0273-2289/97/70-72—0369$8.50

Effect of Surfactants and Zeolites on Simultaneous Saccharification and Fermentation of Steam-Exploded Poplar Biomass to Ethanol

I. Ballesteros, J. M. Oliva, J. Carrasco, A. Cabañas, A. A. Navarro, and M. Ballesteros*

Instituto de Energís Renovables-CIEMAT, Avda. Complutense, 22 28040-Madrid-Spain

ABSTRACT

In this work, the effect of the addition of different concentrations of Tween-80 and three different zeolite-like products on enzymatic hydrolysis, ethanol fermentation, and simultaneous saccharification and fermentation (SSF) process has been investigated. The ability of these products to enhance the effectiveness of the SSF process to ethanol of steam-exploded poplar biomass using the thermotolerant strain *Kluyveromyces marxianus* EMS-26 has been tested.

Tween-80 (0.4 g/L) increased enzymatic hydrolysis yield by 20% when compared to results obtained in hydrolysis in absence of the additive. Zeolite-like products (ZESEP-56 and ZECER-56) (2.5 g/L) improved rates of conversion and ethanol yields in the fermentation of liquid fraction recovered from steam-exploded poplar. The periods required for the completion of fermentation were approx 10 h in the presence of zeolite-like products and 24 h in the absence of additives. The probable mode of action is through lowered levels of inhibitory substances because of adsorption by the additive.

Index Entries: Simultaneous saccharification and fermentation; lignocellulose biomass; zeolite; surfactant.

INTRODUCTION

The increase of the cellulose-conversion level and reduction of SSF fermentation time have significant impact on estimated production cost of fuel-ethanol *(1)*. One means of increasing cellulose-conversion yield is to

* Author to whom all correspondence and reprint requests should be addressed.

carry out the SSF process at the cellulase optimum temperature. Increasing hydrolysis temperature should decrease reaction time, assuming that yeasts are capable of performing well at higher temperatures.

The inhibitory action of ethanol produced in the course of fermentation has effects on the cell growth, cell viability, and fermentation of yeasts. With increasing temperature, some of the effects of ethanol on yeast cells may become more severe *(2–4)*. Ethanol enhances thermal death by acting in a nonspecific way on membrane lipids. It has been shown that the target of thermal death and of ethanol-enhanced thermal death in *Saccharomyces cerevisiae* is a macromolecular site in the inner mitochondrial membrane *(5,6)*. There is considerable evidence in the pertinent literature *(7–10)* indicating that unsaturated fatty acids and sterols in the membrane are important for enhancing ethanol tolerance in yeasts, and several groups have reported improvements in alcoholic fermentation and final ethanol concentration by broth supplementation with lipids, proteins, and vitamins *(11–15)*.

During pretreatment of the poplar biomass by steam explosion, aromatic monomers that have inhibiting effects on the bioconversion process to ethanol are produced *(16)*. The elimination of these toxic compounds would be desirable in order to increase the yield of the process of enzymatic hydrolysis and simultaneous fermentation.

Some natural *(17)* and synthetic *(18)* zeolites have been reported to enhance the fermentation rate of sugarcane molasses to ethanol through protection against the inhibitory effects of substrates and products. Zeolites are crystalline, hydrated aluminosilicates, and all applications of zeolites are related to their physical and chemical properties: ion-exchange, adsorption, and related molecular sieve properties. The three-dimensional crystal structure of zeolites contains pores and channels of uniform size on a molecular scale. Their shape selectivity seems to play a role in removing inhibitory substances from the fermentation medium and in favoring yeast sorption onto zeolite crystals. Their characteristics in cation-exchange capacity and cation selectivity have led to their use in waste-water treatments *(19,20)*.

In this work, the effect of media supplementation with unsaturated fatty acids and zeolite-like products on the SSF process of steam-exploded poplar biomass to ethanol has been tested.

MATERIALS AND METHODS

Substrate and Pretreatment

Poplar biomass was ground at 5-mm mesh size. The biomass was pretreated in a steam-explosion pilot unit operated by batches and equipped with a reaction vessel of a 2-L working volume which was filled with 200 g of dry biomass. The plant description and working methodol-

ogy are described in a previous paper *(21)*. The temperature (210°C) and residence time (4 min) conditions of the biomass pretreatments were selected with regard to the maximum glucose recovery after 72 h of enzymatic hydrolysis.

