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The Conversion of Lignocellulosics to Ethanol and/or Microbial Biomass Product: The Intermediate Acid Approach

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FINAL GRANT REPORT

INNOVATIVE SCIENTIFIC RESEARCH

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The Conversion of Lignocellulosics to Ethanol and/or Microbial Biomass Product: The Intermediate Acid Approach

Presentado por el ICAITI a

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INTRODUCTION

This final report describes the research carried out on the conversion of lignocellulosic biomass to organic acids by microorganisms, and the subsequent conversion of the intermediate acids into ethanol or microbial biomass. The research was carried out in Guatemala by the Applied Research Division of the Central American Research Institute for Industry (ICAITI) under a grant from the Office of Science, US-AID, contract No. 932-5542-G-00-2041-00 from the Program in Science and Technology Cooperation (PSTC). As instructed by the Office of Science, a major emphasis was placed on the physical and chemical pretreatment of the lignocellulose to foster microbial degradation. Three lignocellulosic substrates were chosen: EX-FERMented sugarcane, lemon grass bagasse and citronella bagasse.

The Final Report has been organized so that the reader may obtain an overview of the results obtained in the research, recommendations which the investigators feel are pertinent to the results obtained, a general description of the activities carried out, a financial statement, and detailed descriptions of the research efforts in the form of scientific papers already published, accepted for publication, or submitted to leading scientific journals in the field for publication. Such publications undergo peer review prior to publication and assure divulgation of the research on an international level with ample credit being given to the generous support by AID's Program in Science and Technology Cooperation.

One final comment should be made addressing the interest which this research has provoked internationally, resulting in requests for additional

publications and oral presentations in the Americas and Europe. Locally in Guatemala, the sugar industry has shown interest in immediate application of the research to animal feeding. Also, the project provided an opportunity for training young scientists in the technical aspects of pretreatments, chemical analysis and the particularly difficult techniques for handling anaerobic microorganismis. The team of ICAITI scientists, a total of seven, was joined by eight young scientists who were finishing their university training or had recently done so, and a group of experienced technicians from ICAITI's staff.

Again special acknowledgement should be given to the Office of Science for the generous support of the project.

CONCLUSIONS

1. Pretreatment of EX-FERMented sugarcane[a]

1.1 Pretreatment of EX-FERMented sugarcane improves enzymatic digestibility.

1.2 The convertional full soda cook gave the highest value of digestibility (IVDMED = 98%), 5.1 times greater than the untreated control bagasse (IVDMED = 19%).

1.3 Of the innovative pretreatments on EX-FERMented sugarcane, highest digestibility was achieved by the alkaline organosolv pretreatment (IVDMED = 90%) followed by solid state sodium hydroxide (72%), phenol extraction (69%), steam explosion (57%), solid state calcium hydroxide-sodium carbonate (33%), gaseous ammonia (30%), and gaseous sulfur dioxide (26%).

1.4 When the sugarcane samples were fermented with the anaerobic bacteria, the co-cultures of mesophilic and of thermophilic microorganisms degraded the full soda cook sample to the greatest extent, giving 80% weight loss.

1.5 The inocula from the natural sources gave highest digestion values on the solid state calcium hydroxide-sodium carbonate treated sample, giving 55-60% weight loss.

1.6 Acid production by the mesophilic co-culture on the sugarcane substrates reached high levels when the alkaline organosolv, gaseous sulfur dioxide, solid state sodium hydroxide, and untreated samples were fermented; highest yields of acid were 0.7 g/g substrate.

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a. EX-FERMented sugarcane is the lignocellulosic residue leftover after the bioconversion of sucrose present in cane into ethanol by yeasts employing the EX-FERM (simultaneous extraction and fermentation) process.

1.7 Acid accumulation by the natural mixed inocula was lower, due to conversion to methane.

2. Pretreatment of citronella and lemon grass bagasse

2.1 Pretreatment of lemon grass bagasse improved enzymatic digestibility.

2.2 Pretreatment of citronella bagasse gave similar, although slightly lower, results.

2.3 Highest digestibility value was observed in the alkaline organosolv lemon grass, (IVDMED = 74%), 5.3 times greater than the untreated bagasse.

2.4 The alkaline organosolv treated citronella gave a digestibility of 70%, 3.9 times greater than the untreated bagasse.

2.5 The alkaline organosolv pretreatment was followed by (in order of decreasing digestibilities): solid state sodium hydroxide, gaseous sulfur dioxide, steam explosion, gaseous ammonia, and solid state calcium hydroxide-sodium carbonate. The same order was observed in both bagasses.

2.6 When the lemon grass bagasse samples were fermented by anaerobic bacteria, the mixed natural inocula digested the samples to greatest extent, with highest digestion values observed in the solid state calcium hydroxide-sodium carbonate treated sample (up to 68% weight

loss) followed by gaseous sulfur dioxide treated samples (up to 58% weight loss).

2.7 When the citronella bagasse samples were fermented by anaerobic bacteria, the same tendencies held out: highest digestion in the solid state calcium hydroxide-sodium carbonate treated sample (up to 73% weight loss), followed by the sulfur dioxide treated sample (up to 58% weight loss).

2.8 Slightly lower digestion values were observed with the co-cultures in both substrates.

2.9 Acid production on lemon grass was highest by the mesophilic co-culture, giving an average of 0.7 g acid equivalents/g substrate.

2.10 The highest individual value of 1.0 g/g was observed when the mesophilic co-culture fermented the untreated lemon grass bagasse.

2.11 Similar but lower results were observed with citronella bagasse, with a maximum of 0.7 g/g produced by the mesophilic co-culture on untreated citronella bagasse.

2.12 Again, acid accumulation by the natural mixed inocula was lower due to methane production.

2.13 No inhibition was observed due to residual essential oils which might have been present.

3. **Biological delignification**

3.1 Of the twelve white-rot fungi tested only one, **Coriolus versicolor** impr - digestibility of EX-FERMented sugarcane.

3.2 Lignin and hemicellulose were degraded to a greater degree than cellulose.

3.3 In citronella and lemon grass bagasse, nine out of the twelve same white-rot fungi tested produced a residue with a higher digestibility than the original bagasses.

3.4 The best, **Bondarzewia berkeleyi**, increased the digestiblity (IVDMED) of lemon grass to 22% and of citronella to 24%.

3.5 Again hemicellulose and lignin were preferentially degraded.

4. **Conversion of organic acids to ethanol and/or biomass**

4.1 The yeasts, **Saccharomyces cerevisiae** and **Candida utilis** were not capable of converting the organic acids into ethanol. This may be due to biological deficiencies or thermodynamic considerations.

4.2 Both yeasts produced biomass from the organic acids, which included: acetic acid, propionic acid, lactic acid, and butyric acid.

4.3 Aerobic growth was more efficient in terms of biomass production and substrate consumption.

4.4 Butyric acid alone was not used by **S. cerevisiae** under aerobic conditions. It was used by **Candida utilis.**

4.5 Under anaerobic conditions, butyric acid appears to be inhibitory.

5. Process considerations

5.1 Ultrasound field enhances enzymatic saccharification of lignocellulose.

5.2 Lactic acid is an important acid product in the fermentation of lignocellulosics by anaerobic microorganisms. When this acid is considered, total acid production is 1 g/g or better when the mesophilic co-culture is used on any of the substrates. The same is not observed with the other inocula sources.

5.3 The use of monensin increased acid production by the mixed natural flora; however, methanogenesis was not inhibited.

5.4 Agitation does not improve digestion rate nor acidogenesis rate.

5.5 Gas atmosphere, N_2 or CO_2, does not affect digestion or acidogenesis, as long as anaerobic conditions are maintained.

5.6 With the co-cultures, little or no methane production is observed.

SUMMARY OF THE RESEARCH ACTIVITIES AND FINDINGS

GENERAL DESCRIPTION OF THE PROJECT ACTIVITIES

1. Characterization of lignocellulosic substrates

Citronella bagasse and lemon grass bagasse are byproducts from the essential oil industry, being the fibrous residue remaining after the oil is removed by steam stripping. Recently steamed bagasse was collected in the Pacific piedmont area of Guatemala, sun-dried in Guatemala City, and evaluated with respect to composition, in vitro digestibility, and saccharification by enzymes. Untreated bagasses of both grasses were very similar with 11.0 - 11.1% lignin, 29.9 - 28.5% cellulose, 28.5 - 30% hemicellulose, and 11.0 - 9.3% ash for lemon grass and citronella bagasse, respectively. In vitro enzymatic digestibilities were 13.8 and 18.2%.

Sugarcane was obtained during two different harvest seasons from three sugar mills. The cane was chopped in the Institute, EX-FERMented with commercial yeast to remove sugar and to produce ethanol; the remaining fiber was washed, pressed and dried. The samples were analyzed with respect to composition, in vitro digestibility and saccharification by enzymes.

Commercial sugarcane bagasse samples were obtained from the mills in the Guatemalan Pacific piedmont, and analyzed as above to serve as controls for the EX-FERMented samples. Sugarcane bagasse average values were as follows: lignin, 13.9%; cellulose, 43.9%; hemicellulose, 37.5%; ash 2.1%; and in vitro enzymatic digestibility, 16.91%. Little difference was observed among bagasse samples from different points in the milling process, and sugarcane whose sugar had been extracted through fermentation in ICAITI using the EX-FERM process.

2. Pretreatment methods, physical and chemical

In order to enhance the degradation of the lignocellulosic substrates, they were pretreated using conventional and experimental chemical and physical methods. The pretreatments should make the cellulose more available to enzyme attachment and attack. This is achieved by physically opening up the fibrous structure or by removal of components which restrict the intimate contact or inhibit enzyme activity.

The methods used in this project are:

Alkaline pretreatments:	solid state sodium hydroxide
	solid state mixture of sodium carbonate and calcium hydroxide
	gaseous ammonia
Acid pretreatment:	gaseous sulphur dioxide
Steam explosion	
Organosolv	ethanol and water with sodium hydroxide
	ethanol and water with sodium hydroxide and anthraquinone
	aqueous phenol

Conventional soda pulp with 15% sodium hydroxide.

All samples were again analyzed with respect to composition, **in vitro** digestibility and saccharification by enzymes. Details for the pretreatment methods, the yields obtained, the evaluation of the effectiviness, and results of the chemical analysis as well are found in papers 1, 2 and 5.

3. Biological pretreatment of the lignocellulosic substrates

Delignification was sought through biological means employing white-rot fungi. This group of fungi is active in the natural decomposition of lignocellulosic matter, degrading holocellulose and lignin to lower molecular weight compounds. The fungi were obtained from international culture collections. Myceliar growth was inoculated on sterile moist substrate. Forced aeration through the substrate each week fostered fungal growth. The samples were dried and analyzed after four to six weeks, when considerable fungal biomass had penetrated the substrate. At this time weight loss was determined as well as composition of the resulting mass. Details of the results as well as experimental technique are found in papers 3 and 4. The fungal strains tested were:

Phanerochaete crysosporium
Ischnoderma resinosum
Bondarzewia berkeleyi
Coriolus versicolor
Agrocybe aegerita
Dichomitus squalens
Flammulina velutipes
Ganoderma applanatum
Pycnoporus sanguineus
Coprinus fimetarius
Pleurotus flabellatus

4. Fermentation of the lignocellulosic substrates with mixed natural flora

Semi-continuous anaerobic digestors were initially innoculated with flora from rumen of beef cattle from a local slaughter house, swamp muds from an area rich in decaying reeds, thermophillic muds from a volcanic-active area in Guatemala, and the acidogenic muds from the first phase of a two-phase biogas unit developed for the production of methane from coffee pulp. Each unit was fed weekly with ground sugarcane bagasse and rumen extract.

For the fermentation of the lignocellulosic substrates, weighed portions of the substrates were fermented anaerobically with filtrates from the digestors described with an appropriate buffer as the only additive. Samples were taken at 5, 15, and 30 days, with pH, gas volumen and composition, substrate weight loss, volatile acid production and composition determined, and the respective yields calculated. Details of the research as well as the results can be found in papers 5 and 6.

5. Fermentation of the lignocellulosic substrates with the mixture of pure cultures

Mesophillic anaerobes were used as a mixed culture for the degradation of the lignocellulosic substrates. The strains, obtained from international culture collections, were: Ruminococcus albus (two strains), Bacteroides ruminicola subs. brevis, and Bacteroides ruminicola subs. ruminicola. A second mixed culture of anaerobic thermophiles was also used, this mixture being composed of Clostridium thermoaceticum var Ljungdahl, Clostridium thermoaceticum var. Wood, Clostridium thermocellum and Clostridum thermo-saccharolyticum. A standardized inoculum of each microorganism was prepared in the medium recommended for its growth, the cultures were mixed and added to a weighed portion of the lignocellulosic substrate, together with sterile basal medium. After 5, 15, and 30 days of incubation, the samples were tested for volatile acid production, gas production and composition, pH, and weight loss or degradation of substrate. The results for these trials, as well as details for the procedures used are found in papers 5 and 6.

6. Effect of monensin on acidogenesis

The acidogenesis of lignocellulose by bacteria is composed of many steps, the first being degradation of the polymers to simpler compounds such as sugars. These are then converted to organic acids, and, in nature, the degradation is complete to carbon dioxide and methane according to the environment and flora present. In order to improve the yield of the organic acids in this project, the following approaches were tried: improved pH control using standardized buffer system with initial pH of near 8.0, and addition of monensin, and antibiotic studied in ruminant nutrition for improved feed utilization by blockage of methane production in the animal. The following eight substrates were included in this study: full soda cook sugarcane bagasse, solid state alkaline trated sugarcane bagasse with calcium hydroxide and sodium carbonate, ammonia treated citronella, steam exploded citronella, citronella treated in the solid state with a mixture of calcium hydroxide and sodium carbonate, lemon grass treated in the solid state with calcium hydroxide and sodium carbonate, lemon grass treated with sulphur dioxide, and organosolv plus anthraquinone treated lemon grass. The microorganisms used were the mesophilic mixture of pure cultures, the thermophilic mixture of pure cultures, mixed flora from the reactor containing swamp mud, and mixed flora from the reactor containing thermal muds.

Substrate did not affect degree of digestion or acid production. Volatile fatty acid and lactic acid production were higher when the mesophilic co-culture was used. Monensin increased acid production only in the case of the swamp mud inoculum, but this increase was not correlated with inhibition of methanogenesis.

7. Kinetics of acidogenesis

The rate of acid production was studied in order to establish the parameters which would indicate the direction to focus future optimization and scale up activities. Two sources of microorganisms were used, the mesophilic swamp mud digestor, and the moesphilic mixture of pure cultures. Two substrates were included, the full soda cook sugarcane bagasse and the solid state calcium hydroxide-sodium carbonate treated citronella bagasse. Two different gas atmospheres were used, carbon dioxide and nitrogen, and the experimental vials were either shaken or left stationary for 2, 4, 7, 9, 11, 14, and 16 days. At that time they were analyzed for volatile fatty acids, weight loss, pH, gas production and composition.

Analysis of the results showed that with the soda cooked sugarcane bagasse, an average of 79.5% digestion was achieved with mixed flora from swamp mud. Agitation had little effect on the results, as did the gas atmosphere. The swamp muds produced a greater degree of digestion than the mesophilic co-culture, and tended to produce higher levels of methane earlier than the pure cultures. The treated sugarcane bagasse was digested more completely than the citronella bagasse. Both substrate and inoculum had a significant effect on digestion. Acid production was not significantly affected by any of the parameters studied.

Results of these trials as well as details of experimental procedures employed are found in paper 8.

8. Production of biomass and or alcohol from volatile acids by yeasts

The ability of Saccharomyces cerevisiae and Candida utilis to convert organic acids to biomass or ethanol was studied using continuous fermentation techniques. Acetic, butyric, propionic and lactic acids alone or in mixture were used as carbon sources. Anaerobic conditions were used to foster ethanol production, and aeration was initiated to foster biomass generation. Change in pH, acid utilization, biomass production, viable cell counts, and ethanol production were measured. The results for these trials are found in paper 9.

9. Ultrasound effect on enzymatic saccharification

An intimate relationship between active enzyme and reactive holocellulose is essential to enzymatic saccharification. For improved reaction rates, the adsorption-desorption mechanism must be functioning effectively. Otherwise the enzyme remains tightly bound to unreactive sites, thus losing its biocatalytic effectiveness. The use of an ultrasound field to facilitate the adsorption-desorption mechanism was evaluated with sugarcane bagasse at the laboratory level.

Details are found in paper 11.

10. Mechanism of holocellulolysis

The research carried out in this project was accompanied by extensive literature reviews. An interesting area of research at the present moment is the adsorption-desorption mechanisms of the biocatalyst with respect to the holocellulose substrate surface. C. Rolz prepared a brief but timely review of the various current concepts involved in the regulation of the holocellulolysis.

This interesting review has been included as paper 10.

RECOMMENDATIONS

The results from the work carried out under the present grant have led the researchers to make the following recommendations for immediate application of the research, for further technological evaluation, and for future scientific research.

1. **Pretreated bagasse as animal feed**

The excellent improvement in digestibility of the lignocellulose leads to the first and most immediate application: use of the pretreated bagasse as animal feed. Great interest has been demonstrated by the sugar industry in Central America who is looking for alternate sources on income to supplement the depressed world sugar market. In this area, feedlots for fattening beef cattle are being developed adjacent to the sugar mills. The mills are very interested in the pretreatment of the cane to provide a material which would be readily digestible by the animals. Alkaline treatments or steam explosion pretreatments appear to be highly appropriate for rumen nutrition. The results from the EX-FERMented sugarcane bagasse can be applied to sugarcane bagasse in general. At present, with the modernization of the sugar mills and strict internal control with energy conservation, there is a surplus of bagasse which is usually burned. For this application, forage acceptability and weight trials would be of interest and could be carried out in the conjunction with the Department of Animal Husbandry at the University of San Carlos, Guatemala, if funding were made available.

2. **Use of white-rot fungi as animal feed**

Again in the area of animal feeding with immediate application is the use of white-rot fungi for biological delignification. The results from this

research show that white-rot fungal growth improved digestibility of lemon grass and citronella bagasse. The material remaining after fungal growth has been enriched due to the presence of the fungal mycelia and removal of lignin. At present, use of this approach on wheat straw is being studied in ICAITI financed by the United Nations University.

3. Rural technology: edible mushrooms and improved animal feed

The development of this system applied to a rural technology is extremely attractive. A system can be envisioned whereby the fungal fruiting bodies are consumed as edible mushrooms. The by-product, the lignocellulose-mycelial mat can also be commercialized as animal feed. Here biological processes can be united with commercial benefit at the rural level. The Pleurotus mushrooms among others, would be particularly suited to this process. The Pleurotus enjoy such a great demand that immediate commercialization should be feasible. It should be pointed out that the excellent growth on the citronella and lemon grass bagasse was unexpected, showing no inhibition by the essential oils. For commercial application yield of fruiting bodies on each substrate, as well as optimization of aeration and humidity, would be of interest.

4. Use of organosolv in industrial cellulose processes

The pretreatments studied have indicated that the holocellulose-lignin structure has been modified considerably. The organosolv pretreatment, in particular, appears to be an attractive alternative for preparing cellulose derivatives for other uses besides animal feeding and fermentation. At present, the pulp and paper industry employs sulfur for the purification of

cellulose in a process which leads to considerable contamination of the environment. Use of the organosolv process would provide quality cellulose with the possibility of recovery of the solvent and lignin, tremendously reducing the polution load in terms of both volumen and concentration.

5. Feasibility studies

The information provided in the specific reports is presented with sufficient detail so that interested researchers can carry out the preliminary economic feasibility studies according to each need.

6. Specific recommendations for further scientific research

The research carried out under this grant has indicated new areas where further research should be particularly fruitful. Among them, the following can be mentioned:

6.1 Use of ultrasound field

Ultrasound appears to be very promising for enhancing biocatalysis of substrates.

