provides more than the conventional physical contact between enzyme and substrate. The first step in the cellulose hydrolysis is the enzyme adsorption to the cellulose. The reason of this adsorption depends on the viscosity and the agitation rate of the system. The adsorption equilibrium is described by Longmuir isotherm adsorption equation 2:

$$P_{ads} = K_p*P_{máx}*P_L/1 + K_p*P_L \qquad (2)$$

P_{ads} = adsorbed protein, mg/g cellulose
K_p = bonding constant ml/mg.
P_{max} = saturation constant, mg/g cellulose
P_L = protein in solution mg/ml

Enzyme inhibition model

The velocity of many catalyzed reactions is reduced specifically due to the presence of inhibitors. The principal two types of inhibition Are: competitive and no competitive.

The cellulase is inhibited by its final products, cellulbiose and glucose. In case of *Trichoderma cellulase*, the inhibition by final products is no-competitive. This behavior is expressed by the following equation 3 (RABINOVICH, M. L. et. al., 1985a, RABINOVICH, M. L. et. al., 1984)

$$4r_i/r = 1/1 + 4[I]/K_i \quad (3)$$

r = hydrolysis rate g glucose L/h.
r = inhibited hydrolysis rate gL/h.
[I]= inhibitor concentration g/L.
K_i = inhibition constant g/L.

For the Trichoderma cellulase, Ki of glucose = 69 g/L, and for cellubiose Ki= 3.3 g/L. The action of cellulase is inhibited o inactivated by the presence of different compounds, including strong oxidants o reducing agents, metallic ions, salts, solvents, etc.

Beta-glycosidase is a soluble enzyme which acts over a soluble substrate (celliobose), transforming it to glucose with a respective increase in the process yield. Beta-glycosidase is well represented by Michaelis-Menten classical equation, with the glucose action as a competitive inhibitor. Its kinetics parameters are defined in the following equation 4 and calculated in table II, for the case of Aspergillum Niger (TIKHOMIROV, D. F. et. al., 1989, TIKHOMIROV, D. F. et. al., 1988):

$$V = V_{max}*G_2/G_2 + K_m (1+4G/K_i) \quad (4)$$

V = reaction velocity g/Lh.
V_{max} = maximum velocity g/Lh.
G_2 = celliobiose concentration g/L.
K_m = Michaelis constant g/L.
G = glucose concentration g/L.
K_i = inhibition constant g/L.

Endoglucanases of cellulase complexes from different sources. These data indicate that the endoglucanases can be subdivided tentatively into three groups that are (KLOYOSOV, A. A., 1988): cellobiose (K_i 100-120 g/l, or 0.29-0.35 M),

(ii) Of moderate sensitivity (K_i 50-90 g/l, or 0.15-0.26 M)

(iii) Of high sensitivity (K_i 20-40 g/l, or 0.06-0.12 M).

Since the products of the reaction of cellulases inhibit the catalytic efficiency of the native enzymes, decreasing the inhibition constant is beneficial. However, it is teleologically difficult to design such an enzyme since the product of the cellulase reaction is a hydrolyzed monomer of a polymeric substrate. It is probably easier to maintain the product formed at low concentrations so that it will not significantly inhibit the rate of reactions.

Conclusions

The shortage and high price of fossil fuel has become increasingly serious in many developing countries as a result of increased population density. From this point of view the enzymatic saccharification of cellulosic materials, primarily renewable agricultural residues, presents a possible partial solution to this problem, especially given the extremely large quantity of cellulosic resources being produced each year in subtropical and tropical developing countries (EMERT, G. H. et. al., 1980, HINMAN, N. D. et. al., 1989).

In those developing countries where the technique of microbial technology is not fully established, enzymatic hydrolysis of cellulosic materials and fermentation of the resulting sugars into ethanol should be performed on a relatively small scale with facilities that are already available.

Renewable cellulosic resources will be used as industrial raw material for producing sugar, yeast, and liquid fuel in the near future. Importantly, education may well prove to be the critical determinant for the future developments foreseen in this treatise

Acknowledgment

The authors would like to thank FAPESP and CNPq for their financial support.