Cellulase Source

The cellulolytic complex employed (Celluclast 1.5 L) and β-glucosidase (Novozyme 188) were a donation from NOVO Nordisk (Bagsvaerd, Denmark).

Surfactants and Zeolitic Products

Tween-80 (polyoxyethylenesorbitan monooleate) was purchased from Sigma (St. Louis, MO). The zeolite clinoptilolite is a natural mineral with ion-exchange and gas-adsorption properties, and it was obtained from Mineral Research (Clarkson, NY). The zeolite-like products ZESEP-56 (SiO_2/Al_2O_3) (R = 8.3) and ZECER-56 (SiO_2/Al_2O_3) (R = 12.1) were prepared by the University of Cadiz (Spain) from Sepiolite 120NF and ceramic residues, respectively, after alkaline treatment. In ceramic residues, there is a great quantity of B_2O_3 along with TiO_2. These compounds, when subjected to the alkaline treatment proper to zeolitization, can give rise to zeolitic structures in which silica and aluminum are partially substituted by boron and titanium in the crystalline framework *(22)*. Sepiolite is a magnesium silicate with a low aluminum content. Its structure includes internal channels that provide zeolitic properties.

Microorganisms and Growth Conditions

Kluyveromyces marxianus EMS-26, a thermotolerant mutant yeast strain obtained in our laboratory *(23)* was used in fermentations and SSF experiments. Active cultures for inoculation were prepared by growing the organism on a rotary shaker at 180 rpm for 16 h at 42°C in a growth medium containing (g/L): yeast extract (Difco, East Molesley, Surrey, UK), 5; peptone (Oxoid), 5; NH_4Cl, 2; KH_2PO_4, 1; $MgSO_4$ $7H_2O$, 0.3; and glucose, 30.

Enzymatic Hydrolysis, Simultaneous Saccharification, and Fermentation Assays

Experiments were carried out in 100-mL Erlenmeyer flasks, each containing 50 mL of the fermentation medium (initial pH 4.1) described above, which were agitated at 150 rpm. Glucose was substituted with the lignocellulose biomasses at 10% (w/v) substrate concentration, and the cellulolytic complex (Celluclast 1.5 L at 15 FPU/g substrate and Novozyme 188 at 12.6 IU/g substrate enzyme loading, respectively,) was also added.

Tween-80 and the zeolite-like products were added at the stated levels at the beginning of fermentation.

Enzymatic hydrolysis, SSF, and fermentation assays were conducted at 42°C for 72, 144, and 24 h respectively pH was not controlled throughout assays.

In the SSF experiments, flasks were inoculated with 10% (v/v) of yeast cultures and periodically checked during the assays for ethanol and glucose.

Initial enzyme concentration (Eo) was measured as the amount of protein in the reaction medium before substrate addition. By taking the difference between the initial concentration and the concentration at a particular time during the hydrolysis, the amount of adsorbed enzyme was calculated.

Analytical Procedures

Composition of substrate and residues in potential glucose and lignin have been determined by total hydrolysis with H_2SO_4 *(24)*.

Enzymatic activities (filter paper and β-glucosidase) were measured according to the methods described by Mandels et al. *(25)* and Bailey and Nevalainen *(26)*, respectively.

Glucose was quantified by HPLC in a 1081B Hewlett Packard (HP) (Palo Alto, CA) apparatus with differential refractometer detector at the following conditions: column, AMINEX HPX-87P (Bio-Rad, Hercules, CA); temperature, 85°C; eluent, water at 0.1 mL/min.

Ethanol was measured by gas chromatography, using a HP 5890 Series II apparatus, with flame ionization detector and a column of Carbowax 20 *M* (2 m × 1/8 in) at 95°C. Injector and detector temperature: 150°C.

Protein was measured using the Lowry reaction *(27)*.

RESULTS

To analyze the effects that the supplementation of culture mediums with Tween-80 and zeolite-like products have on the process of enzymatic hydrolysis and simultaneous fermentation, some preliminary experiments about the effects of these additives on each one of these processes were carried out separately.

Effect of Tween-80 and Zeolite-Like Products on Hydrolysis Efficiency

Figure 1 shows the effect of different concentrations of Tween-80 and zeolite-like products on the enzymatic hydrolysis yield of steam-exploded poplar biomass. The results are expressed as percentages referring to the control set-up. The 100% hydrolysis yield corresponds to the glucose produced in the control experiment in which no supplementation was effected.