6.2 Toxic effect of accumulated acids

Acidogenesis appears to be limited in part by an accumulation of acid or protons in the medium. A system could be developed which would permit gradual removal of the acids while leaving the microorganisms. Alternatives such as semipermeable membranes, porous ceramic barrier, etc. could be evaluated to separate the insoluble lignocellulose and microorganisms from the soluble products, sugars and acids.

6.3 Mechanism of holocellulolysis under anaerobic conditions

How do the microorganisms separate the hemicellulose from the lignin? Lignin and hemicellulose are present in intimate contact with one another. Does the lignin remain untouched? How do the microorganisms get at the hemicellulose?

6.4 Anaerobic interaction

In the natural mixed flora digestors, a complex mixture of microorganisms was observed, including protozoa, bacteria and yeast-like fungi. The interaction of these microorganisms, with a study of the contribution of each to holocellulolysis would provide important basic information which could be used for control of the entire process.

6.5 Microbiology of volcanic environments

The microorganisms present in the volcanic ponds represent an untapped resource for germplasm and biocatalysts. Characterization of the microorganisms from this thermophilic source, and their interactions, should be a most productive area of research.

6.6 Osmotic effect of buffer system on acidogenesis

The use of buffer systems to cushion the toxic effects of the generation of acids can contribute to an environment whose osmotic pressure adversely affects the microbial metabolism. In this study, the ionic load of the buffer system system was aumented in same cases by the salts used for solid state and gaseous pretreatment. What effect does this have on total acid production and the profile of individual acids produced?

6.7 Production of lactic acid

The unexpectedly high production of lactic acid under the conditions used in this study merits further investigation. Why was lactic acid produced and not the volatile fatty acids as expected? What controls the acid profile and how can it be manipulated?

SPECIFIC RESEARCH REPORTS

1. Effects of some physical and chemical pretreatments on the composition, enzymatic hydrolysis and digestibility of lignocellulosic sugarcane residue.

   C Rolz, MC de Arriola, J Valladares and S de Cabrera

   Submitted for publication in Process Biochemistry

2. Effects of some physical and chemical pretreatments on the composition and enzymatic hydrolysis and digestibility of lemon grass and citronella bagasse.

   C Rolz, MC de Arriola, J Valladares and S de Cabrera

   Accepted for publication in Agricultural Wastes

3. White-rot fungal growth on EX-FERMented sugarcane

   C Rolz, R de León, MC de Arriola and S de Cabrera

   Accepted for publication by Applied Microbiology and Biotechnology

4. Biodelignification of lemon grass and citronella bagasse employing white-rot fungi

   C Rolz, R de León, MC de Arriola and S de Cabrera

   Accepted for publication by Applied and Environmental Microbiology

5. Biodegradation of pretreated EX-FERMented sugarcane chips by fungal enzymes and mixtures of anaerobic bacteria

   C Rolz, S de Cabrera, MJ Valdez, MC de Arriola and J Valladares

   Accepted for publication in Biotechnology Progress

6. Biodegradation of pretreated citronella and lemon grass by mixtures of anaerobic bacteria

S de Cabrera, C Rolz, MJ Valdez, MC de Arriola and J Valladares

Submitted for publication in Canadian Journal of Microbiology

7. Effect of monensin on acidogenesis of lignocellulosic biomass by mixed anaerobic cultures

S de Cabrera, C Rolz, MJ Valdez, SM Saravia, F de Micheo and J Valladares

Submitted for publication in Enzyme and Microbiol Technology

8. Kinetics of lignocellulosic degradation and acidogenesis by mesophilic anaerobic bacteria on alkali treated sugarcane bagasse and lemon grass bagasse

S de Cabrera, C Rolz, SM Saravia, MJ Valdez and F de Micheo

Submitted for publication in Journal of Chemical Technology and Biotechnology

9. Conversion of organic acids to biomass by yeasts

S Ortiz-Jaramillo, S de Cabrera, F de Micheo and C Rolz

Submitted for publication in Applied Environmental Microbiology

10. Regulation of adsorption-desorption mechanism during holocellulosis of biomass

C Rolz

Trends in Biotechnology 4 (6) 135-136 (1986)

11. Ultrasound effect on enzymatic saccharification

C Rolz

Biotechnology Letters 8 (2) 131-136 (1986)

PAPER No. 1

"Effects of some physical and chemical pretreatments on the composition, enzymatic hydrolysis and digestibility of lignocellulosic sugarcane residue"

(Submitted for publication in Process Biochemistry)

EFFECTS OF SOME PHYSICAL AND CHEMICAL PRETREATMENTS ON THE COMPOSITION, ENZYMATIC HYDROLYSIS AND DIGESTIBILITY OF LIGNOCELLULOSIC SUGARCANE RESIDUE

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SUMMARY

The following pretreatments were done on sugarcane chips previously fermented with yeast according to the EX-FERM process: sodium hydroxide, sodium carbonate plus calcium hydroxide gaseous ammonia, SO2, steam explosion, alkaline organosolv, aqueous-phenol and a full soda cook. Greater than 87% delignification was obtained in one of the organosolv samples and the aqueous-phenol pretreatment. Practically all hemicelluloses were hydrolyzed during aqueous-phenol and steam explosion pretreatments. In vitro dry matter enzymatic digestibilities were increased 4.7 and 3.6 fold for one of the organosolv and aqueous-phenol samples. The rate and extent of saccharification was higher for the steam-exploded and the non-washed organosolv samples. The IVDMED values for the alkaline pretreated samples were correlated with the amount of soluble solids produced. Of these the solid state sodium hydroxide was superior to the sodium carbonate plus calcium hydroxide and ammonia pretreatments. SO2 was inferior to NaOH, steam-explosion or the organosolv variants.

INTRODUCTION

The fibrous residue remaining after juice extraction from the sugarcane stalk is commonly referred as bagasse. It consists mainly of the lignocellulosic polymer matrix and small amounts of ash and water soluble solids which are mostly sucrose. In the sugar industry mills most of the bagasse is used as fuel, and although the figure varies within each factory, energy efficient units can have 2t of surplus bagasse on a dry basis for 100t of fresh cane processed (1). In independent fuel-ethanol distilleries part of this surplus is used for ethanol purification making the overall system net energy-positive, either for mechanized or for labor intensive agriculture (2-5). Nevertheless, even here, a surplus is possible if a new demand for bagasse is developed, either by applying energy saving options in the ethanol separation or new technologies are employed for processing cane chips directly into ethanol like the EX-FERM process (6-8). EX-FERM process developed at ICAITI features the simultaneous extraction and fermentation of the sugars in chipped sugarcane to ethanol by Saccharomyces cerevisiae, with two end products, the ethanol-rich beer and the spent bagasse or fibrous residue. Specially important for bagasse use would be the hydrolysis of the holocellulose for

monomer production or the separation of lignin or lignin-derivatives. Henceforth the research efforts to make bagasse more susceptible for biological processing are worth pursuing. It must be remembered that sugarcane has been improved by plant breeding techniques for other purposes, for example, higher sucrose content, resistance to pests, drought and adverse soil conditions without a major concern for fiber content and polymer characteristics. This causes then a large variation in bagasse composition depending on cane variety and ecological conditions, a fact that many workers have not taken into account and which is undoubtedly the source of much of the variations in the technical data so far reported in the literature. Henceforth we should consider sugarcane bagasse as a heterogeneous raw material.

Lignocellulosic biomass in general is relatively refractory to bioconversion and sugarcane bagasse is no exception. In natural soil ecosystems its biodegradation rate and extent of reaction are low (9-12). In ruminant nutrition, bagasse has been found to be a low quality roughage, with its feeding value fitting in between wood and the cereal straws (13); it will only support cattle maintenance when fed as whole-mature-chopped sugarcane (14, 15). Polysaccharide degradation in the rumen takes place as the result of the action of a consortium of anaerobic bacteria, protozoa and phycomycete fungi and even so its bioconversion is far from complete (16, 17).

Hence in order to increase the rate and extent of holocellulose hydrolysis a pretreatment of the substrate is required in order to alter significantly the structural characteristics of the lignocellulosic matrix. Such a pretreatment must enhance the close contact between microbe and fibers to have an efficient enzyme action. There seems to be little doubt that the various pretreatments tested so far, alone or in combination (in a recent review there was a list of 10 physical, 18 chemical and one biological treatments) (18) enhance enzyme or microbically catalyzed holocellulose breakdown (19-28). There seems to be, however, only a limited understanding of how these pretreatments enhance biodegradation. The mode of holocellulolysis may be quite species-specific, both in terms of the substrate and the microorganism. It is also an heterogeneous reaction and it is therefore influenced by the structural features of the substrate, the interaction between microbe and substrate surface and the complexity of the associated enzymatic system. Most of the research work done so far has employed enzymatic preparations of filamentous fungi grown in submerged culture and temperate zone cereal straws as substrates. It seems that the rate and extent of hydrolysis depends on the amount of substrate surface exposed, the proper adsorption of the biocatalyst,the degree of polymerization of the polysaccharide and a minimum lignin, phenolic acid and acetyl contents (29-47).

The objective of the present work was to evaluate the effect of some physical and chemical pretreatments on the composition, susceptibility to enzymatic hydrolysis and in vitro enzymatic digestibilities of EX-FERMented sugarcane residue and discuss the

results obtained in comparison to data for sugarcane bagasse published in the literature.

MATERIALS AND METHODS

. Lignocellulosic substrate

Sugarcane was sampled in three sugar mills in the south coast of Guatemala located around 300 meters above sea level, during the month of April which is the latter part of the crop during the dry season. The sugarcane sample came from the receiving yard and usually one or more cane varieties were being processed on the same day. The cane had been cut by hand and was not burned in the field. Samples were also taken of cane which had been chopped in the sugar mill before sugar extraction and of the bagasse after sugar extraction by a tandem of roller mills. The bagasse samples were solar dried to a final moisture around 6-7%, milled in a pilot Fitz Mill Comminutor (The Fitzpatrick Co.) employing a 0.84 mm sieve and packed in plastic bags. The whole and chopped sugarcane samples were chipped in a pilot wood chipper (Type 9-72, OY Santsalo). The chips were placed in 7000 l tanks, water was added to just cover them and 2% of commercial baker's yeast was mixed. The EX-FERM cycle to convert all the sucrose into ethanol took 48h (6-8). The fermented chips were pressed in a cointinuous screwpress (Model VP-6F, Vincent Corp.), washed with water in a mechanically agitated tank and screw pressed again. They were finally sun dried to a final moisture of around 6-7% and packed in plastic ventilated bags. The CAST-1 sample, spent EX-FERMented sugarcane chips prepared as described here, was used for pretreatments. The control-unpretreated samples were milled as indicated above. A summary of the various samples taken, their identification and description are given in Table I.

- Substrate pretreatments

All of the treated samples came from CAST-1. The following pretreatments were done: a) solid state sodium hydroxide: the amount of sodium hydroxide added to the ground material was 80g kg-1 of dry matter. It was added as a concentrated solution in a 1:4 solid to liquid ratio and the amount of raw material used in all cases was 250g dry matter. The mixture was throughly mixed in a ribbon blender and placed in a polyethylene bag for 10 days at room temperature. Then it was solar dried and stored again in the same bag. The sample was identified as CANA; b) solid state mixture of sodium carbonate and calcium hydroxide: the amount of both chemicals added was 146g per kg of dry matter of which 73% was sodium carbonate. Due to chemical reaction the effective charge determined by an ash mass balance was 80g per kg. The same procedure as in CANA was followed. The sample was identified as CACANA; c) gaseous ammonia: 250g bagasse was moistened with water using a solid to liquid ratio of 1:3; placed in a Buchner funnel and a stream of gaseous NH_3 was passed until saturation. Then the bagasse was transferred to a polyethylene bag and maintained for 10 days at room temperature. Then it was solar dried and stored in

the same bag. The amount of chemical used determined by weight difference of the substrate after treatment was 290.5g per kg of dry matter. However most was lost during storage as the final ratio was 21.7g NH_3 per kg dry matter. The sample was identified as CAAM. The samples following the alkaline pretreatments were milled as indicated before.

One gaseous pretreatment was done employing SO2. 250g of dry material was moistened with water using a solid to liquid ratio of 1:3, placed in a Buchner funnel and a stream of gaseous SO2 was passed through the sample for 15-20 min until saturation. Then the material was placed in a sealed jar and maintained at 70C for 72h, after which it was solar dried and transferred to a polyethylene bag. The amount of chemical used determined by weight difference of the substrate after treatment was 185.7g per kg of dry matter. Again, most of it was lost during storage as the final ratio was 36.4g SO_2 per kg of dry matter. The sample was identified as CASO. After treatment it was milled as indicated before.

One 200g sample was steam exploded by Dr. J.N. Saddler of the Eastern Laboratory of Forintek Canada Corp, Ottawa. Their procedure consisted of an exposure to steam at 240C for 60s in a small steam explosion gun (48). The yield on dry basis was 77.87% and the steam explosion was done on the previously milled sample. It was identified as CAEX.

Various samples were pretreated employing a modified organosolv process, in which a 60% ethanol-40% water alkaline solvent plus a catalyst were placed in contact with a sample in a solid to liquid ratio of 1:6, cooked in a pressurized rotating digestor for 4h at 175C, the liquors drained and the solid residue water washed, solar dried, milled and stored in polyethylene bags. Four different samples were obtained, CAOK-1, CAOK-2, CAOA-1 and CAOA-2 as described on Table II.

Another sample was treated by Dr. J.P.Sachetto from Battelle-Geneve employing an aqueous phenolic solvent (49). The yield obtained was 42% on dry basis. The sample was identified as CABG.

Finally as a comparison a full soda pulp was obtained from a sample of depithed sugarcane bagasse, employing 15% NaOH (as Na2O on bone dry bagasse) in a cooking cycle of 3h at 165C and a yield of 50.60%. The sample was identified as BCSODA.

In summary, the identifications used were: sodium hydroxide, CANA; sodium carbonate plus calcium hydroxide, CACANA; ammonia, CAAM; sulfur dioxide, CASO; steam explosion, CAEX; alkaline organosolv, CAOK-1, CAOK-2, CAOA-1 and CAOA-2; organosolv-phenol, CABG and full soda cook, BCSODA.

- Analysis

The samples were analyzed for Kjeldahl nitrogen and ash content and

(50) for neutral detergent fiber, acid detergent fiber, permanganate lignin and cellulose following the Van Soest techniques (51, 52). In vitro dry organic matter enzymatic digestibilities (IVDMED), were determined by the methodology suggested by Goto and Minson (53), Adegbola and Paladines (54), McLeod and Minson (55, 56) and Dowman and Collins (57). Briefly, 200 mg of the material were suspended in 20 ml of a 2 gl-1 pepsin solution (No. P-7125 Sigma Chemical Co.) in 1N HCl and incubated at 40C for 48h. Samples were centrifuged, washed once with distilled water, centrifuged and suspended in 20 ml of a 2.5% by weight solution of fungal cellulase Onozuka FA (Maruzen Chemical Co. Ltd.) in 0.2M acetate buffer pH 4.8 and incubated at 40C for 48h. The suspension was filtered in sintered glass crucibles, washed with water and weighed. Controls were done with distilled water and no enzyme. During incubation the tubes were agitated twice a day. Results are expressed in % dry matter digestibility.

- Susceptibility to enzymatic hydrolysis

100 g of the material were suspended in 9 ml of 0.2M acetate buffer pH 4.8 containing 1 mg thimerosal per 100 ml as preservative. One ml of a 1% by weight solution of cellulase Onozuka FA in 0.2M acetate buffer pH 4.8 was added and the sealed tubes were placed in a rotary shaker (New Brunswick Co.) at 40C and 300 rpm. Samples were taken at 8, 24, 48 and 72h, the solids discarded and sugars determined in the liquid by the dinitrosalysilic acid method (58) Controls were done with distilled water and no enzyme. The filter paper activity of the enzyme solution was 0.04 units per mg of protein (59). Soluble protein was determined by the Lowry method (60). The results are expressed as % saccharification on the basis of the initial amount of cellulose plus hemicellulose present in each sample after pretreatment.

RESULTS AND DISCUSSION

. Chemical Analysis The chemical analysis of the EX-FERMented whole and chopped-in-the mill sugarcane samples and the bagasse samples are shown in Table III. An average for all samples shows that the lignocellulosic substrate had 43.9% cellulose, 37.5% hemicellulose and 13.9% lignin. Cellulose and acid detergent fiber showed the highest sample standard deviation values, followed by lignin and neutral detergent fiber. If one considers the samples in the blocks only and realizes that CAST, CPST and BEST are really three different samples of the same cane variety obtained from different parts of the sugar mill, an ANOVA analysis between samples 3 and 4 gives the results shown on Table IV. They show that the only macrocomponent statistically different between samples 3 and 4 was lignin, assuming of course, that no drastic effects on the lignocellulosic components were caused by crushing and heating in the sugar mill, as shown by Gartside et al (61). The average lignin value found of 13.9% is slightly higher than most cereal straws (62, 63). The IVDMED of the samples has been put in the last column of Table III; an overall value of 16.91 1.74% has been obtained. This value is much lower than legumes, most common grasses and cereal straws which are around 70, 54 and

29% respectively (64). Nevertheless it should increase if the digestibility is determined by **in vitro** dry matter digestibility (IVDMED) procedures based on the Tilley and Terry (65) technique (or similar ones employing rumen fluid or the nylon bag technique) or if the **in vivo** digestibility (IVD) in ruminants is measured. However the repeatibility of the IVDMED determination is superior (53, 57, 65). The IVDMED values between samples 3 and 4 were statistically the same as shown in Table IV. This clearly shows, that lignin content, although important (33, 66-70), is not the only major parameter affecting the extent of enzymatic digestion. In Table V a summary of a comprehensive literature review on bagasse samples from many countries, cane maturity and variety is given. From these data the following pertinent observations can be made: a) the higher standard deviations were found for the two biological tests: the **in vitro** digestibilities; b) of the two, the enzymatic method was better; c) the IVDMED data reported in Table VI for the Guatemalan canes and bagasse fell within the confidence interval reported in Table V; d) the IVDMD was on the average about 9 units higher than IVDMED as was expected; e) the data for permanganate or Klason lignin showed the lowest standard deviations; f) the lignin average value shown on Table III for the Guatemalan canes is slighty higher than the reported average of Table V; g) there exists, however, a large discrepancy between the permanganate and Klason values, the latter being about 8 units higher. There is no easy explanation for this fact. For example, Hartley (109) mentioned that, contrary to what has been the case for sugarcane bagasse, the permanganate lignin values are sometimes higher than those from the Klason method. However as shown by Theander and Aman (110) this is not necessarly so for all plant species; h) the hemicellulose average value of 26.2% is much lower than the one reported for the Guatemalan samples in Table III which is equal to 37.5%. As shown by Theander and Aman (110), Windham et al (111) and McAllan and Griffith (112), the hemicellulose contents ohtained by NDF-ADF usually are overestimated compared with the summation of hemicellulose constituent sugars; i) the presence of a lignin-carbohydrate complex (LCC) stabilized by chemical linkages has been proven in wood (113); in gramineous plants this linkage might involve phenolic acids such as ferulic and p-coumaric acids (114-118); the existance of LCCs in sugarcane bagasse have been reported (99, 104, 119, 120) and Atsushi et al (101) reported in detail the isolation of three fractions different from each other in contents of lignin, carbohydrate, phenolic acids and molecular weight. Usually the LCCs are not recovered in the acid detergent residue (121), hence this could be an explanation of the low permanganate lignin values and the overestimated hemicellulose figures; j) cellulose values showed a large standard deviation; k) the cellulose content reported in Table III for the Guatemalan canes and bagasse fell within the confidence interval reported in Table V.