References

- Klyosov, A. A., and Sinitsyn, A. P.. Enzymatic Hydrolysis of Cellulose. IV. Effect of Major Physicochemical and Structural Features of the Substrate. ***Bioorgan. Khimiya***, V.7, p. 994-1004, 1981.

- Klyosov, A. A., Chernoglazov, V. M., Rabinovich, M. L., and Sinitsyn, A. P.. The Role of Endoglucanase Adsorptivity in Degradation of Crystalline or Amorphous Cellulose. ***Bioorgan. Khimiya***. V.8, p.643-651. 1982a.

- Klyosov, A. A., Rabinovich, M. L., Churilova, I. V., Martyanov, V. A., Gusakov, A. V., and Elyakova, L. A. Enzymatic Hydrolysis of Cellulose. V. Cellulases in Marine Organisms of the Sea of Japan. ***Bioorgan. Khimiya***, V.8, p.1490-1496. 1982b.

- Klyosov, A. A., Mitkevich, O. V., and Sinitsyn, A. P. Role of the Activity and Adsorption of Cellulases in the Efficiency of the Enzymatic Hydrolysis of Amorphous and Crystalline Cellulose. ***Biochem,*** V.25, p. 540-542. 1986a.

- Klyosov, A. A., Grigorash, S. Yu., and Gromov, A. I. Mathematical Modelling of the Kinetics of the Multienzyme Hydrolysis of the Insoluble Cellulose.

Prikl. Biokhim. Mikrobiol., V.22, p. 612-621. 1986b.

- Klyosov, A. A. Cellulases of Third Generation. In: Aubert, J.P., Beguin, P. and Millet, J., (Eds). **Biochemistry and Genetics of Cellulose Degradation**, pp 87-99, Academic Press, London. 1988.

- Fan, L. T., Gharpuray, M. M. and Lee, Y.H. Evaluation of Pretreatments for Enzymatic Conversion of Agricultural Residues. ***Biotechnol. Bioeng. Symp***. V.11, p.29-45, 1981.

- Lynd, L. R. Large-Scale Fuel Ethanol from Lignocellulose. Potential, Economics, and Research Priorities. ***Appl. Biochem. Biotechnol.*** V.24/25, p. 695-719. 1990.

- Spano, L., Tassinari, T., Ryu, D. D. Y., Allen, A., and Mandels, M. Producing Ethanol from Cellulosic Biomass. In: **Biogas and Alcohol Fuels Production. Proceedings, Seminar on Biomass Energy for City, Farm and Industry**, pp 62-81, The J.G. Press, Inc., E-aus, PA. 1980.

- Rabinovich, M. L., Klyosov, A. A., and Berezin, I. V. The Mechanism of the Transfer of an Enzyme Adsorbed on an Insoluble Substrate Surface. ***Dokl. Akad. Nauk.*** V. 274, p. 758-763. 1984.

- Rabinovich, M. L., Melnick, M. S., Badalov, A. B., Klyosov, A. A., and Berezin, I. V. Evolution of the Enzyme-Substrate Interactions in the Course of the Enzymatic Hydrolysis of Cellulose. ***Dokl. Akad. Nauk***. V. 282, p. 1013-1017. 1985a.

- Tikhomirov, D. F., Nuzubidze, N. N., Lakhtin, V. M., and Klyosov, A. A. Isolation of Multiple Forms of *Trichoderma reesei* Endoglucanase Possessing High Hydrophobicity. ***Biokhimiya***, V. 52, p.1097-1106. 1987.

- Tikhomirov, D. F., Stolbova, V. V., and Klyosov, A. A. Endo-1,4-beta-Glucanases of the Anaerobic Bacterium *Clostridium thermocellum* St. No. 3 with a High Heat Stability. ***Prikl. Biokhim. Mikrobiol***.V.25, p.48-55. 1989.

- Emert, G. H., Katzen, R., Fredrickson, R. E., and Kaupisch, K. F. Economic Update of the Gulf Cellulose Alcohol Process. CEP September, 47-52. 1980.

- Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. ***Appl. Biochem. Biotechnol.*** V.20/21, p.391. 1989.