As may be observed, on the addition of Tween-80 to the medium in which enzymatic hydrolysis is effected, the saccharification yield increases with the corresponding incrementation of the concentration of surfactant,

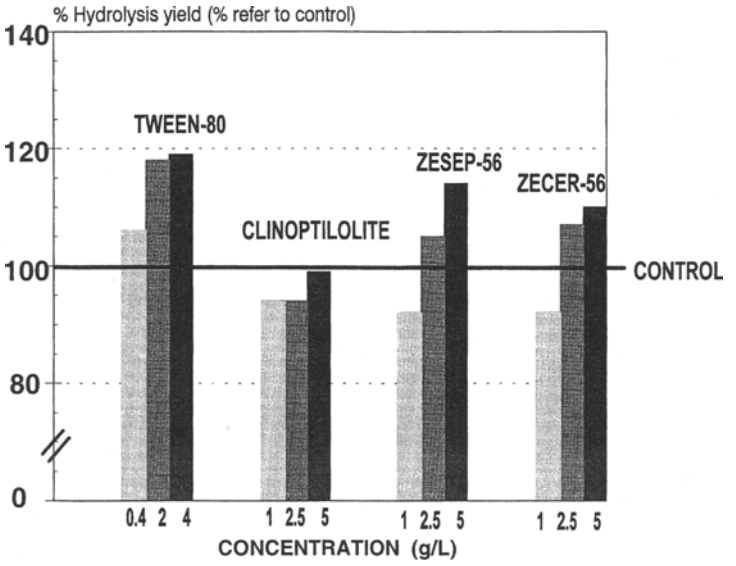

Fig. 1. Effect of varying levels of Tween-80 (0.4, 2, and 4 g/L), clinoptilolite (1, 2, and 5 g/L), ZESEP-56 (1, 2, and 5 g/L), and ZECER-56 (1, 2.5, and 5 g/L) on the final hydrolysis yield of 10% steam-exploded poplar biomass. Hydrolysis yields refer to control without supplementation

achieving gains of 6, 18, and 19% in hydrolysis yield when supplements of Tween-80 at 0.4, 2, and 4 g/L respectively, are applied.

The addition of zeolite-like products has uneven effects on the enzymatic hydrolysis yield of pretreated poplar. The addition of a natural zeolite such as clinoptilolite does not improve hydrolysis yields, lower glucose productions being obtained in these tests by comparison with the control experiments. Zeolite-like products prepared from sepiolite (ZESEP-56) and ceramic residues (ZECER-56) increase the yield of hydrolysis by 5 and 15% when concentrations of 2.5 and 5 g/L, respectively, are used.

For the purpose of determining if the supplementation of culture media with Tween-80 (4 g/L), clinoptilolite (5 g/L), and zeolite-like products (5 g/L) modify the adsorption of cellulolytic enzymes, the concentration of soluble protein throughout the process of enzymatic hydrolysis was analyzed. The results of these tests are shown in Fig. 2.

As can be seen, the extent of cellulase adsorption was significantly affected by the addition of the different products tested.

The course of enzyme adsorption during the first 8 h of fermentation followed a similar profile for all cases tested. A rapid initial adsorption of cellulose was observed followed by an equally rapid desorption.

In the media that were used as controls (no supplements added) it is observed that, 8 h after the beginning of hydrolysis, a slow but constant increase of soluble protein is generated. The addition of Tween-80 and clinoptilolite produces an increase in free-protein concentration. However,