. Changes due to pretreatments

With the analytical data of the solid samples from each pretreatment and the corresponding solid yields a mass balance of the lignocellulosic fraction was made. The results are shown in

Figure 1 where they are expressed as % change with respect to the values reported in Table III for the CAST-1 sample, which was the only one employed for pretreatments as explained in the Materials and Methods section. The data for IVDMED are included. The digestibilities represent weight loss due to enzymatic action in terms of initial total dry matter, but taking into account the weight loss of the control (no enzyme) samples. Hence these figures represent true insoluble matter resistant to enzyme attack. All the pretreaments were effective in increasing the IVDMED values; specially the organosolv variants, sodium hydroxide, the aqueous phenol and the steam exploded samples. Lignin and hemicellulose contents decreased in all samples; the former was higher for the organosolv, aqueous phenol and steam exploded samples. Hemicellulose was fully hydrolyzed in the aqueous phenol and steam exploded samples.

The expected effects of the alkali treatments on the substrate were to partially dissolve hemicelluloses and lignin with a parallel increase in their in vitro or in vivo digestibilities (62, 122, 123). The important chemical reaction in this pretreatment seems to be the saponification of esters of uronic acids associated with the xylan chains, which induces an extensive swelling of the solid matrix (123) and promotes the penetration of microorganisms and makes possible a more favorable enzyme-fiber contact. There is recent experimental evidence that both alkali-labile and alkali-resistant lignin-carbohydrate bonds exist and that their ratio in a particular plant material governs the decision to use alkali as a pretreatment to increase its digestibility (124). The alkali-labile bonds include the hemicellulose-phenolic acid and acetyl constituents of the cell walls, (109), both of which affect holocellulose hydrolysis (116, 124-126).

In Table VI the data for CANA, CACANA and CAAM are compared with values reported in the literature. There is a widespread variation in conditions used for the pretreatment which makes difficult any quantitative comparison, nevertheless there are some general trends worth commenting on: a) our percent IVDMED increases for NaOH are higher than those usually reported, with exception of the data of Cabello et al(85), Awad and Abel-Mottaleb (89), Cabello and Conde (105), and Ismail et al (107), which are in the same order of magnitude; b) the one obtained for NH3 compares well with those reported for NH3 and NH4OH pretreatments, with the exception of the data of Awad and Abdel-Mottaleb (89) which is rather than high; c) our data for Ca(OH)2+Na2CO3 is lower than the one by Matei and Playne (98), Playne (103) and Martin et al (75) ; d) we found higher digestibility values for NaOH than for NH3 and Ca(OH)2+Na2CO3, which coincides with Ibrahim and Pearce (96); e) as shown by Cabello et al (85) IVDMED values are usually lower than IVDMD values, as a matter of fact the authors report a linear relationship among the two; f) there was only one datum for in vivo digestibility using wethers as testing animals and these are low compared to in vitro values. Alkaline pretreatments seem to induce a faster rate of passage through the digestive tract of ruminants (128) and the alkali increases osmotic pressure which also reduces microbial activity in the rumen (129). Hence the lower value was

not unexpected; g) our data on lignin losses for NaOH were usually on the high side, they compare only with the data of Dekker and Richards(74) for cane bagacillo (fines) and of Ellenrieder and Castillo (92) . For ammonia our value is within values reported by Ibrahim and Pearce (96) and for calcium ours is higher; h) the same comments are more or less are applicable to hemicellulose losses. less are applicable to hemi- cellulose losses.

Due to ammoniation the nitrogen content of CAST-1 (which was 0.12%) increased to 2.0% (for CAAM), which is equivalent to a 941% increase. Chang et al (72) reported 707% and Hamad and El-Saied (130) 513% increases. The authors have shown that ammonia reacted mainly with hemicelluloses. The induced changes were typical of what has been found on cell walls of graminaceous plants (131).

Cabello et al (85) found linear correlations between IVDMD and IVDMED and between IVDMD and soluble solids for bagasse pretreated with 4,5,8 and 12g NaOH 100 g-1 dry sample, added as solutions employing a liquid:solid ratio of 2:1, at 30C with no subsequent washing. Later the same authors reported similar results (105) and both sets of data have been presented on Figure 2. The IVDMED and soluble solids have been calculated on a relative basis as a ratio between the pretreated and the unpretreated or control sample. Note that there are two possible correlations one for each set of data. This might be explained as the different response of sugarcane varieties to sodium hydroxide pretreatments. Correlations of this type could be used to select cane varieties more susceptible to alkaline pretreatments. We have included in the figure our data for CANA, CACANA and CAAM samples. It can also be observed that the linear relationship holds for one cane variety even if different alkalies at various concentrations are employed. Again more experimental data are needed to see if this conclusion holds.

Early work by Millet et al (21) at the FPL in Madison showed that after pretreatment with gaseous SO2 under pressure for 2-3h at a temperature of about 120C, samples of hardwoods and softwoods increased their enzymatic digestibility substantially. The Klason lignin values of the pretreated hardwoods decreased 67% from the initial content and 29.50% for the pretreated softwoods, indicating an extensive depolymerization of the original lignin. Conner (132) treated pure cellulose and cotton linters and found that sulfur dioxide caused a dramatic decrease in the degree of polymerization but did not affect the enzymatic digestibility or the crystallinity of the substrate. This might indicate that the pretreatment effects were geared towards delignification, hemicellulose hydrolysis and LCCs breakdown. In the CASO sample there was a 23% lignin decrease and 56% hemicellulose decrease as shown in Figure 1. The IVDMED was increased 1.38 times over the control. This is equivalent to a 38% increase over the control as shown in Figure 1. These results are compared with those obtained by other researchers employing bagasse and other lignocellulosic residues on Table VII. Our data is in agreement with that reported by scientists from the Volcani Center (133-135) in Israel for wheat straw, although they used about seven times less chemical per unit dry weight of

lignocellulosic residue. Ibrahim and Pearce (96-97) showed rather slight effects of SO2 on barley and pea straws and cane bagasse. Although they tried several temperatures and holding times it seems that they did not use intermediate temperatures (about 70C) and large holding times (3 days), which are necessary to induce the required benefits.

Steam pretreatment was originally developed in 1925 and has been extensively used in the manufacture of hardboard by the Masonite processes employing wood. In the late seventies Iotech Corp Ltd and Stake Technology Ltd in Canada started using this process for the production of feed for ruminants. Treatment of wood chips up to 185C also is practiced commercially in the first stage of the manufacture of dissolving pulp by the prehydrolyzed kraft process. Hence wood steam pretreatment studies have shown that the lignocellulosic matrix is modified drastically and that the remaining solids are more susceptible to enzymatic hydrolysis. The structural changes and chemical reactions taking place seem to be a function of temperature and time of the pretreament. The following can be summarized: a) during a short prehydrolysis (about 30-60s) taking place at temperatures of 220-240C lignin was extensively depolymerized to low molecular weight polymers that were soluble in aqueous alkali or ethanol-water solutions and a small amount to low molecular wieght phenol related compounds, although recondensation reactions could eventually take place; b) hemicelluloses were easily hydrolyzed into mono and oligosaccharides of low molecular weights, in addition, some degradation products were formed that apparently condensed with lignin, thereby increasing the lignin content; c) cellulose was the least hydrolyzed component maintaining its crystallinity degree but reducing its degree of polymerization, and c the rapid steam decompression or explosion defibrated the cellular structure and caused a large increase in accessibility of the cellulose to enzymatic hydrolysis (136-145). The resulting solids contained inhibitors which interferred with growth of microorganisms and anaerobic fermentations (146-150). Washing the solids with water or dilute alkali removes the inhibitors. However it seems that anaerobic mixed cultures are less prone to inhibition, both in in vitro systems (150), as also in vivo ruminant feeding studies (84, 151-155). However the chemical nature of the inhibitors is unknown (156) , as also is any detoxifying mechanism existing in mixed cultures existing in mixed cultures of anaerobic bacteria.

The solid yield for cane bagasse after steam explosion at 240C (3.35MPa) and 60s was about 78%. This figure was within expected values: Cheong et al (157) found that yield decreased with exposure time at fixed pressure and with increasing pressure at a constant exposure time, minimum yield was 70.7% at 0.16 MPa (199C). Hart et al (158) found higher values, 92-95% at 2.07 MPa for 5 min and 84-94% at 2.76 MPa for 0.5-1.5 min. A minimum yield of 79.2% has been reported by Rangnekar et al (91) for 60 min at 0.88 MPa, and he found the same yield dependence with time and pressure. Morjanoff et al (159) explored various steam pretreatment techniques; the yield was between 69 to 74% after 1h at 160-180C.

The solid fraction remaining was treated for 5 min at 208C and the corresponding yield was 87.6%. Hence a combined minimum yield of 60.4% was obtained. Dekker and Wallis (160) reported a yield of 58% after a pretreatment of 4-20 min at 200C and 6.9 MPa obtained by nitrogen pressurization. The yield varied from 63.5 to 71.9% by 5 min at 200C and a pressure from 3.45 to 13.8 MPa employing pure CO2 or in mixtures with N2 (46, 161). Lower yield values can be obtained if an explosion soda pulping is employed, for example Mamers et al (162) reported a figure of 52.5% for 5 min at 3.4-13.8 MPa with 7% Na2O.

In Figure 1 it is easily seen that this pretreatment caused one of the largest decreases in hemicellulose and lignin from bagasse, 95.9 and 64.5% of the original values respectively. The IVDMED increased over the control about 3 fold. The extent of hemicellulose hydrolysis has been reported as 77% (at 0.88 MPa and 60 min) (91), 34-59% (at 194-224C for 1 min) (143) and 26% (140C for 90 min) (90). In the case of lignin very small losses have been found previously, 11% (140C for 90 min) (90) and 3% (0.88 MPa and 60 min) (91). However Ghose et al (163) have reported a value of 88% delignification with 44% yield employing aqueous butanol with an unspecified aromatic acid catalyst. In Table VIII are summarized the increase in **in vitro** digestibilities for steam-exploded bagasse samples. There have been a wide range of experimental conditions explored and there is also a large variation in the response obtained. Our data is comparable to the one by Hart et al (158) in terms of experimental conditions used but our results are about 50% better. Here the aspects of cane varieties might be partly responsable for these differences. Some data in Table VIII have been obtained with a combination of chemical and steam-explosion pretreatments; in all cases there has been an improvement over the one obtained separately but nothing spectacular. The digestibility improvement has been caused by a partial delignification (66-70) but also probably more important to a decrease in degree of polymerization of the cellulose (94) and an increase in surface area (142) caused by a physico-chemical disruption of the lignocellulosic matrix. It is important to point out that cellulose crystallinity remains unchanged during this process (94, 164), although due to the partial hemicellulose and lignin (and probably LCCs) hydrolysis and loss of soluble monomers caused by the acid medium generated due to the cleavage of acetyl groups, there is an actual crystallinity increase of the remaining solids. Hence it seems that, as Gharpuray et al (41) have suggested, the enzymatic hydrolysis is mainly affected by the substrate surface area, lignin content and cellulose crystallinity in that order. Although for other chemical pretreatments crystallinity seems to be an important parameter (33), and moreover recrystallization during pretreatment might be a factor (165).

The modified organosolv pretreatments either with ethanol or phenol caused the greatest delignification of all methods tested, accompanied with a substantial loss of hemicelluloses as shown in Figure 1. This behavior is typical in this process as clearly shown, among others, by Phillips and Humphrey (166,167) for poplar in wood, in which regardless of the treatment conditions used, the

lignin removal was always accompanied by hemicellulose removal in a linear correlation among the two. The IVDMED increases were very high, from 3.2 times for CAOK-1 to 4.7 fold for CAOA-2. In terms of relative percentual increases over the control value, these figures correspond to 217% and 372% respectively. The sample treated with phenol (CABG) responded similar to CAOK-1 but was 23% below the best response obtained with a 4% NaOH:60 ETOH:40 H2O:0.05 AQ mixture (CAOK-2).

The susceptibility of EX-FERMented chips to the hydrolysis by the fungal cellulase enzyme are presented in Figure 3. The data are expressed as percent saccharification of the holocellulose fraction of each sample. Similar information is presented for the various bagasse samples and this is compared with data from the literature (87, 106, 160, 168). The reducing sugars detected in the liquid fraction of the samples minus the ones in the control (no enzyme) were employed to make the calculations, hence these values represent true products of enzyme hydrolysis and do not take into account the different soluble sugars residues present in the various cane and bagasse EX-FERMented samples. The hydrolysis behavior of the CAST samples is similar especially after the first 20h where the variation between duplicates was large. It seems that the initial rates are different, CAST-1 and CAST-3 showing the highest rates. The bagasse sample shown in Figure 4 showed in general a higher initial rate of hydrolysis and a higher extent of hydrolysis after 72h than the corresponding cane chip samples. Although it is tempting to say that these differences might have been caused by the action of the crushers and roller mills during sucrose extraction, more samples would be needed in order to confirm it. The data of Dekker and Wallis (87, 160) obtained with a cellulase preparation obtained from Trichoderma reesei RUT-C-30, and the one by Rao et al (168) obtained with an enzyme from Penicillium furiculosum, fall nicely with the data for BEST-2, 3 and 4. However the data of Trevedi and Ray (106) employing a high-strength cellulase from a mixed culture of Scytalidium lignicola and Trichoderma longibrachiatum was far superior in extent of hydrolysis after 48h. However the differences might be caused not only due to a different enzyme but also because of a different cane variety.

The percent saccharification and the enzymatic digestibility data are not equal as they are expressed on a different basis and have been obtained measuring unequal parameters and employing a different amount of enzyme. Nevertheless a comparison can be made at 72h between the two. On the average the CAST samples showed a 28% less saccharification than the IVDMED values; the BEST samples showed the same average for the two values. The fact that the percent saccharification was lower in the unprocessed cane chips might reflect indirectly the hydrolyzing action of acid-pepsin in the two-enzyme digestibility assay. Dowman and Collins (57) comment that either the use of acid-pepsin or neutral detergent solution is recommended before the use of cellulases for determining forage digestibility, since in this way more insoluble material is hydrolyzed by the holocellulolytic enzymes. The protein polymer might protect the holocellulosic fiber in sugarcane

and this fact persists even after an EX-FERM process of sucrose conversion to ethanol. Cane crushing and pressing in the sugar mill, might alter this situation and hence there is no difference between the two assays. Again more proof is needed to reach a final conclusion, specially since cane has only about 1.2-1.6% protein compared with hardwood leaves, lucerne and ordinary grass hays which have in the range of 8 to 23% (110, 169) and also cereal straws, 2.0-5.0% (110, 170).

The data for the pretreated samples are presented in Figures 5 to 7. In Figure 5 the alkaline pretreated samples are compared to CAST-1. As expected all the pretreated samples were hydrolyzed faster and more completely. The sample most susceptible to hydrolysis was the sample pretreated with NaOH under solid substrate conditions. This sample was even better than the one obtained with a full soda cook. The ammonia and Na_2CO_3+$Ca(OH)_2$ pretreated samples showed a similar behavior but were inferior to the NaOH effect, as was found for the IVDMED data which was shown in Table VI. If one compares the 48h extent of hydrolysis, the relative values in terms of the CAST-1 sample are 376, 128 and 66% increase for the CANA, CACANA and CAAM samples. These figures are better than the IVDMED relative increases. Rao et al (168) reported a 312% increase at 48h for a 4% NaOH pretreated (and washed afterwards) bagasse sample. Trivedi and Ray (106) found only a 86% extent of hydrolysis increase after 48h of 2-10% NaOH pretreatment (with washing and neutralization afterwards).

In Figure 6 the data for the organosolv pretreated samples are given. All organosolv treatments were effective in increasing the initial rate of hydrolysis and the extent of hydrolysis after 48h, specially CAOK-1 and CAOA-2. As specified in Table II, the first one was not water washed after an alkaline (1%) organosolv; in the second one a higher NaOH content (4%) and anthraquinone as catalyst were employed and the sample was water washed. We do not know if washing the sample after pretreament removed easily hydrolyzed complexes from it. This is a possible explanation for the fact that more carbohydrates were detected in the liquid phase in the CAOK-1 sample. The phenol pretreated sample gave a similar behavior as the CAOK-2 and CAOA-1 samples, and all of them were inferior to CAOK-1 and CAOA-2. These two gave hydrolysis values at 48h which were 4.7 fold higher than the untreated CAST-1 sample. Very similar increases were obtained for the IVDMED values as mentioned before.

Figure 7 compares the results for the steam-exploded sample (CAEX) and the SO_2 pretreated sample (CASO) with the untreated (CAST-1) and the best samples from Figures 5 and 6. The CASO sample shows a lower susceptibility to enzymatic hydrolysis than the NaOH and organosolv samples. However the steam-exploded sample gave the highest values for the percent saccharification, close to 85%. This is 5.5 times the extent of hydrolysis for the untreated sample. Rao et al (168) report a 3.1 increase after steam-explosion for 30 min at 0.68 MPa. Both of these values are much higher than those found for the IVDMED parameter and summarized on Table VIII. Again these steam-exploded samples were

not water washed after pretreatment and the possibility of the generation during the steam-explosion of easily hydrolyzable complexes might explain these results.

CONCLUSIONS The results reported in this article indicate the different effects upon chemical composition, **in vitro** enzymatic digestibility and susceptibility to enzymatic hydrolysis of EX-FERMented sugarcane chips subjected to various pretreatments. All the pretreatments were effective in increasing the IVDMED, the initial rate of holocellulose hydrolysis and the saccharification extent. The best pretreatments were the organosolv variants, the steam-explosion, the sodium hydroxide and the aqueous-phenol. Lignin and hemicellulose contents decreased in all samples. Delignification was higher for the organosolv, aqueous phenol and steam exploded samples. Hemicellulose was fully hydrolyzed in the aqueous phenol and steam-exploded samples. Extensive comparison with literature values and several, although limited, Guatemalan samples showed quite a large variation in pretreatment effects. It is quite possible that an explanation for this fact might be the structural differences found in the lignocellulosic matrix of the various cane varieties processed in the tropical sugarcane growing regions of the world.

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Table I. Description and identification for sugarcane EX-FERMented chip samples used as control, for in the mill chopped EX-FERMented chip samples and for bagasse samples

Identification	Description
CAST 1	Whole sugar cane from Concepción Mill EX-FERMented in ICAITI, without pretreatment.
CAST 2	Whole sugar cane from Concepción Mill EX-FERMented in ICAITI, without pretreatment.
CAST 3	Whole sugar cane from Pantaleon Mill EX-FERMented in ICAITI, without pretreatment.
CAST 4	Whole sugar cane from Los Tarros Mill EX-FERMented in ICAITI, without pretreatment.
CPST 3	Sugar cane chopped in the sugar mill (Pantaleón), EX-FERMented in ICAITI, without pretreatment.
CPST 4	Sugar cane chopped in the sugar mill (Los Tarros),EX-FERMented in ICAITI, without pretreatment.
BEST 2	Whole sugar cane bagasse, collected in the Concepción Mill, without pretreatment.
BEST 3	Whole sugar cane bagasse, collected in the Pantaleõn Mill, without pretreatment.
BEST 4	Whole sugar cane bagasse, collected in the Los Tarros Mill, without pretreatment.

Table II. Description, characteristics and identification of EX-FERMented chip samples pretreated by a modified organosolv process

Sample	Weight ratio Ethanol : Water	NaOH,%[a]	Cooking cycle,h	Temperature,°C	AQ,%[b]	Cook yield,%
CAOK-1[c]	60 : 40	1	4	175	0	86.84
CAOK-2	60 : 40	2	4	175	0	80.50
CAOA-1	60 : 40	2	4	175	0.05	71.84
CAOA-2	60 : 40	4	4	175	0.05	67.38

a As Na_2O on bone dry bagasse

b Anthraquinone as a catalyst on bone dry bagasse

c Sample CAOK-1 was water washed after the pretreatment

Table III. Chemical analysis of sugarcane and bagasse samples

Sample	NDF[a]	ADF[b]	Permanganate lignin	Cellulose	Ash	Protein	Hemicellulose[c]	IVDMED[d]
CAST 1	92.8	54.8	12.5	43.3	2.4	1.2	38.0	19.04
CAST 2	95.2	52.0	14.1	46.7	2.4	1.8	43.2	18.60
CAST 3	95.0	55.6	16.0	41.9	2.2	1.2	39.4	14.51
CAST 4	94.1	60.3	11.7	44.8	1.4	1.6	33.8	15.81
CPST 3	92.5	54.6	15.0	41.7	2.1	2.4	37.9	13.89
CPST 4	95.5	61.6	11.8	50.4	1.8	1.9	33.9	16.54
BEST 2	93.4	55.7	12.8	42.7	2.1	2.3	37.7	20.68
BEST 3	91.3	55.1	18.4	40.8	2.9	3.5	36.2	19.59
BEST 4	93.5	56.4	12.5	42.9	2.0	2.0	37.1	13.55
Sample mean, $\bar{x}$	93.7	56.2	13.9	43.9	2.1	2.0	37.5	16.91
Sample standard deviation, s	1.4	3.0	2.3	3.0	0.4	0.7	-	2.65
95% confidence interval on population mean	±.9	±2.0	±1.5	±2.0	±0.3	±1.3	-	±1.74

a Neutral detergent fiber
b Acid detergent fiber
c Obtained as: NDF-ADF
d *In vitro* dry matter enzymatic digestibility

Table IV. Analysis of variance (ANOVA) of samples 3 and 4

	Sum of squares	Mean square	F test	$F = S^2_B / S^2W = 7.71$
For Lignin:				
between samples	29.9	29.9	18.5	18.5 >7.71[a]
within samples	6.5	1.6		
For ADF				
between samples	28.2	28.2	7.44	7.44 <7.71[b]
within samples	15.2	3.8		
For Cellulose				
between samples	31.3	31.3	4.02	4.02 <7.71[b]
within samples	31.3	7.8		
For NDF				
between samples	3.1	3.1	1.33	1.33 <7.71[b]
within samples	9.2	2.3		
For IVDMED				
between samples	0.7	0.7	0.12	0.12 <7.71[b]
within samples	24.4	6.1		

a Null hypothesis of equality rejected
b Null hypothesis of equality accepted

Table V. Chemical analysis of sugarcane bsgasse - literature values[1,46,62,69,71-108]

	NDF[a]	ADF[a]	Permanganate lignin	Klason lignin	Cellulose	Hemicellulose	IVDMED[a]	IVDMD[b]
1. Number of samples, n	15	14	18	35	41	48	13	21
2. Sample mean, $\bar{x}$	87.0	59.2	12.5	20.5	41.7	26.2	17.2	26.0
3. Sample standard deviation, s	3.9	6.2	1.7	2.4	6.6	4.6	9.1	11.8
4. 95% Confidence interval on population mean	±1.8	±3.0	±0.7	±0.7	±1.7	±1.1	±4.5	±4.4
5. Maximum value	94.5	68.3	15.5	27.5	53.1	34.2	29.0	52.3
6. Minimum value	81.6	49.2	10.0	14.3	26.6	10.0	1.5	3.8

a See footnote Table III
b *In vitro* dry matter digestibility measured by employing ruminal fluid or the nylon bag technique

Table VI. Comparison of changes induced on bagasse by alkaline pretreatments.
Figures expressed in % change over control.
Lignin and hemicellulose are losses.
All the digestibilities are increases.

Sample	Lignin	Hemicellulose	IVDMED[a]	IVDMD[a]	IVD[b]
work	53.6	56.8	279.6	-	-
A	31.2	32.4	75.6	-	-
AKA	25.6	34.7	55.4	-	-
M					
l[73]	-	-	-	60.2	-
OH, 24h, washing					
r and Richards[74]	58.2	-	-	174.1	-
OH, 1-1.5h, washing					
in et al[75]	-	-	-	181.5-737.0	-
% NaOH, 40 min, 90C	-	-	-	185.1-577.2	-
% NaOH, 30 min, 100C	-	-	-	340.2-378.3	-
% $Ca(OH)_2$, 72h					
as et al[79]	14.9-22.1	4.7-48.5	-	-	-
NaOH, 45 days ensilage					
9	23.2	34.5	60.3	84.7	-
NaOH, Danish process					
t al[82]	-	-	83.3	-	-
NH_4OH, 24h, washing					
llo et al[85]	-	-	30-280.7	129.4-569.6	-
% NaOH					
and Abdel-Mottaleb[89]	-	-	289.1-991.5	-	-
% NaOH	-	-	230.5-406.0	-	-
% NH_4OH					
nrieder and Castillo[92]	54.5	32.5	-	-	-
OH, 3h, 80C, washing					
ina et al[96]	2.6-23.7	15.3-64.3	-	-	24.5-43.1[c]
% NaOH					
hin and Pearce[96]	11.4-35.2	2.6-43.0	-	28.3-66.9[d]	-
2% NaOH, 24h	1.1- 2.8	18.6-31.3	-	16.7-28.9	-
5% $Ca(OH)_2$, 24h	1.1-14.8	1.2-22.8	-	0.4-14.5	-
-7.7% NH_4OH, 30 days	25.0-34.7	10.1-10.2	-	26.4-31.7	-
-5.8% NH_3, 5-10 days					
ahim and Pearce[97]	-	-	-	103.7[d]	-
% NaOH, 24 h	-	-	-	43.4	-
% NH_3, 5 days					
him and Pearce[127]					
% NaOH, 24h					
ray, Beckman method and	-	-	-	48.5-116.1[d]	-
k and press method)					
ei and Playne[98]	-	-	153.8	-	-
% $Ca(OH)_2$ + Na_2CO_3, 1h, 121C	-	-	73.1	-	-
% NH_3, 35 days					
yne[103]	-	-	61.8-252.9	-	-
11% NaOH, 18h	-	-	96.1-243.1	-	-
12% $Ca(OH)_2$ + Na_2CO_3, 18h	-	-	47.0- 96.1	-	-
11% NH_3, 18h					
bello and Conde[125]	11.0-21.9	1.1-5.6	241.6-491.6	-	-
6% NaOH					
ail et al[127]	-	-	400.5-566.7	-	-
5% NaOH, 24h, washing	-	-	273.3-506.7	-	-
oiling, 4h), washing					

See footnote
In vivo digestibility
Employing wethers
In vivo organic matter digestibility

Table VII. Changes induced on agricultural residues by SO_2 pretreatment

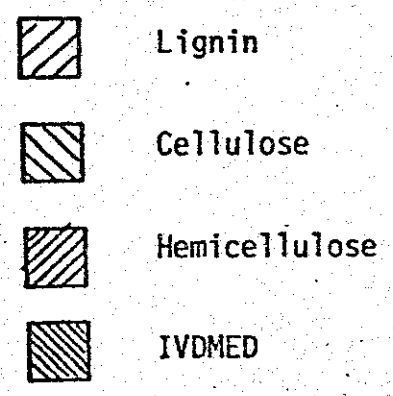
Sample	SO_2 %	Pretreatment Time,h	Pretreatment Temperature,C	Decrease (% untreated sample) Permanganate lignin	Decrease (% untreated sample) Hemicellulose	IVDMED increase (times the untreated sample)
Other agricultural residues						
Wheat straw[133-135]	5	72	70	17.7	63.9	1.7 - 2.0
Barley straw[96]	-	1-3	20	0	18.7	1.1
		1-3	60	0	10.7	1.1
		1-3	120	0	20.0	1.2
Barley straw[97]	-	72	20	0	11.0	1.2
Pea straw[96]	-	1-3	20	0	36.5	1.1
		1-3	60	0	29.0	1.1
		1-3	120	0	36.9	1.1
Sugarcane bagasse						
Ibrahim and Pearce[96]	-	1-3	20	12.4	2.2	1.2
		1-3	60	15.2	4.8	1.2
		1-3	120	6.5	9.1	1.2
Ibrahim and Pearce[97]	-	72	20	5.3	1.5	1.5
This work	36.4	72	70	23.2	55.5	1.4

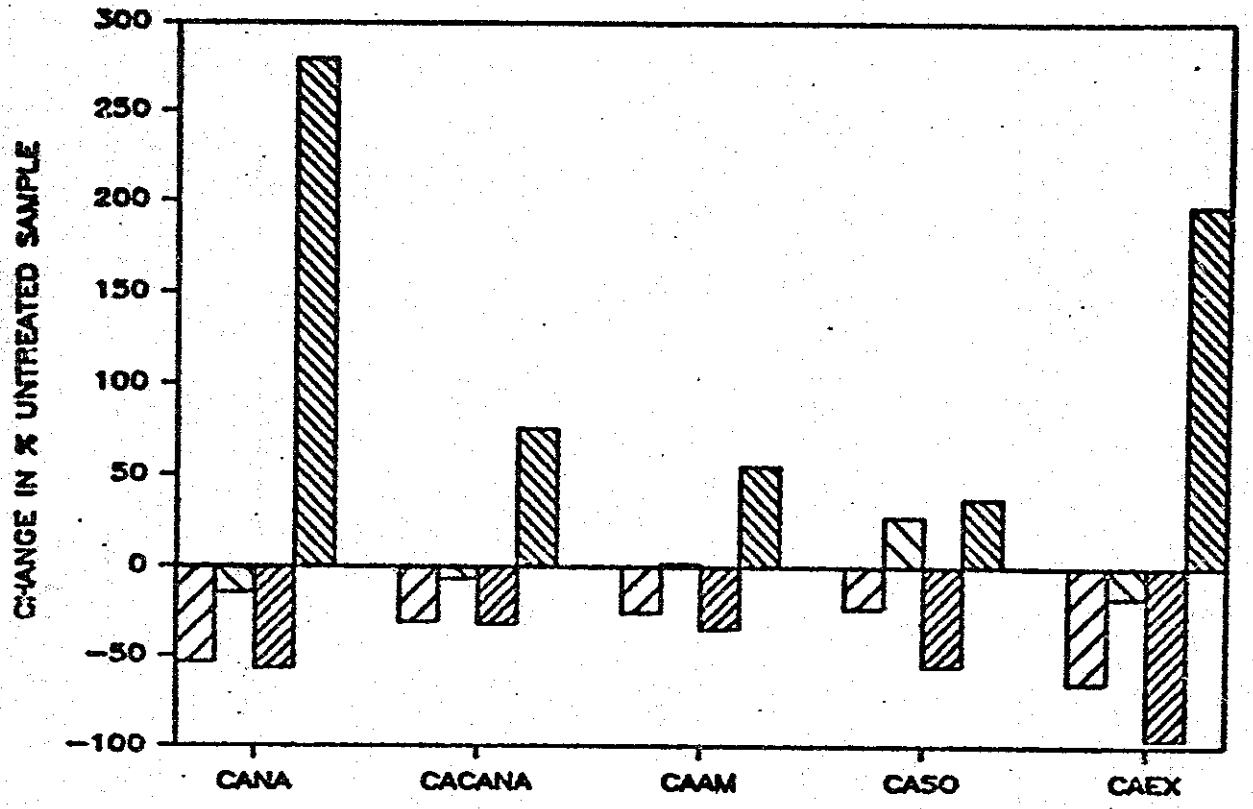
Table VIII. Increase in *in vitro* digestibilities by steam/explosion pretreatments of bagasse

References	Pretreatment Pressure MPa	Pretreatment Time min	Temperature °C	IVDMD (ratio over control)	IVD (nylon bag) (ratio over control)	IVDMED (ratio over control)
Cheong et al[157]	0.13	10	-	1.78	-	-
	0.13	30	-	1.28	-	-
	0.16	10	-	1.73	-	-
	0.16	30	-	0.95	-	-
Martin et al[75]	0.39	15	-	2.48	-	-
	0.39	30	-	2.64	-	-
	0.59	15	-	2.62	-	-
	0.59	30	-	3.94	-	-
Preston[76]	(0.90)	15	175	-	2.30	-
	(0.90)	60	175	-	2.60	-
	(1.50)	15	198	-	3.30	-
	(1.50)	60	198	-	3.20	-
Hart et al[158]	1.38	5	-	-	-	1.96
	2.07	5	-	-	-	2.40
	2.76	0.5	-	-	-	2.59
	2.76	1.5	-	-	-	2.45
	3.45	0.3	-	-	-	2.42
	3.45	1.5	-	-	-	2.09
	4.14	0.3		-	-	2.07
	4.14	1.5	-	-	-	2.09
	1.38	5	-	-	-	2.45[a]
	2.76	1.5	-	-	-	2.79[b]
Ibrahim and Pearce[98]	(0.20)	90	120	1.18	-	-
	(0.36)	90	140	1.43	-	-
	(0.80)	90	170	1.49	-	-
	(0.20)	90	120	1.30[c]	-	-
	(0.80)	90	170	1.49[c]	-	-
Rangnekar et al[91]	0.49	30	-	1.23	-	-
	0.49	60	-	1.47	-	-
	0.69	30	-	1.48	-	-
	0.69	60	-	1.46	-	-
	0.88	30	-	1.58	-	-
	0.88	60	-	1.64	-	-
Morjanoff et al[159]	(1.6)	5	203	-	-	2.88
	(2.0)	5	213	-	-	2.35
	(3.3)	5	236	-	-	1.82
	(3.4)	5	241	-	-	1.75
Ibrahim and Pearce[97]	(0.80)	60	170	1.82	-	-
	(0.80)	60	170	2.13[d]	-	-
	(0.80)	60	170	1.97[e]	-	-
	(0.80)	60	170	1.83[f]	-	-
Puri et al[56]	(1.6)	5	200	-	-	2.79[g]

a Combined pretreatment with 3.5% NH_3
b Combined pretreatment with 4.0% NaOH
c Combined pretreatment with 3.0% NaOH
d Combined pretreatment with 6.0% NaOH
e Combined pretreatment with 5.2% NH_4OH
f Combined pretreatment with SO_2
g CO_2 explosion

Figure 1. Percent change of the lignocellulosic fraction and the IVDMED for the pretreated samples




CHANGE IN % UNTREATED SAMPLE
300
250
200
150
100
50
0
−50
−100
CANA
CACANA
CAAM
CASO
CAEX

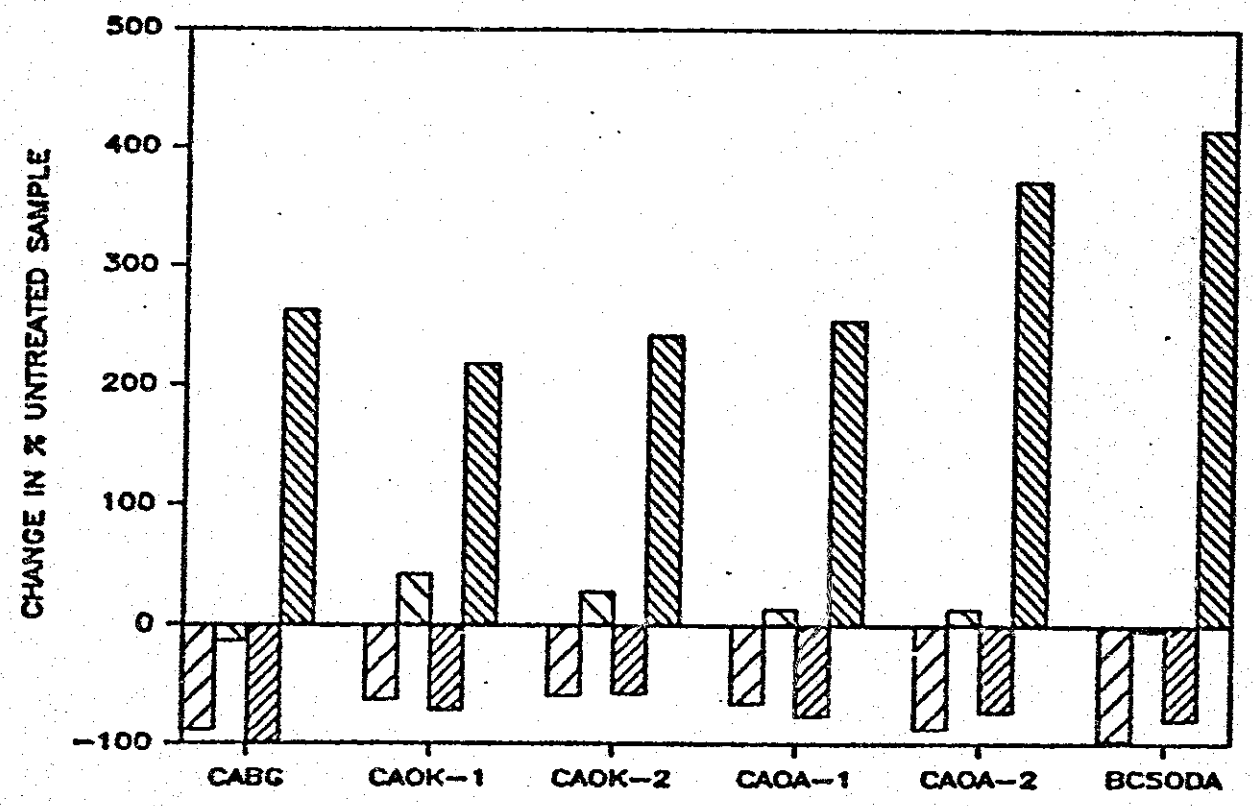

CHANGE IN % UNTREATED SAMPLE
500
400
300
200
100
0
−100
CABG
CAOK−1
CAOK−2
CAOA−1
CAOA−2
BCSODA

Figure 2. Relationship between IVDMED and soluble solids.

Both parameters are ratios between the value for the pretreated substrate and the material without any pretreatment

△ Sugarcane bagasse pretreated with NaOH
Correlation: IVDMED = 0.05 + 1.78 (x)

▲ Sugarcane bagasse pretreated with NaOH
Correlation: IVDMED = 0.07 + 1.06 (x)

□ CANA, CACANA and CAAM
Correlation: IVDMED = -0.19 + 0.41(x)

(x = soluble solids)

RELATIONSHIP BETWEEN IUDMED AND SOLUBLE SOLIDS

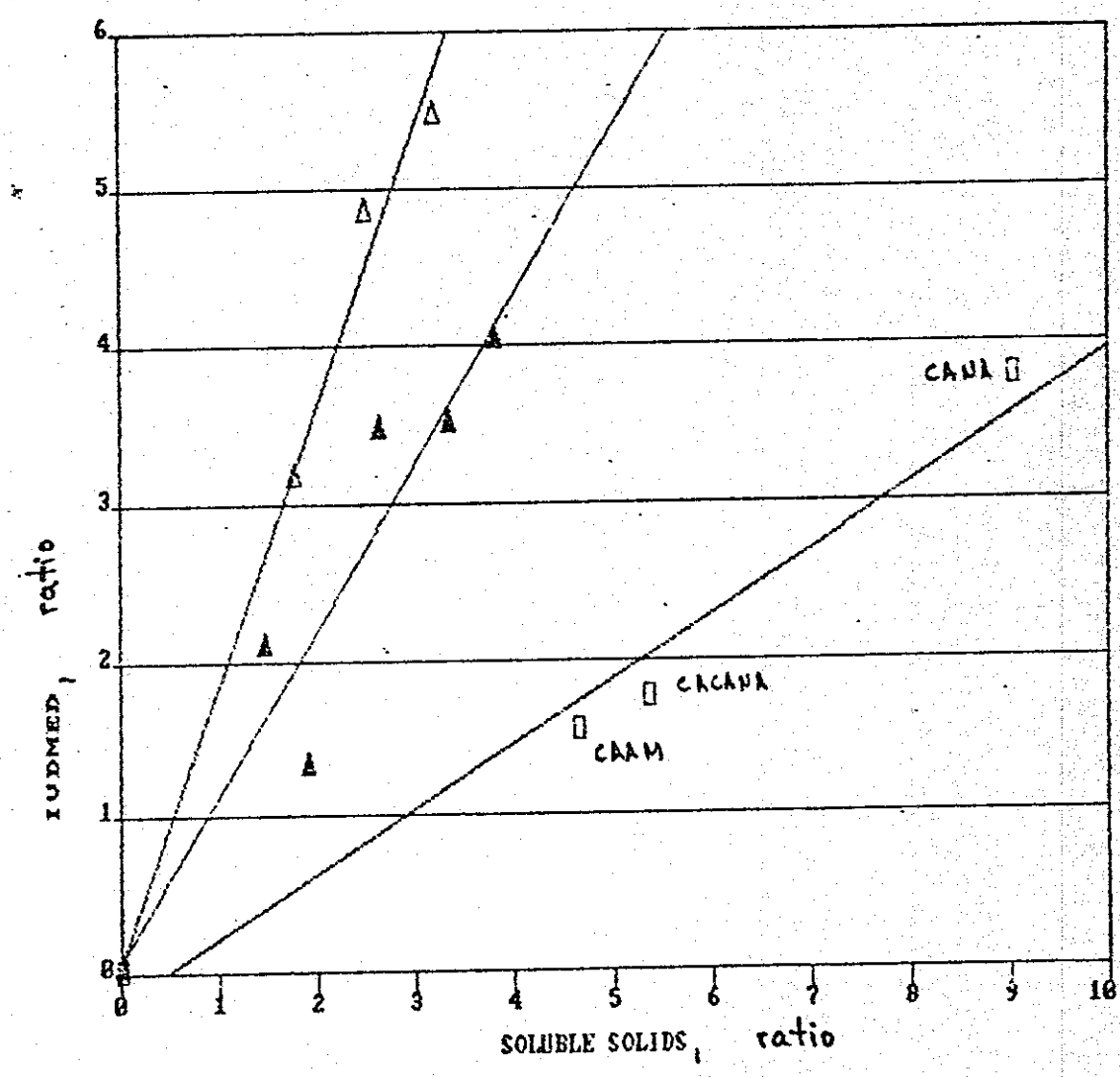


Figure 3. Enzymatic hydrolysis of the holocellulose from EX-FERMented cane chips. See Table I for sample identification

The % saccharification is based on the initial amount of cellulose plus hemicellulose present in each sample.