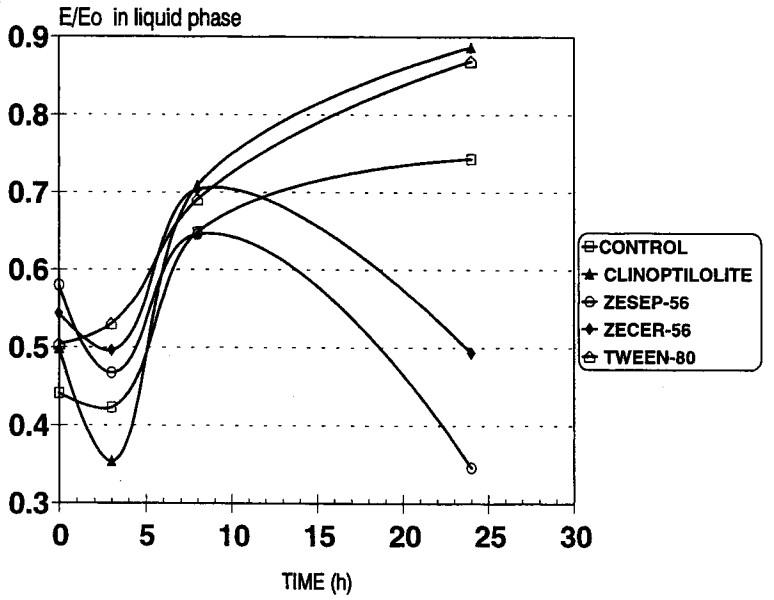

Fig. 2. Protein concentration in solution during the hydrolysis of 10% (w/v) steam-exploded poplar biomass supplementation with Tween-80 (4 g/L), clinoptilolite (5 g/L), ZESEP-56 (5 g/L), and ZECER-56 (5 g/L). Eo = 3.81 g/L.

with the presence of the zeolite-like products ZESEP-56 and ZECER-56, the adsorption profiles during enzymatic hydrolysis changed significantly and the amount of cellulose adsorbed increased with time.

Effect of Tween-80 and Zeolitic Products on Ethanol Fermentation

The effects of the supplementation of Tween-80, clinoptilolite, ZESEP-56, and ZECER-56 at different loadings on the ethanol yield of *Kluyveromyces marxianus* EMS-26 fermentations (growth conditions described in the subheading MATERIALS AND METHODS) are shown in Fig. 3.

No positive effects are observed with regard to the supplementation of Tween-80 on the production of ethanol at the assayed concentrations. The addition of zeolite-like products, ZESEP-56 and ZECER-56, likewise had no positive effect on the yield of ethanol in the fermentations. On the contrary, by supplementing the culture media with a natural zeolite such as clinoptilolite slightly increased the production of ethanol (between 5 and 7%) in the fermentation process.

For the purpose of evaluating the effect on the production of ethanol of the addition of zeolites to a natural medium containing the toxic products that are generated during the pretreatment of lignocellulose biomass, some supplementation tests (5 g/L) were carried out using as fermentation media those liquids obtained during the pretreatment of the poplar biomass by steam explosion, to which glucose was added in order to complete the initial 55 g/L. The results of these experiments are shown in Fig. 4.

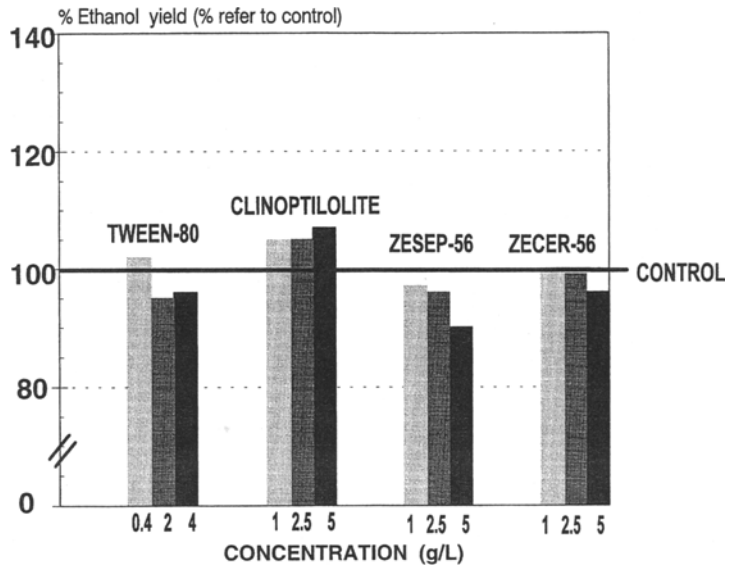

Fig. 3. Effect of varying levels of Tween-80 (0.4, 2, and 4 g/L), clinoptilolite (1, 2, and 5 g/L), ZESEP-56 (1, 2, and 5 g/L), and ZECER-56 (1, 2, and 5 g/L), on the ethanol yield of growth medium containing 55 g/L initial glucose. Ethanol yields refer to control without supplementation.