□ CAST - 1

+ CAST - 2

◇ CAST - 3

△ CAST - 4

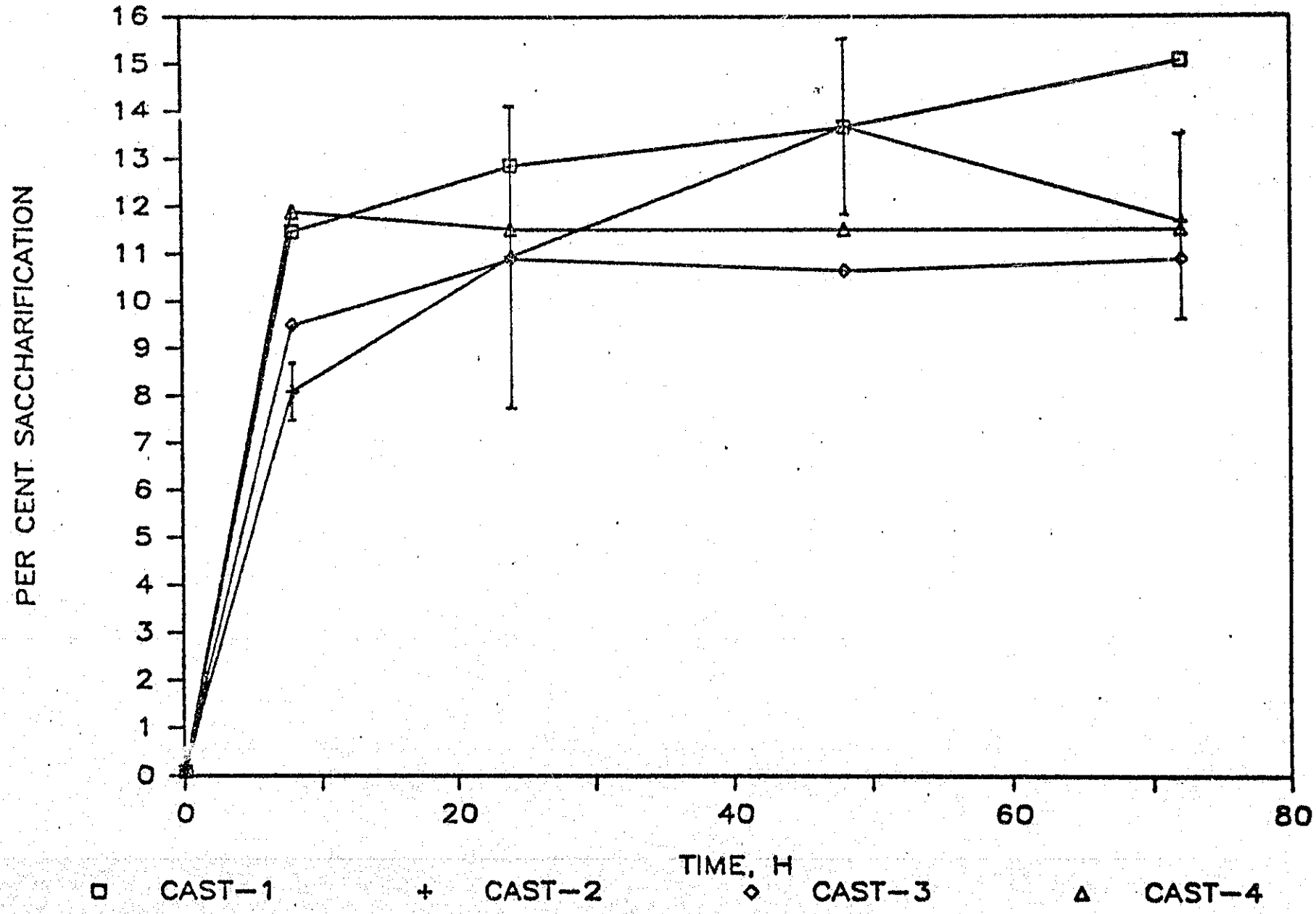

PER CENT SACCHARIFICATION
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
TIME, H
CAST-1
CAST-2
CAST-3
CAST-4

Figure 4. Enzymatic hydrolysis of the holocellulose from bagasse samples. See Table I for identification

The % saccharification is based on the initial amount of cellulose plus hemicellulose present in each sample.

□ BEST - 2

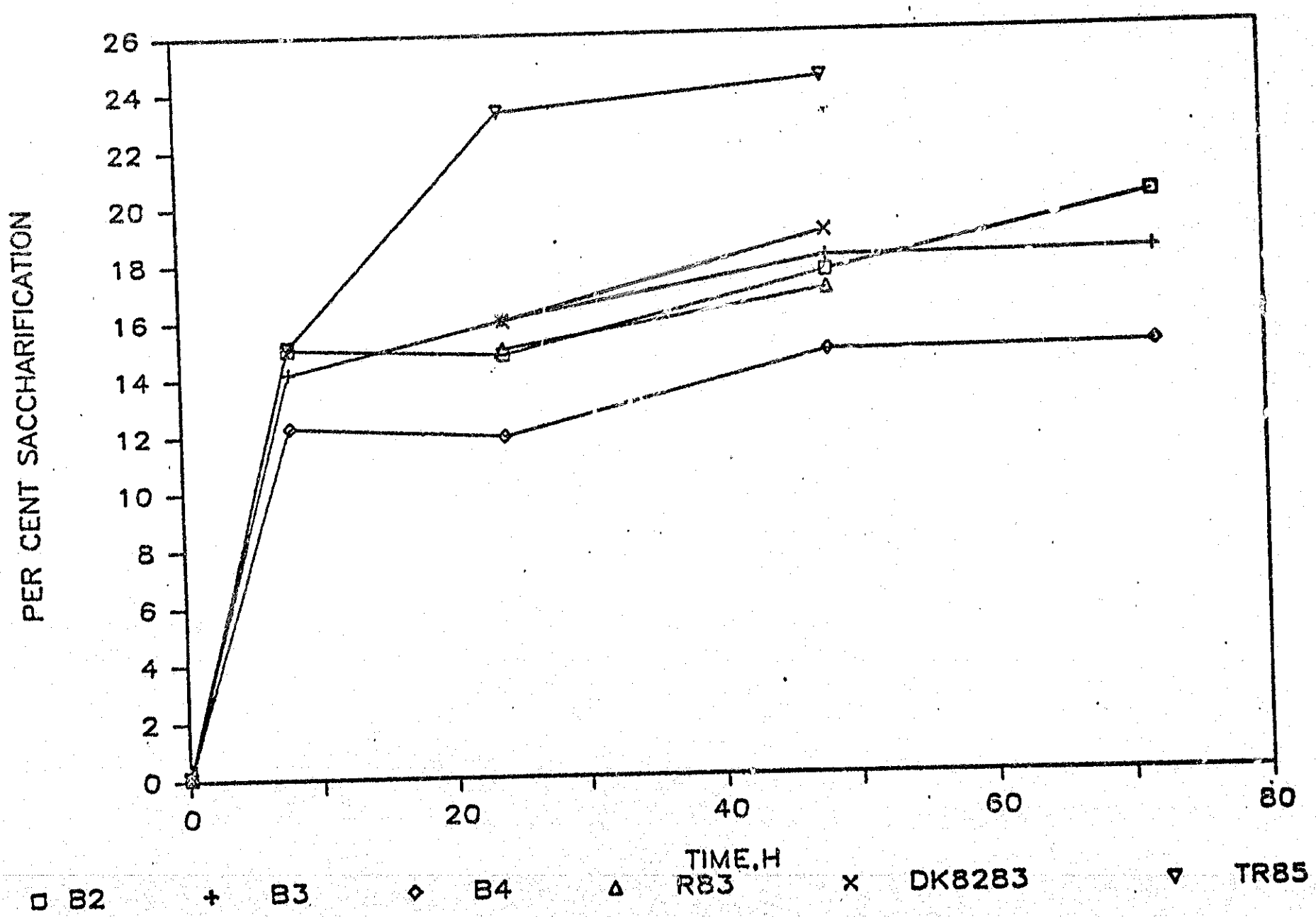
+ BEST - 3

◇ BEST - 4

△ Bagasse[168]

x Bagasse[87,160]

▽ Bagasse[106]


PER CENT SACCHARIFICATION
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
TIME,H
B2
B3
B4
R83
DK8283
TR85

5.

Figure 5. Enzymatic hydrolysis of the holocellulose from alkaline pretreated EX-FERMented sugarcane chip samples.
See text for sample identification.
The % saccharification is based on the initial amount of cellulose plus hemicellulose present in each sample.

□ CAST - 1 (no pretreatment, only used as control)

+ CANA

◇ CACANA

△ CAAM

x BCSODA

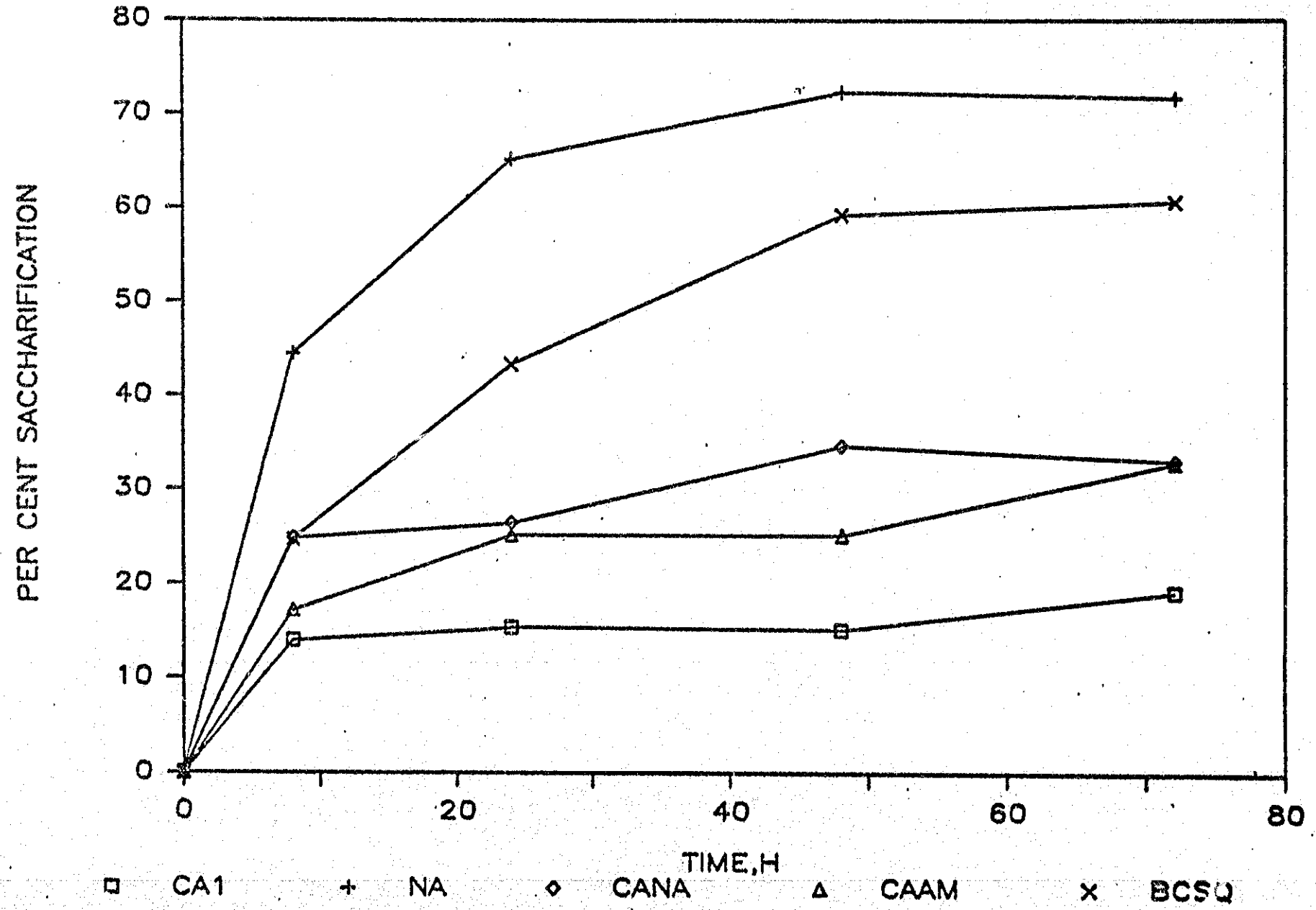

PER CENT SACCHARIFICATION
80
70
60
50
40
30
20
10
0
0
20
40
60
80
TIME,H
CA1
NA
CANA
CAAM
BCSQ

Figure 6. Enzymatic hydrolysis of the holocellulose from solvent pretreated EX-FERMented sugarcane chip samples.
See text for sample identification.
The % saccharification is based on the initial amount of cellulose plus hemicellulose present in each sample.

□ CABG

△ CAOA - 1

+ CAOK - 1

x CAOA - 2

◇ CAOK - 2

▽ CAST - 1
(no pretreatment, only used as control)

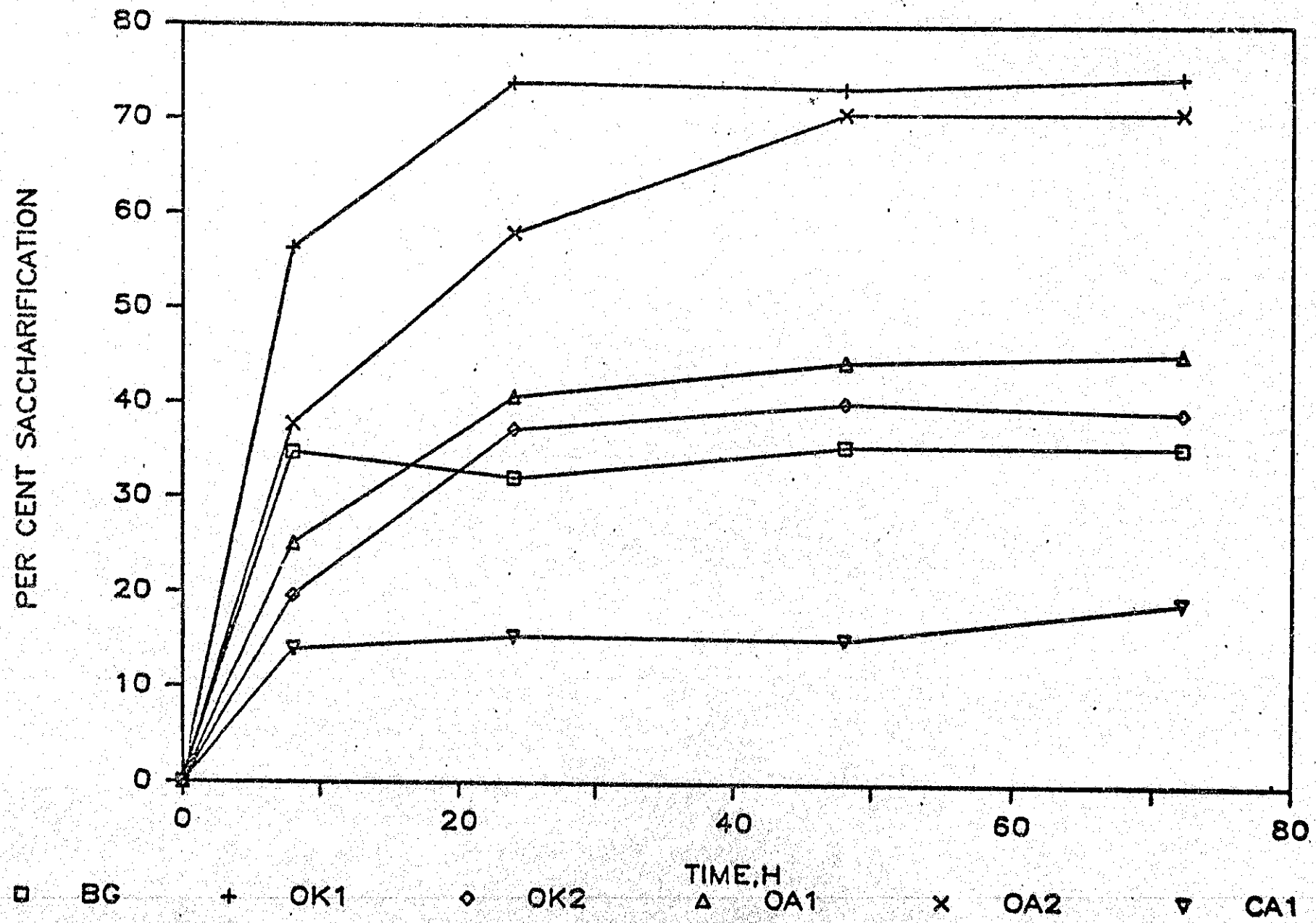

PER CENT SACCHARIFICATION
80
70
60
50
40
30
20
10
0
0
20
40
60
80
TIME,H
BG
OK1
OK2
OA1
OA2
CA1

Figure 7. Enzymatic hydrolysis of the holocellulose from selected pretreated EX-FERMented sugarcane chip samples.
See text for sample identification.
The % saccharification is based on the initial amount of cellulose plus hemicellulose present in each sample.

□ CAST - 1 (no pretreatment, only used as control)

△ CAEX

+ CANA

x CAOK - 1

◇ CASO

▽ CAOA - 2

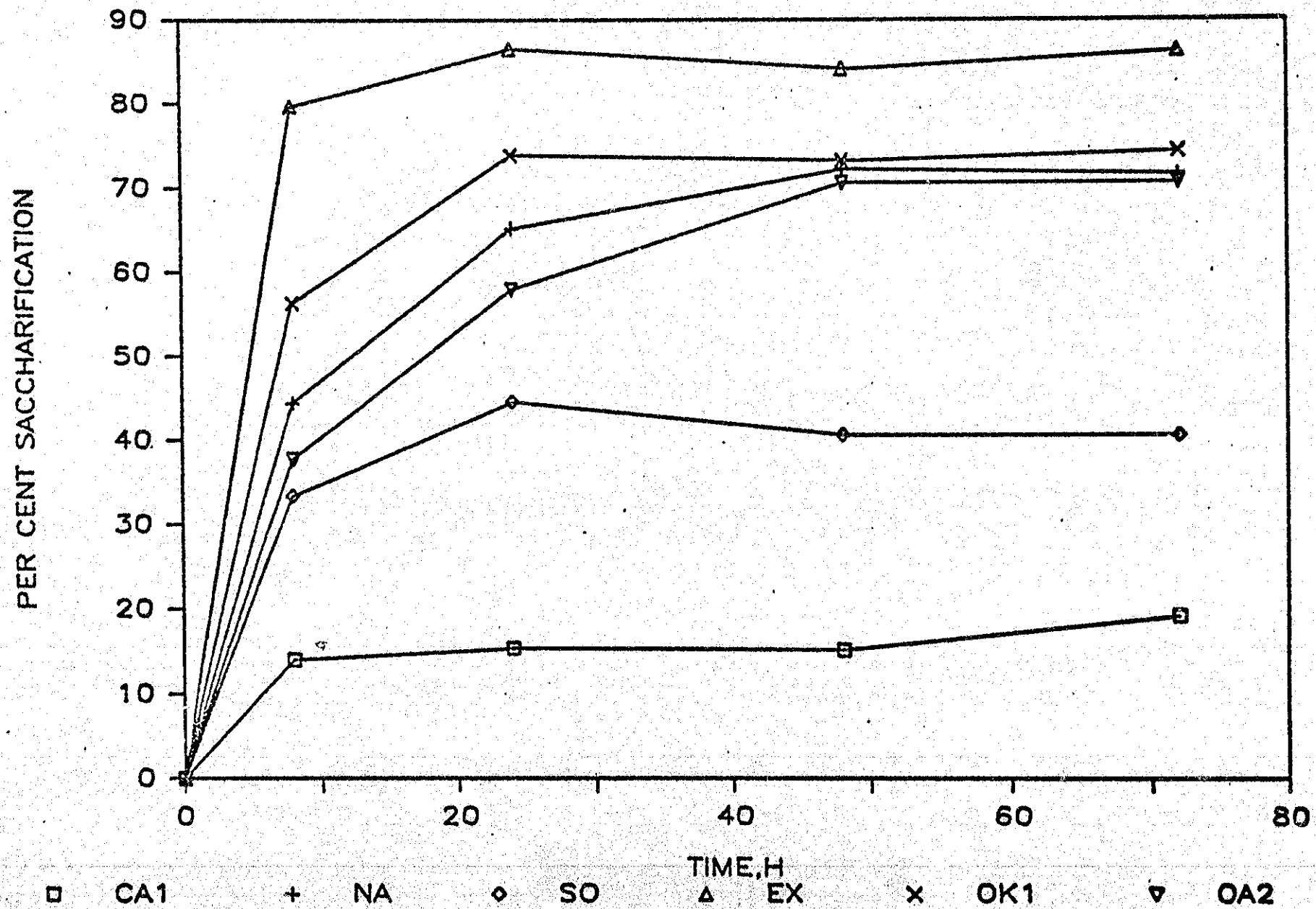

PER CENT SACCHARIFICATION
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
TIME,H
CA1
NA
SO
EX
OK1
OA2

Fig. 4

PAPER No. 2

"Effects of some physical and chemical pretreatments on the composition and enzymatic hydrolysis and digestibility of lemon grass and citronella bagasse"

(Accepted for publication in Agricultural Wastes)

EFFECTS OF SOME PHYSICAL AND CHEMICAL PRETREATMENTS ON THE COMPOSITION AND ENZYMATIC HYDROLYSIS AND DIGESTIBILITY OF LEMON GRASS AND CITRONELLA BAGASSE

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ABSTRACT

The effect of some physical and chemical pretreatments of lemon grass and citronella bagasse on the composition changes, susceptibility to enzymatic hydrolysis and in vitro enzymatic digestibilities was evaluated. Three alkaline chemical pretreatments were tested (NaOH, Na_2CO_3 + $Ca(OH)_2$, NH_3), one acidic (SO_2) and two physical pretreatments (steam explosion and a modified organosolv employing alkaline ethanol-water mixtures with anthraquinone as catalyst). The general response to different pretreatments was similar with the two bagasse samples. However, the observed improvements were greater for lemon grass than for citronella, suggesting than improvements were, overall, species-specific Hemicellulose was hydrolyzed extensively: cellulose was chemically stable and higher lignin losses were observed for the organosolv, the sulfur dioxide and the sodium hydroxide methods. With the steam exploded, the organosolv and the NaOH and SO_2 pretreated materials, saccharification values above 70% were obtained with cellulase compared with about 14% for untreated materials. The in vitro digestibilities with pepsin and cellulase were highest for the organosolv and the NaOH pretreatments, more than 5 times the untreated values for lemon grass and around 3.5 for citronella.

INTRODUCTION

Lemon grass and citronella bagasse are the lignocellulosic residues of steam distilling freshly cut lemon grass and citronella leaves for the recovery of the essential oils. The plants belong to the Gramineae family and have been classified as Cymbopogon citratus and Cymbopogon winterianus respectively. The essential oil content is low, 0.3-0.7% by weight of fresh grass, and its recovery is not complete. After steam distillation, the bagasse is partially dried in the fields and a fraction is burned to generate steam for the stripping; the rest is left in the fields where natural biodegradation takes place (Guenther, 1950). Its use as a ruminant feed is limited due to animal rejection because of the residual aroma and flavor. Several attempts have been made to use it as a source of fiber for paper and board products but the process, at least in Guatemala, has only been taken to pilot plant tests. Lemon grass oil production is around 1000 t per year, being produced by

India, Guatemala, the Republic of China and Sri Lanka. Citronella oil production is around 3000 t per year produced by Indonesia, Sri Lanka, the Republic of China, Taiwan, Guatemala and Brazil (Robbins, 1983). This means that there is a possible worldwide availability of about 200 000 t of dry bagasse per year that could be used as a source of lignocellulosic biomass. The material has been already subjected to a low pressure steam treatment which not only sterilizes it but induces physical and chemical changes in the lignocellulosic matrix.

In order to increase the rate and extent of holocellulose hydrolysis for production of sugars for fermentation or digestible fibre for use as animal feed, a pretreatment of the substrates is required in order to alter significantly the structural characteristics of the lignocellulosic matrix. Such a pretreatment must enhance the close contact between microbe and fibers to give an efficient enzyme action. There seems to be little doubt that the various pretreatments tested so far,alone or in combination enhance enzyme or microbially catalyzed hollocellulose breakdown. In a recent review there was a list of 10 physical, 18 chemical and one biological treatments (Fan et al, 1982). There seems to be, however, only a limited understanding of how these pretreatments enhance biodegradation.
The mode of hollocellulolysis may be quite species-specific, both in terms of the substrate and the microorganism. It is also an heterogeneous reaction and it is therefore influenced by the structural features of the substrate, the interaction between microbe and substrate surface and the complexity of the associated enzymatic system. Most of the research work done so far has employed enzymatic preparations of filamentous fungi grown in submerged culture and temperate grown cereal straws as substrates. It seems that the rate and extent of hydrolysis depends on the amount of substrate surface exposed, the proper adsorption of the biocatalyst, the degree of polymerization of the polysaccharide and miminum lignin, phenolic acid and acetyl contents (Cowling and Brown, 1969; Cowling, 1975; Cowling & Kirk, 1976; Tsao et al, 1978; Sasaki et al, 1979; Fan et al, 1980a,b; 1981a,b; Losyakova et al, 1980; Lee et al, 1982, 1983; Gharpuray et al, 1983; Sinitsyn & Klesov, 1982; Lin et al, 1981; Ryu & Lee, 1982; Gilhert & Tsao, 1983; Puri, 1984; Grethlein, 1984).

The objective of the present work was to evaluate the effect of some physical and chemical pretreatments on the composition susceptibility to enzymatic hydrolysis and **in vitro** enzymatic digestibilities of lemon grass and citronella bagasse and discuss the results as these are, as far as we know, the first data published for such lignocellulosic residues.

METHODS

- Lignocellulosic substrates

Lemon grass and citronella bagasse were obtained from plantations located in the Guatemalan Pacific lowlands at 420 meters above sea level, in June at the start of the rainy season and the harvest of the leaves. They were collected as they came out of the essential oil steam distillery, sun dried to a final moisture around 6-7% and packed in plastic ventilated bags. The control samples were prepared by milling the dried raw materials in a pilot Fitz Mill Comminutor (The Fitzpatrick Co.) with a 0.84 mm sieve. The final powders obtained were packed in plastic bags.

- Substrate pretreatments

The following alkaline pretreatments were done: a) solid state sodium hydroxide: sodium hydroxide was added to the ground dried material at 80 g kg-1 of dry matter. It was added as a concentrated solution in a 1:4 solid to liquid ratio and the amount of raw material used in all cases was 250 g dry matter. The mixture was throughly mixed in a ribbon blender and placed in a polyethylene bag for 10 days at room temperature (25C). Then it was solar dried and stored again in the same bag. b) solid state mixture of sodium carbonate and calcium hydroxide: the amount of both chemicals added was 146 g kg-1 of dry matter of which 73% was sodium carbonate. Due to chemical reaction the effective charge determined by an ash mass balance was 80 g kg-1. The same procedure as before was followed. c) gaseous ammonia: 250 g of dry bagasse was moistened with water to a solid to liquid ratio of 1:3; placed in a Buchner funnel and a stream of gaseous NH_3 passed until saturation. Then the bagasse was transferred to a polyethylene bag and maintained for 10 days at room temperature (25C). It was then solar dried and stored in the same bag. The amount of chemical used determined by weight difference of the substrate after treatment was 290.5 g kg-1 of dry matter. However most was lost during storage as the final ratio was 21.7 g NH_3 kg-1 dry matter.

One gaseous pretreatment was done employing SO_2. 250 g of dry material was moistened with water using a solid to liquid ratio of 1:3, placed in a Buchner funnel and a stream of gaseous SO_2 was passed for 15-20 min until saturation. Then the material was placed in a sealed jar and maintained at 70C for 72 h, after which it was solar dried and transferred to a polyethylene bag. The amount of chemical used determined by weight difference of the substrate after treatment was 185.7 g kg-1 of dry matter. Again, most of it was lost during storage as the final ratio was 36.4 g SO_2 kg-1 dry matter.

One set of 200 g samples was steam exploded by Dr J.N. Saddler of the Eastern Laboratory of Forintek Canada Corp, Ottawa. Their procedure included exposure to steam at 240C for 60s in a small steam explosion gun (Saddler et al, 1982). The yields on dry basis were 75.41% for lemon grass and 75.75% for citronella.

The final pretreatment employed was a modified organosolv process, in which the materials were placed in contact in a solid to liquid ratio of 1:6 with a mixture of 60% ethanol-40%water by weight with 40 g of Na2O kg-1 of dry material and 0.05% of anthraquinone by weight of dry material. The mixture was cooked in a pressurized rotating digestor for 4 h at 175C. After that the liquors were drained, the solid material was washed with water, solar dried and stored in polyethylene bags. The yield was 55.66% for lemon grass and 56.66% for citronella.

The identifications used for samples were: untreated lemon grass TEST; untreated citronella CIST; solid state sodium hydroxide treated lemon grass TENA, and citronella CINA; solid state sodium carbonate plus calcium hydroxide treated lemon grass TECANA, and citronella CICANA; gaseous ammonia treated lemon grass TEAM, and citronella CIAM; gaseous sulfur dioxide treated lemon grass TESO, and citronella CISO; steam exploded lemon grass TEEX, and citronella CIEX; and alkali organosolv treated lemon grass TEOA, and citronella CIOA.

- Analysis

The samples were analyzed for Kjeldahl nitrogen, ash content and residual essential oil (AOAC, 1975) and for neutral detergent fiber, acid detergent fiber, permanganate lignin and cellulose following Goering & Van Soest (1970) and Van Soest & Robertson (1980). In vitro dry and organic matter enzymatic digestibilities were determined by following the methodology suggested by Goto & Minson (1977); Adegbola & Paladines (1977); McLeod & Minson (1978, 1980); Dowman & Collins (1982). In summary, 200 mg of the material were suspended in 20 ml of a 2 gl-1 acid pepsin solution (No. P-7125 Sigma Chemical Co.) and incubated at 40C for 48h. Samples were centrifuged, washed once with distilled water, centrifuged and suspended in 20 ml of a 2.5% by weight solution of fungal cellulase Onozuka FA (Maruzen Chemicals Co. Ltd) in 0.2M acetate buffer pH 4.8 and incubated at 40C for 48h. The suspension was filtered in sintered glass crucibles, washed with water and weighed. Controls were done with distilled water and no enzyme. During incubation the tubes were agitated twice a day.

- Enzymatic hydrolysis

100 g of the material were suspended in 9 ml of 0.2M acetate buffer pH 4.8 containing 1 mg thimerosal 100 ml-1 as preservative. One ml of a 1% by weight solution of cellulase (Onozuka FA in 0.2M acetate buffer pH 4.8) was added and the sealed tubes were placed in a rotary shaker (New Brunswick Co) at 40C and 300 rpm. Samples were taken at 8, 24, 48 and 72 h, the solids discarded and sugars determined in the liquid by the dinitrosalicylic acid method (Hodge

& Hofreiter, 1962). Controls were done with distilled water in place of enzyme. The filter paper activity of the enzyme solution was 0.04 units mg-1 protein (Canevascini & Gattlen, 1981). Soluble protein was determined by the Lowry method (1951).

RESULTS

The analyses of the raw bagasse samples are given in Table 1. The two Cymbopogon plants are similar. The hollocellulose fraction, amenable to enzymatic hydrolysis, was 58.4% for lemongrass and 58.5% (dry wt.) for citronella. The corresponding figures on ash free basis were 65.2 and 64.1% respectively.

With the analytical data of the solid samples from each pretreatment and the corresponding solid yields a mass balance of the lignocellulosic fraction was made. The results are shown in Figure 1 where they are expressed as % change with respect to the values reported in Table 1. There was a similar general response to different pretreatments with the two bagasse samples (Figure 1). Most the pretreatments increased the water soluble substances substantially, the sodium hydroxide pretreatment being the most effective. The only exceptions were the organosolv samples. However only these samples were water washed after pretreatment. Highest delignifications were observed for the organosolv, the sodium hydroxide the sulfur dioxide and the sodium carbonate-calcium hydroxide mixture treatments, with the highest value a decrease of 61% for TEOA. Practically the only pretreatment that produced a significant decrease in the cellulose fraction was the steam explosion technique in which 13-17% was hydrolyzed. With exception of the ammonium and sodium carbonate-calcium hydroxide pretreatments, the pretreatments induced a rather high hemicellulose hydrolysis of above 50%; it was practically complete for the steam-explosion method.

The results of the determinations of fungal cellulase hydrolysis are in Figures 2-5. The data are expressed as per cent saccharification of the hollocellulose fraction of each pretreated sample. The reducing sugars in the enzyme digests minus the ones in the control (no enzyme) were determined, hence these values represent true products of enzyme hydrolysis and do not take into account the soluble sugars produced during the pretreatment step. Again, there were more similarities than differences in the effects of the different pretreatments on both substrates. All the pretreatments were effective in enhancing the enzymic hydrolysis. The steam exploded, the organosolv and the sodium hydroxide and sulfur dioxide pretreated materials were most extensively attacked and in which above 70% saccharification values were obtained early in the digestion.

The digestibilities in Table 2 represent weight loss due to enzymatic action in terms of initial total dry matter (IVDMED) and

initial total organic matter (IVOMED), but taking into account the weight loss of the control (no enzyme) samples. Hence, again these figures represent true digestible matter susceptible to enzyme attack. All the pretreatments were again effective; the observed changes for lemon grass were superior to the ones for citronella, probably due to the lower digestibility for the untreated sample of the former. However, in both cases the organosolv and sodium hydroxide treated samples showed the greater increases in digestibility: more than 5 times for lemon grass and around 3.5 for citronella.

The percent saccharification and the enzymatic digestibility data are not equal. Nevertheless it is easy to transform one into the other. If the percent saccharification data at 72 h from Figures 2-5 are converted into a digestibility, they are, on the average, 33% lower for lemon grass and 41% lower for citronella relative to the IVDMD values of Table 2. The fact that they are lower might reflect the hydrolyzing action of acid-pepsin in the two-enzyme digestibility assay. Dowman & Collins (1982) comment that the use of either acid-pepsin or neutral detergent solution is recommended before the use of cellulases for determining forage digestibility, since in this way more insoluble material is hydrolyzed by the enzymatic action and the results resemble more digestion in vivo.

DISCUSSION

The research described in this paper is part of an effort to convert lignocellulosic residues to organic acids through anaerobic fermentation. The resistance of the untreated residues to attack by microorganisms and enzymes led to the investigation of pretreatment methods, with the results of the work on lemon grass and citronella bagasse described here. The data in Table 1 are, to our knowledge, the first of its kind reported for these materials, however they must be taken with caution as individual components may vary within Cymbopogon species cultivated under different ecosystems and of course with plant maturity. The results of the chemical analysis show similarity to the leaves (sheath and blade) of common straws (Aman & Nordkvist, 1983). In terms of their NDF and ADF contents they show some similarity to the common straws and they are of intermediate lignin content, again being close to the straws and sugarcane bagasse (Jackson, 1977; Klopfenstein, 1981; Ohlde & Becker, 1982).

The effect of the alkali treatments on the chemical composition of the bagasse as shown in Figure 1 was to dissolve hemicellulose and lignin, to drastically increase the soluble solids and to slightly decrease cellulose. The effects of sodium hydroxide on the in vitro dry and organic matter enzymatic digestibilities shown in Table 2 are extremely favorable, an improvement ratio of 2.7 to 4.8 over the untreated samples. There is experimental evidence that both alkali-labile and alkali-resistant lignin-carbohydrate bonds exist and that their ratio in a particular plant material governs

the decision to use alkali as a pretreatment to increase its digestibility (Chesson, 1981). In lemon grass and citronella bagasse these bonds seemed quite labile to alkaline conditions; since water soluble compounds increased drastically as shown in Figure 1, and also the NDF values decreased from 72.6 and 72.0 to 48.2 and 44.7 respectively. Recently Scalbert et al (1985) found for wheat straw that ferulic acid ethers cross-link betwen hemicelluloses and lignin. These are alkali labile and might explain the high solubility of straw lignins in soda. It could also be argued that the alkaline treatment modified the small amount of essential oils still present in the bagasse, hence removing any inhibitory effects upon the enzymes. In order to check this matter bagasse samples were extracted with ether for 48h, removing 2.13 g 100 g-1 dry matter for citronella and 1.34 g 100 g-1 dry matter for lemon grass. The corresponding IVDMED and IVOMED values were 19.45, 26.06 for citronella and 16.06 and 22.28 for lemon grass respectively. These were not statistically different so that the inhibition hypothesis seems not valid.

The effects of calcium hydroxide were of the same general trend as sodium hydroxide, but were less marked. Less soluble solids were produced and less lignin and hemicellulose were degraded. As shown in Table 2 the IVDMED and IVOMED values were from 1.6 to 1.9 times less for the calcium hydroxide pretreatments relatively to sodium hydroxide.

The ammonia pretreatment were also typical and did not differ in their basic effects upon cell walls of graminaceous plants as suggested by Van Soest et al (1984); however it was different for both raw materials, illustrating again with this the variable response obtained with different materials. As shown in Figure 2, soluble solids increased much more in lemon grass (above 70%) than in citronella; hemicellulose was degraded about the same (around 25%), but lignin loss was much higher in citronella (about 20%). Modification, including hydrolysis of the lignin-polysaccharide bond has been shown to be the major result of alkaline pretreatments (Klopfenstein, 1978). The extent of saccharification obtained for lemon grass was five times the control; a similar figure for citronella was four times (Figures 2 and 3).