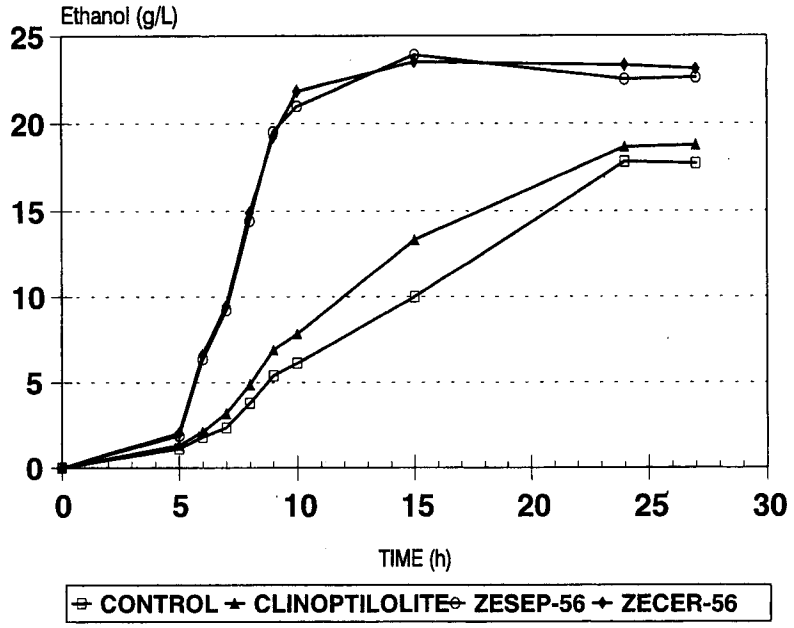

Fig. 4. Effect of 5 g/L supplementation of clinoptilolite, ZESEP-56, and ZECER-56 on the progress of batch fermentation of liquid medium from steam-explosion pretreatment of poplar biomass. Initial glucose: 55 g/L.

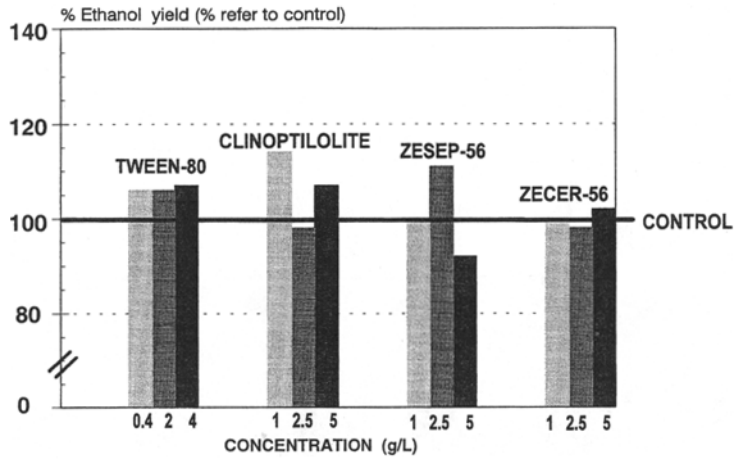

Fig. 5. Effect of varying levels of Tween-80 (0.4, 2, and 4 g/L), clinoptilolite (1, 2, and 5 g/L), ZESEP-56 (1, 2, and 5 g/L), and ZECER-56 (1, 2, and 5 g/L) on ethanol yield in the SSF process of 10% (w/v) steam-exploded biomass. Yields refer to control experiments without supplementation.

Supplementation with zeolite-like products resulted in markedly enhanced ethanol production and in a shortening of fermentation times in all the cases tested, as compared to those obtained in the control. The supplementing of the fermentation with a natural zeolite such as clinoptilolite did not produce improvements in ethanol production as spectacular as those obtained in the experiments in which ZESEP-56 and ZECER-56 were added.

In the control tests and in the tests supplemented with clinoptilolite, maximum concentrations of ethanol of approx 18 g/L are obtained after 24 h of fermentation. The presence of zeolite-like products increases the production rate of ethanol in a drastic manner. After 10 h, 23 g/L of ethanol had been produced and almost all of the substrate had been consumed.

Effect of Tween-80 and Zeolitic Products on the Simultaneous Saccharification and Fermentation Process of Steam-Exploded Poplar Biomass to Ethanol

The addition of Tween-80 increases the yield of the SSF process by approx 6% in all the concentrations assayed (Fig. 5). The yield results of ethanol obtained in the SSF process when zeolite-like products are added do not follow a defined tendency. The best results are obtained in the following conditions: clinoptilolite, 1 g/L (7% increase); ZESEP-56, 2.5 g/L (11% increase); and ZECER-56, 5 g/L (2% increase).