The results for SO2 pretreated lemon grass and citronella are shown in Figure 1. Cellulose remained unchanged, soluble solids increased at the expense of hemicellulose hydrolysis; permanganate lignin decreased substantially, 25 to 40%. The per cent saccharification values shown in Figures 2 and 3 were among the highest obtained comparable with the ones obtained with sodium hydroxide. Improvements in IVDMED and IVOMED were relatively high as shown in Table 2.

The modified organosolv pretreatment caused the greatest delignification of all methods tested, accompanied with a substantial loss of hemicelluloses, as shown in Figure 1. This behavior is typical in this process as clearly shown, among others, by Phillips and Humphrey (1983a,b) for poplar wood, in which

regardless of the treatment conditions used, the lignin removal was always accompanied by hemicellulose removal in a linear correlation among the two. In Figure 4 and 5 it is shown that for both lemon grass and citronella steam explosion solids were 100% enzymatically saccharified, reflecting an improvement of 10 times on the cellulose susceptibility to enzymatic action. It is easily seen on Table 2 that the in vitro enzymatic digestibilities were increased from 4 to 5 fold for lemon grass and from 3 to 4 fold for citronella. These figures are the highest of all pretreatments. To our knowledge there are no data for this parameter for other organosolv pretreated lignocellulosic materials.

Steam pretreatment has been used with wood for the production of hardboard and feed for ruminants. Studies with wood have shown that the lignocellulosic matrix is modified drastically and that the remaining solids are more susceptible to enzymatic hydrolysis. The structural changes and chemical reactions taking place are a function of temperature and time of pretreatment. In Figure 1 it is easily seen that this pretreatment caused the largest decrease of hemicelluloses of all those tested, being practically 100%. There were also lignin and cellulose losses. No data have been reported to our knowledge in the technical literature for similar materials; Guggolz et al (1971) treated with steam without explosion nine grasses but no chemical composition changes were reported. The solid yields obtained for steam exploded lemon grass and citronella at 240C (242 MF[illegible] and 60s were about 75%, within expected values. Figures 4 and [illegible] show saccharification values of 100% for both citronella and lemon grass. These results can be attributed to the opening of the cellulose matrix, permiting free contact by the enzymes.

The results of the pretreatments indicate that the organosolv pretreatment should be the most effective in preparing the substrates for further enzymatic and biological degradation, not only because of high degree of delignification but also due to the increased susceptibility of cellulose to enzymatic hydrolysis. Steam explosion and solid state sodium hydroxide also appear promising. Lemon grass should be the more favorable starting material.

CONCLUSIONS

The three alkaline pretreatments, the SO_2, steam explosion and organosolv fractionation were quite effective in inducing the necessary physical and chemical changes in lemon grass and citronella bagasse that substantially increased their susceptibility to enzymatic hydrolysis. The solids yields were around 75% and 55% for steam explosion and organosolv respectively. All the pretreatments increased the water soluble substances, with exception of organosolv, which however was the only water washed sample. Higher delignifications were observed for organosolv, SO_2 and the sodium hydroxide methods. In most pretreatments a 50% loss of hemicelluloses was observed; it was practically complete for the steam explosion method. The steam exploded, organosolv, NaOH and SO_2 pretreated materials were more extensively attacked by enzymatic action. The observed changes were in general better for

lemon grass than the ones for citronella. Results show that pretreatment effects are indeed species-specific, and clearly point out that the enhancement to enzymatic hydrolysis is also not only due to the extent of delignification of the raw material but also to the effect on physical properties of the hollocellulose.

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TABLE 1

CHEMICAL ANALYSIS OF RAW BAGASSE

	TEST *	CIST *
Neutral detergent fiber (NDF)	72.6	72.0
Acid detergent fiber (ADF)	44.1	42.0
Permanganate lignin	11.0	11.1
Cellulose	29.9	28.5
Hemicellulose (NDF-ADF)	28.5	30.0
Ash	11.0	9.3
Crude protein (Kjeldahl nitrogen x 6.25)	5.1	4.5
Essential oil	Traces	Traces
Water soluble substances[a]	16.2	23.3

* For abbreviation see Methods.
a Determined in the controls of the *in vitro* dry and organic matter enzymatic digestibilities (see Table 2).
All results % dry matter.

TABLE 2

IN VITRO DRY MATTER (IVDMED) AND ORGANIC MATTER (IVOMED) ENZYMATIC DIGESTIBILITIES*

SAMPLE[a]	IVDMED, %	CHANGE[b]	IVOMED, %	CHANGE[b]
Lemongrass:				
TENA	66.14	4.79	71.03	3.85
TECANA	40.80	2.96	38.66	2.10
TEAM	41.12	2.98	54.18	2.94
TESO	49.49	3.59	62.18	3.37
TEEX	47.76	3.46	62.90	3.41
TEOA	73.57	5.33	79.63	4.32
TEST	13.80±1.60[c]	1.00	18.44±3.40	1.00
Citronella:				
CINA	67.10	3.68	65.80	2.67
CICANA	37.87	2.08	34.49	1.40
CIAM	45.86	2.51	57.16	2.32
CISO	56.86	3.12	68.28	2.77
CIEX	47.12	2.58	69.10	2.40
CIOA	70.44	3.86	73.51	2.98
CIST	18.24±2.02[c]	1.00	24.63±3.81	1.00

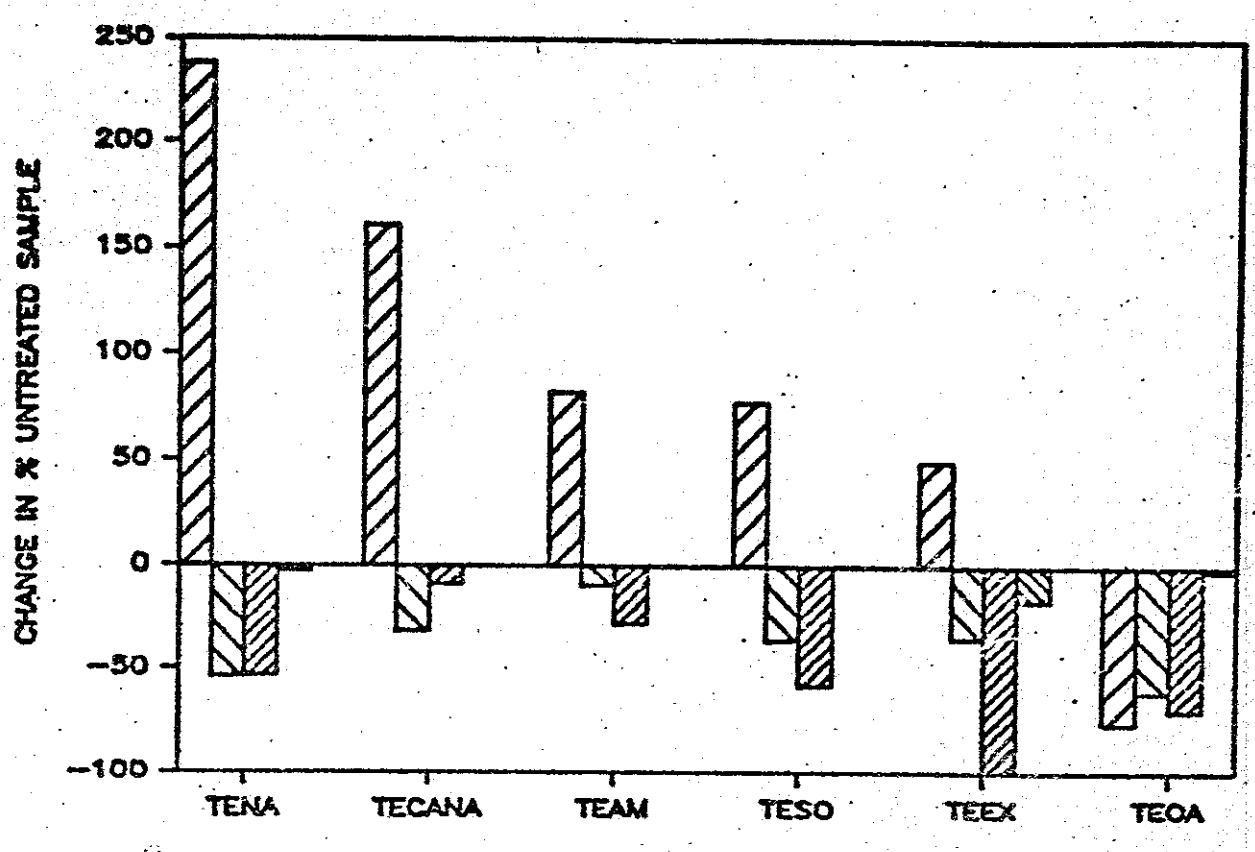
* Pepsin-cellulose treatment, see Methods.
a For abbreviations, see Methods.
b Ratio between pretreated and unpretreated (TEST and CIST) samples
c 95% confidence interval

FIGURE 1

PER CENT CHANGE OF THE LIGNOCELLULOSIC FRACTION AND SOLUBLE SOLIDS FOR THE PRETREATED SAMPLES. FOR ABBREVIATIONS SEE METHODS. FOR ANALYSIS OF UNTREATED SAMPLES SEE TABLE 1.

, soluble solids. , hemicellulose.

, lignin. , cellulose.


CHANGE IN % UNTREATED SAMPLE
250
200
150
100
50
0
-50
-100
TENA
TECANA
TEAM
TESO
TEEX
TEOA

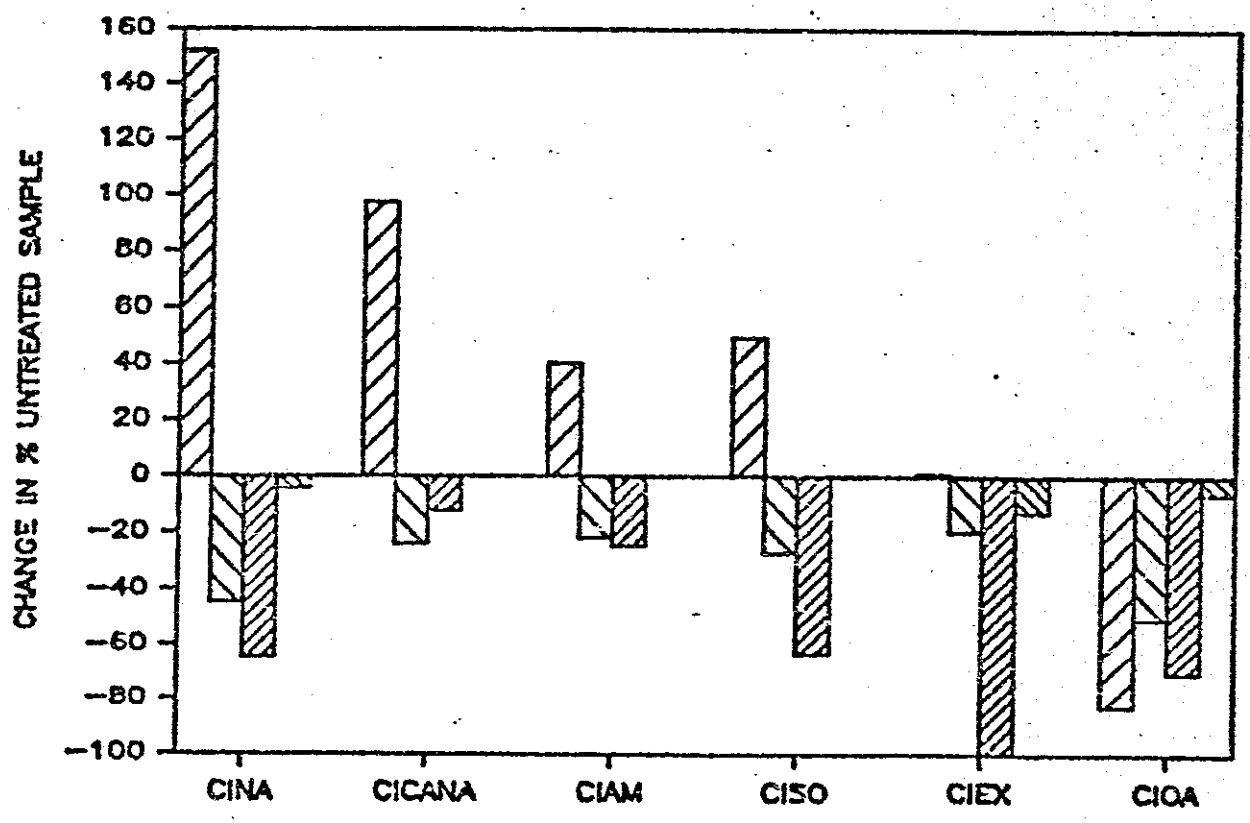

CHANGE IN % UNTREATED SAMPLE
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
CINA
CICANA
CIAM
CISO
CIEX
CIOA

FIGURE 2

CELLULASE HYDROLYSIS OF THE HOLOCELLULOSE FROM UNTREATED AND PRETREATED LEMON GRASS SAMPLES. FOR ABBREVIATIONS SEE METHODS. ◇ TEST. X TEAM. + TECANA. △ TESO. □ TENA.

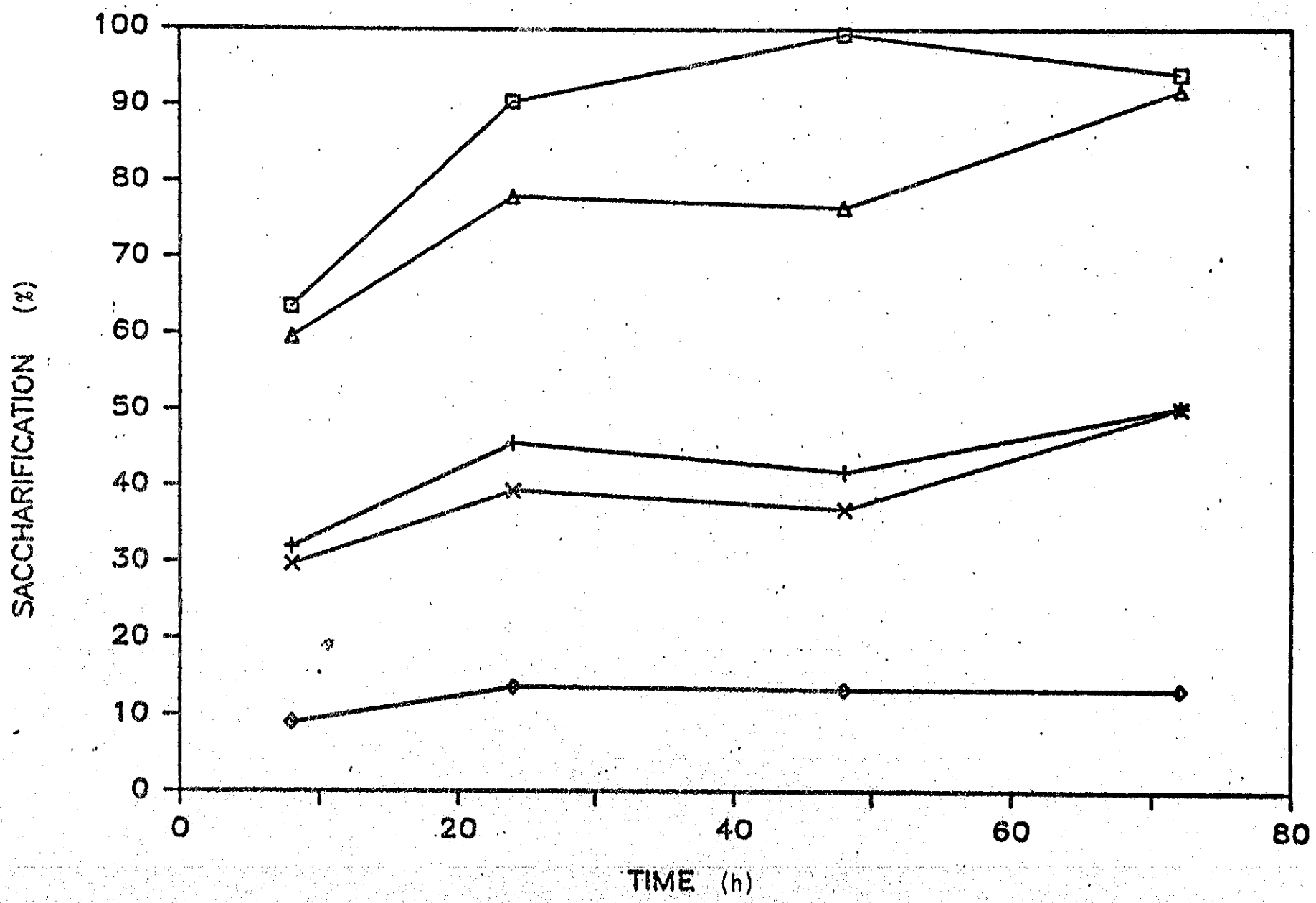

SACCHARIFICATION (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
TIME (h)

FIGURE 3

CELLULASE HYDROLYSIS OF HOLOCELLULOSE FROM PRETREATED CITRONELLA SAMPLES. FOR ABBREVIATIONS, SEE METHODS.

◇ CIST. X CIAM. + CICANA □ CINA. △ CISO.

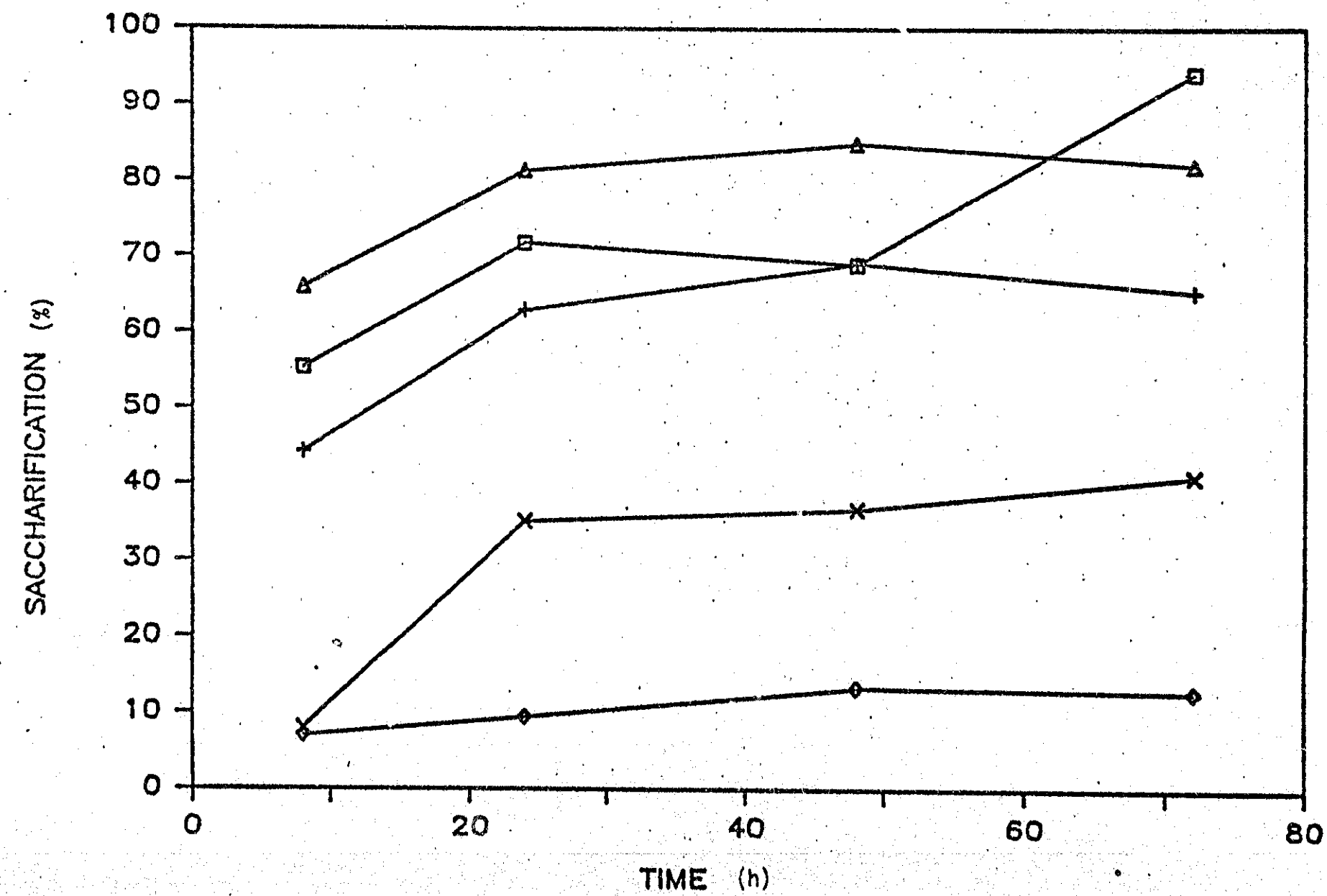

SACCHARIFICATION (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
TIME (h)

FIGURE 4

CELLULASE HYDROLYSIS OF THE HOLOCELLULOSE FROM PRETREATED LEMON GRASS SAMPLES. FOR ABBREVIATIONS, SEE METHODS.