The different zeolite-like products, at these concentrations, along with 0.4 g/L of Tween-80 were assayed for the purpose of establishing the effect that the simultaneous addition of surfactants and zeolite-like products

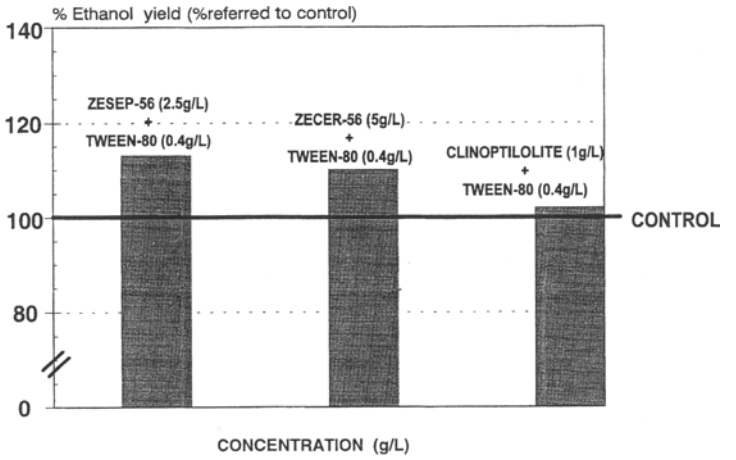

Fig. 6. Effect of different supplementations on ethanol yield in the SSF process of 10% (w/v) steam-exploded biomass. Yields refer to control experiments without supplementation.

would have on the SSF process. The results of these experiments are shown in Fig. 6. In the experiment using clinoptilolite and Tween-80, better results were not obtained than those yielded by the experiments in which each one of these products was added separately. The synthetic zeolitic products ZESEP-56 and ZECER-56, along with Tween-80, produce increases of 20 and 14% in the yields of ethanol in comparison with control. These increases are superior to those obtained by supplementing separately with each one of these products.

DISCUSSION

The simultaneous saccharification and fermentation of cellulose to ethanol is a heterogeneous reaction with a soluble catalyst and an insoluble substrate. The first step in this reaction is the adsorption of cellulolytic enzymes from the reaction medium on the surface of the cellulose substrate. Endoglucanases and exoglucanases adsorb tightly to the cellulose substrate and do not readily desorb until the substrate is degraded. Thus, large amounts of cellulases become bound to inactive sites and, consequently, this severely limits the extent of saccharification *(28)*. Because hydrolysis involves transport of enzymes and soluble sugars between the solid substrate and the reaction medium, modifications of interfacial energy may have some impact on this transfer. Surfactants alter the surface and interfacial properties of the reaction system, and therefore the addition of surfactants would result in more intimate contact between the substrate and enzyme.

The increase in hydrolysis yield obtained in tests carried out on pretreated poplar biomass with the addition of Tween-80 suggests that the

adsorption-desorption of enzymes on the substrate is influenced by the presence of this product. Surfactant also affects the disruption of cellulose structure, making the cellulose more accessible to the enzymes *(29)*.

Results obtained are in concordance with those obtained by Helle et al. *(29)*, in which the cellulose hydrolysis yield of steam-exploded poplar was increased by 67% in the presence of surfactants.

The course of enzyme adsorption during the first 24 h of hydrolysis was in agreement with Lee et al. (30). A rapid initial adsorption on steam-pretreated wheat straw was observed, peaking at 3 h. This was followed by an equally rapid desorption of enzymes up to 7–8 h.

The fact that in the experiments with Tween-80 greater hydrolysis was observed with less protein adsorbed (Figs. 1 and 2) indicates that the surfactants somehow protect the enzyme from nonproductive binding. Since free protein cannot be responsible for cellulose hydrolysis, an explanation postulated by Helle et al. *(29)* is that, without surfactant present, much of the enzyme is adsorbed on inactive sites and does not participate in the hydrolysis of cellulose.

In a previous work *(31)* we observed that lipid addition has a negative effect on the enzymatic hydrolysis using Solka-flock as substrate. These results are different from those obtained on lignocellulosic biomass in this work. Lee et al *(30)* studied the adsorption of cellulases on several different substrates. When physicochemically pretreated substrates are used, enzymes become rapidly adsorbed onto the substrate. This is because of good accessibility and is followed by a continuous decrease in adsorption. If the substrate is Solka Floc, the enzyme accessibility is low, and the initial enzyme adsorption is slow but increases with time as the continuing hydrolysis makes the substrate more accessible. The lipid addition on enzymatic hydrolysis with Solka Floc could result in a more limited adsorption of enzymes during the first hours of hydrolysis, decreasing hydrolysis yields. However, when preteated poplar biomass is used, the lipid addition improves enzymatic hydrolysis by decreasing nonproductive binding of enzymes onto substrate.

The addition of the zeolite-like products ZESEP-56 and ZECER-56 provokes changes in the pH of the media (Fig. 7) because of the properties of ion exchange that are characteristic of these materials. The increase in pH that is produced upon increasing the concentration of zeolites could be responsible for the increases in hydrolysis yield. The addition of clinoptilolite does not produce changes either in the pH of the media or increases in hydrolysis yield as compared with the control.

It is known that part of the polysaccharides and lignin are degraded during steam-explosion pretreatment. These compounds inhibit the action of cellulases and the yields of the fermentative process. The removal of inhibitors formed during pretreatment of poplar biomass by zeolites could also improve the enzymatic hydrolysis and fermentation processes.

The presence of ZESEP-56 and ZECER-56 also increased enzymatic hydrolysis yields but, contrary to surfactant addition, a decrease of free

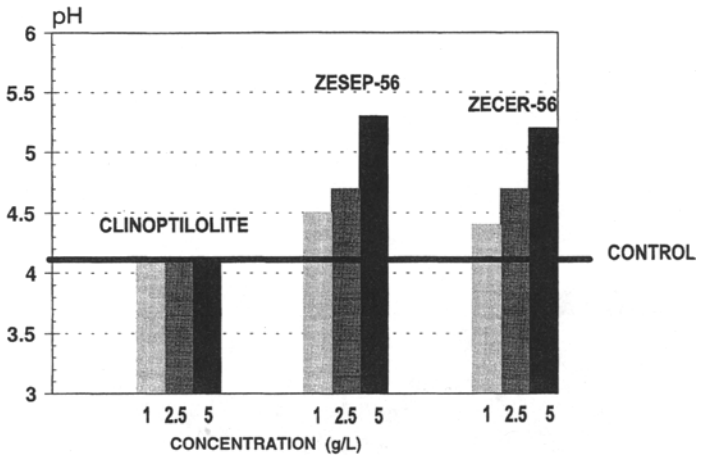

Fig. 7. Effect of varying levels of clinoptilolite (1, 2, and 5 g/L), ZESEP-56 (1, 2, and 5 g/L), and ZECER-56 (1, 2.5, and 5 g/L) on the pH of 10% steam-exploded poplar biomass. pH refers to control without supplementation.

protein in liquid phase was observed. The immobilization of glucosidase on zeolite could be the reason for the lower free-protein content.

The addition of zeolites to the fermentations (5 g/L) which were effected using the liquid fraction obtained in the pretreatment of the lignocellulose biomass favors the production of ethanol. The shortening of fermentation times (10 h vs the 24 h of the control set-up) and the increase in the production of ethanol (superior by 30%) indicate the great capacity of ZESEP-56 and ZECER-56 for adsorbing inhibitors produced during the biomass pretreatment.

The addition of Tween-80, clinoptilolite, and zeolite-like products to the SSF process does not have effects as beneficial as those observed separately in the process of enzymatic hydrolysis and fermentation. This could be because in the SSF process, the presence of yeasts along with the enzyme already favors an increase in the rate and in the yield of saccharification.

When the joint effects of Tween-80 and the zeolites on the SSF process are analyzed, increases of 20 and 14% are obtained with ZESEP-56 and ZECER-56 plus Tween-80, respectively.

CONCLUSIONS

The adsorption of cellulases on pretreated lignocellulose biomass is observed to be modified by the addition of Tween-80, increasing the rate of enzymatic hydrolysis.

The presence of the zeolite-like products ZESEP-56 and ZECER-56 in the fermentation media of the liquid fraction obtained after the pretreatment of poplar biomass by steam explosion significantly increases the rate

and yield of the alcoholic fermentation process. These effects could be caused by the elimination of inhibitors, which in turn is caused by the characteristics of ionic interchange of these products.

Surfactants and zeolite-like products would be useful additives to increase ethanol yields in the SSF process of pretreated wood biomass to ethanol, mainly if the liquid stream recovered from the steam explosion pretreatment is used.

ACKNOWLEDGMENTS

The authors wish to thank J. López Ruiz and his coworkers of the Grupo de Investigación Zeolitas-Acuicultura, Departamento de Construcciones Navales of the University of Cadiz (Spain) for supplying the zeolite samples.

REFERENCES

1. Hinman, N. D., Schell, D. J., Riley, C. J., Bergeron, P. W., and Walter P. J. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 639–649.
2. Nagodawithana, T. W. and Steinkraus, K. H. (1976), *Appl. Environ. Microbiol.* **31,** 158–162.
3. Navarro, J. M. and Durand G. (1978), *Ann. Microbiol.* **129B,** 215–224.
4. Brown. S. W. and Oliver, S. G. (1982), *Biotech. Lett.* **4,** 269–274.
5. Leao, C., and Van Uden, N. (1982), *Biotechnol. Bioeng.* **24,** 1581–1590.
6. Sa-Correia, I. and Van Uden, N. (1986), *Biotechnol. Bioeng.* **28,** 301–303.
7. Ingram, L. O. and Buttke, T. M. (1984), *Adv. Microb. Physiol.* **25,** 253–300.
8. Beaven, M. J., Charpentier, C., and Rose A. H. (1982), *J. Gen. Microb.* **128,** 1447–1455.
9. Thomas, A. S., Hossack, J. A., and Rose A. H. (1978), *Arch. Microbiol.* **117,** 239–245.
10. Ingram, L. O. (1986), *Trends Biotechnol.* **4,** 40–44.
11. Casey, G. P., Magnus, C. A., and Ingledew, W. M. (1984), *Appl. Environ. Microbiol.* **48,** 639–646.
12. Casey, G. P., Magnus, C. A., and Ingledew, W. M. (1983), *Biotechnol. Lett.* **5,** 429–434.
13. Panchal, C. J. and Stewart, G. G. (1981), *Dev. Ind. Microbiol.* **22,** 711–717.
14. Viegas, C. A., Sa-Correia, I., and Novais J. M. (1985), *Biotechnol. Lett.* **7,** 515–520.
15. Viegas, C. A., Sa-Correia, I., and Novais J. M. (1985), *Appl. Environ. Microbiol.* **50,** 1333–1335.
16. Ando, S., Arai, I., Kiyoto, K., and Hanai, S. (1986), *J. Fermentation Technol.* **64(6),** 567–578.
17. Bernal, M. P. and Lopez-Real, J. M. (1993), *Bioresource Technol.* **43,** 27–33.
18. SivaRaman, H., Chandwadkar, A., Baliga, S. A., and Prabhune, A. A. (1994), *Enzyme Microb. Technol.* **16,** 719722.
19. Tarasevich, I. I. (1988), *Sov. J. Water Chem. Tech.* **10,** 22–32.
20. Liverti, L., Boari, G., Petruzzelli, D., and Passino, R. (1981), *Water Res.* **15,** 337–342.
21. Carrasco, J. E., Martínez, J. M., Negro, M. J., Manero, J. Mazon, P., Saez, F. Cabañas A. and Martín, C. (1989), in *Biomass for Energy and Industry. Fifth E.C. Conference,* Elsevier, New York pp. 38–44.
22. Ferreiro M. S., Ramos Lopez, S. R., and Lopez J. (1994), *Afinidad,* 454–461.
23. Ballesteros I., Oliva, J. M., Ballesteros M., and Carrasco J. (1993), *Appl. Biochem. Biotech.* **39/40,** 201–211.

24. Puls, J., Poutanen, K., Körner, H. U., and Viikari, L. (1985), *Appl. Microbiol. Biotechnol.* **22,** 416–423.
25. Mandels, M., Andreotti, R. and Roche, C. (1976), *Biotech. Bioeng.* **6,** 21–23.
26. Bayle, M. J. and Nevalainem, K. H. H. (1981), *Enzyme Microbiol. Technol.* **3,** 153–158.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **23,** 139.
28. Howell, J. A. and Mangat, M. (1978), *Biotech. Bioeng.* **26,** 936–941.
29. Helle, S. S., Duff, S. J. B., and Cooper, D. G. (1993), *Biotech. Bioeng.* **42,** 611–617.
30. Lee, S. B., Shin, H. S., Ryu, D. D. Y., and Mandels, M. (1982), *Biotech. Bioeng.* **24,** 21–37.
31. Ballesteros, I., Oliva, J. M., Carrasco, J. E., and Ballesteros, M. (1994), *Appl. Biochem. Biotech.* **45/46,** 283–294.