□ TEST. + TEOA. ◇ TEEX.

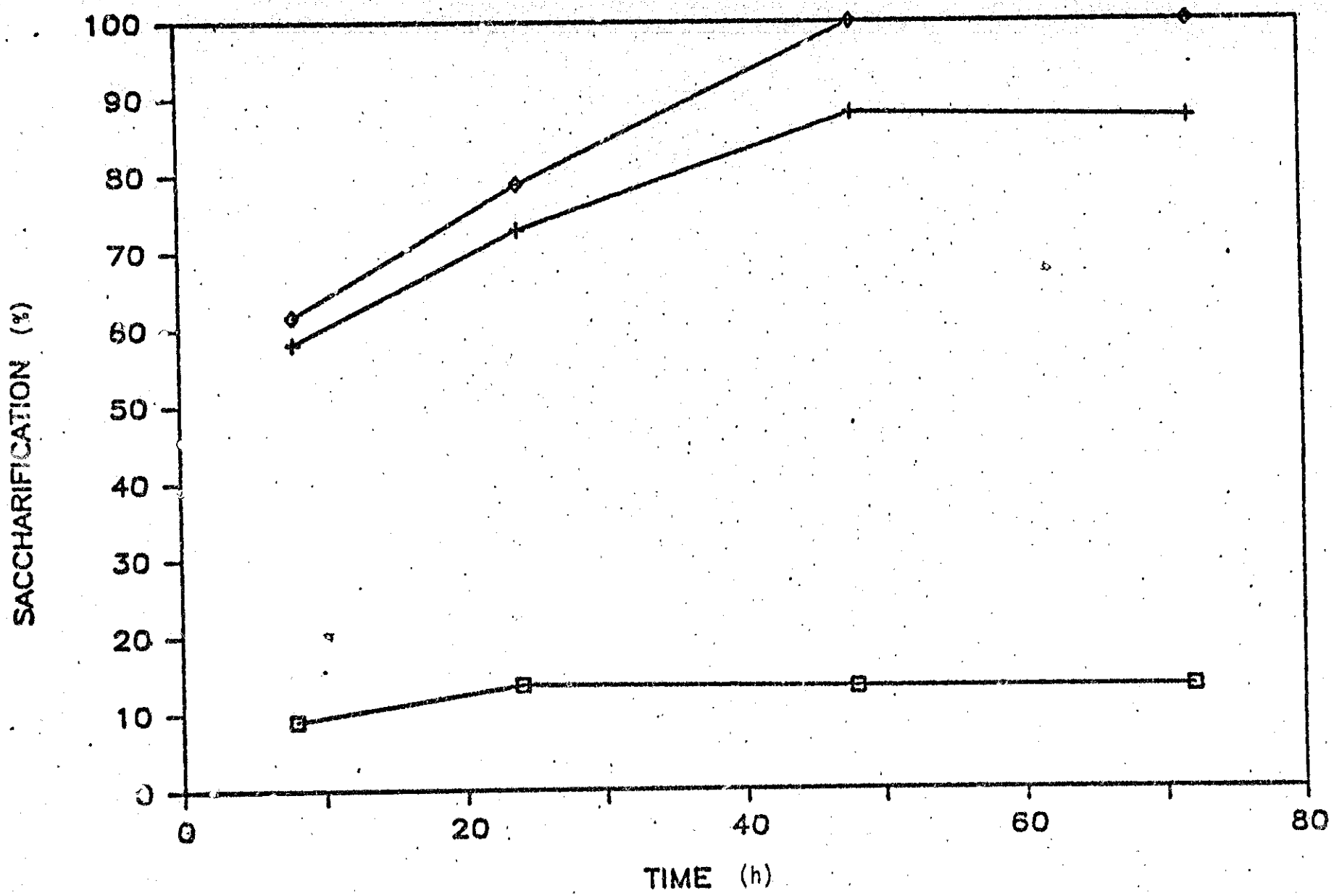
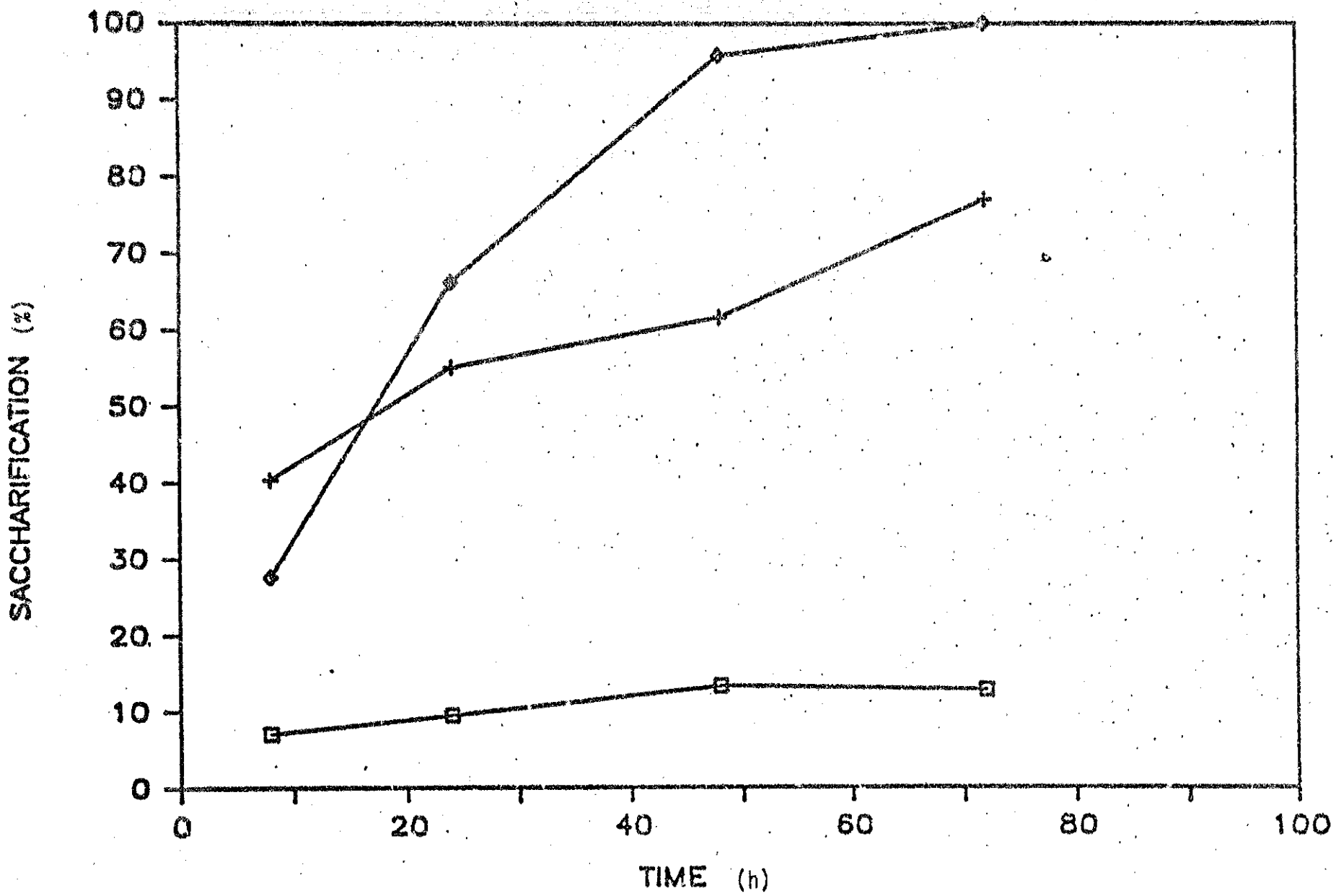

SACCHARIFICATION (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
TIME (h)

FIGURE 5

CELLULASE HYDROLYSIS OF THE HOLOCELLULOSE FROM PRETREATED CITRONELLA SAMPLES. FOR ABBREVIATIONS, SEE METHODS.

□ CIST. ◇ CIEX. + CIOA.


SACCHARIFICATION (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
TIME (h)

PAPER No. 3

"White-rot fungal growth on sugarcane lignocellulosic residue"

(Accepted for publication by Applied Microbiology and Biotechnology)

WHITE-ROT FUNGAL GROWTH ON SUGARCANE LIGNOCELLULOSIC RESIDUE

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Summary

Twelve white-rot fungi were grown in solid state culture on sugarcane chips previously fermented by yeast employing the EX-FERM process. The lignocellulosic sugarcane residue had 12.5% permanganate lignin and 81.3% holocellulose. All fungi after five to six weeks at 20C produced a solid residue which had a lower **in vitro** dry matter enzymatic digestibility than the original bagasse, with the exception of **Coriolus versicolor** which showed a slight increase of six units. Lignin losses were rather similar for all fungi tested, an average value of 38.64% of the original value was obtained. About the same amount of hemicellulose was degraded, 32.22%, indicating a close association between lignin and hemicellulose in sugarcane bagasse probably as a lignin-hemicellulose complex. Most fungi showed a preference for hemicellulose hydrolysis over cellulose degradation. The two fungi that showed greater cellulolytic activity were **Sporotrichum pulverulentum** and **Dichomitus squalens**. No appreciable dry matter losses were detected for **Agrocybe aergerita** and **Flaminulina velutipes**. Three more fungi were below a loss of 10% of the initial dry weight and the rest all were below 17%.

Introduction

In natural decomposition of lignocellulosic matter, both fungi and aerobic bacteria play an important role in degrading holocellulose and lignin to lower molecular weight compounds, some of which are then further metabolized by facultative and obligate anaerobic soil bacteria and actinomycetes (Bazin et al, 1986; Sorensen, 1977; Maccubin and Hodson, 1980). Sugarcane bagasse is no exception (Schmidt and Walter, 1978; Sandhu and Sidho, 1980). Fungi are usually better lignin degraders, particularly the so-called white rots which completely metabolize the complex polymer, exhibit the highest reported rates and have been the most studied (Kirk and Chang, 1981; Crawford and Crawford, 1984). Most of them, however, have the enzymatic capacity to use cellulose, hemicellulose and other components of lignocellulosic matter as a source of carbon and energy; hence total biomass breakdown usually occurs and lignin removal is accompanied by removal of polysaccharides (Kirk and Moore, 1972). Under certain conditions white rots use lignin preferentially, opening up the

cellulose-hemicellulose matrix, making the solid more susceptible to further enzymatic action by other microorganisms, rumen bacteria for example. These conditions, are specific for fungi-substrate combinations. Also some of the fungi produce fruiting bodies when growth occurs under solid substrate conditions. These two points make solid state fungal delignification an attractive alternative for converting lignocellulose into human food (fruiting bodies of edible fungi) and animal feed (solid residue with improved digestibility). Recently an increase of research on this subject has been reported commented briefly by Rolz and Humphrey, 1982;and Rolz, 1984 . Most of the work has been done with cereal straws from temperate countries and very few tropical lignocellulosic residues have been studied. Although the effects of temperature, pH, carbon and energy sources, nitrogen and oxygen availability on the ligninolytic activity have been studied, there is no clear picture on how to enhance its rate or optimize a production system. Moreover fungal responses have varied tremendously; there are reports of little lignin degradation associated with a decrease in substrate digestibility after treatment. There is still controversy if fruiting of the fungi benefits lignin degradation. Fundamental research in lignin degradation has been centred on attempts to identify the microbial catalysts involved in lignin decay, how they are expressed and regulated and what interactions exist with the carbohydrate hydrolytic enzymatic complex (Eriksson, 1984).

The fibrous residue remaining after juice extraction from the sugarcane stalk is commonly referred as bagasse. In the sugar mills most of the bagasse is used as fuel, and although the figure varies within each factory, energy efficient units can have 2 t of surplus bagasse on dry basis for 100 t of fresh cane processed (Paturau, 1982). In independent fuel-ethanol distilleries part of this surplus is used for ethanol purification making the overall system net energy-positive, either for mechanized or for labor intensive agriculture (Hopkinson and Day, 1980). Nevertheless, even here, a surplus is possible if a new demand for bagasse is developed, either by applying energy saving options in the ethanol separation or employing new technologies for processing cane directly into ethanol like the EX-FERM process (Rolz, 1981).

The sugarcane lignocellulosic residue left after sucrose conversion into ethanol by the EX-FERM process will be referred here as EX-FERMented sugarcane chips.

In terms of macrocomponents the EX-FERMented sugarcane chips are very similar to sugarcane bagasse from industrial mills; the average values for cellulose, hemicellulose and lignin for EX-FERMented chips from four cane varieties were 44.8, 37.7 and 13.5% respectively. The corresponding figures for three bagasse samples were 42.1, 37.0 and 14.6% respectively. The lignin content of the samples is slightly higher than most cereal straws (Jackson, 1977), however digestibility is rather

low; an extensive literature search on sugarcane bagasse gave an **in vitro** dry matter enzymatic digestibility (IVDMED) of 17.2 + 4.5% and an **in vitro** dry matter digestibility (IVDMD measured by employing ruminal fluid or the nylon bag technique) of 26.0 + 4.4% (Rolz et al, 1986). Hence sugarcane lignocellulose is rather resistant to enzymatic hydrolysis-delignification.

Ibrahim and Pearce (1980) studied the effects of eleven white rot fungi on the composition and **in vitro** digestibility of bagasse and found that greater delignification and increased digestibility was obtained with **Peniophora gigantea**. Sengupta et al (1984) tested **Termitomyces clypeatus** and Madan and Bisaria (1984), **Pleurotus sajor-caju**. Nigam and Prabhu (1985a,b) isolated three basidiomycetes which showed delignification values up to 59%; unfortunately cellulose and hemicellulose was also degraded extensively.

In this contribution, we report solid state culture of twelve basidiomycetes on EX-FERMented cane chips and their effect on the chemical composition and IVDMED values. None of the basidiomycetes tested were previously studied by Ibrahim and Pearce (1980).

Materials and methods

- Microorganisms. In Table 1 the twelve basidiomycetes are identified and source documented. They were kept on PDA agar plus 3% yeast extract.

- Substrate. Sugarcane was sampled in a sugar mill in the South Coast of Guatemala located around 300 meters above sea level, during the month of April which was within the later part of the crop during the dry season. The sugarcane sample came from the receiving yard and usually one or more cane varieties were being processed on the same day. The cane had been cut by hand and was not burned in the field. It was chipped in a pilot wood chipper (Type 9-72 OY Santsalo). The chips were placed in 7000 l tanks, water was added to just cover them and 2% of commercial baker's yeast was added. The EX-FERM cycle to convert all the sucrose into ethanol took 48 h (Rolz, 1981). The fermented chips were pressed in a continuous screwpress (Model VP-GF, Vincent Corp.), washed with water in a mechanically agitated tank and screwpressed again. They were finally sun dried to a final moisture of around 6-7% and packed in plastic ventilated bags. A chip size between 0.5 - 2.2 cm was separated on circular hole screens (vibrating screen TMI 16-1).

The chemical composition on dry weight basis was: permanganate lignin 12.5%, cellulose 43.3%, hemicellulose 38.0%, ash 2.4% and protein 1.2%. Its neutral detergent fiber value, NDF, or cell wall components, was 92.8. This value checks with the sum of the individual contents for lignin, hemicellulose and

cellulose. Its IVDMED was 19.04% and its saccharification rate at 8 h was 1.51 mg reducing sugars h-1. The soluble solids determined in the IVDMED test were 4.18% of dry weight.

- Inoculation and incubation. A recently inoculated culture tube for each fungi was left for 5 to 6 days at ambient temperature (20C). This was transferred to a petri dish with the same media. Usually it took 9-10 days at ambient temperature to cover the whole surface. If the dish was free of any contaminant, as seen macroscopically, one sixth of its contents were used to inoculate a 700 ml wide mouth glass, jar with metal cap, in which 50 g of dried substrate plus 100 ml of water had been placed and sterilized for 30 min at 125C. Duplicate jars for each fungal culture were left for five to six weeks at ambient temperature until fungal growth had completely covered the substrate surface. The jars were covered loosely with the metal caps and they were oxygenated every week for 15 min by introducing a flow of air through a tube connection installed within the metal cap. The entire jar contents were dried at 60C in a forced air laboratory dryer.

- Chemical analysis. The dried samples were milled in a laboratory Wiley mill employing a 0.84 mm sieve. The following analysis were made: Kjeldahl nitrogen and ash content (AOAC, 1975); neutral and acid detergent fiber, permanganate lignin and cellulose following the Van Soest techniques (Goering and Van Soest, 1970; Van Soest and Robertson, 1980). In vitro dry matter enzymatic digestibilities (IVDMED) were determined as suggested by Goto and Minson (1977), Adegbola and Paladines (1977), McLeod and Minson (1978, 1980) and Dowman and Collins (1982). Briefly, 200 mg of the material were suspended in 20 ml of a 2 g l-1 pepsin solution (No. P-7125 Sigma Chemical Co.) in 1N HCl and incubated at 40C for 48 h. Samples were centrifuged, washed once with distilled water, centrifuged and suspended in 20 ml of a 2.5% by weight solution of fungal Onozuka FA cellulase (Maruzen Chemical Co. Ltd.) in 0.2M acetate buffer pH 4.8 and incubated at 40C for 48 h. The suspension was filtered in a glass filter crucible, washed with water dried and weighed. Controls used distilled water instead of enzyme. During incubation the tubes were agitated twice a day. In order to obtain the saccharification rate, 100 mg of cane lignocellulose were suspended in 9 ml of 0.2M acetate buffer at pH 4.8. One ml of a 1% by weight solution of Onozuka FA cellulase in 0.2M acetate buffer pH 4.8 was added and the sealed tubes were placed in a rotary shaker (New Brunswick Co.) at 40C and 300 rpm. At 8 h the solids were discarded and sugars determined in the liquid by the dinitrosalicylic acid method (Miller, 1959). Controls used distilled water instead of enzyme. The filter paper activity of the enzyme solution was 0.02 units mg-1 protein. Soluble protein was determined by the Lowry method (Lowry et al, 1951). The IVDMED values represent weight loss due to enzymatic action in terms of initial dry matter of the sample but taking into account the weight loss of the control sample. Saccharification rates measured reducing sugars produced in 8 h by enzymatic action again taking into

account any reducing sugars present in the control sample. So both parameters indicate net enzymatic action upon the substrate. Of course the figures can be converted from one to the other, however note that IVDMED was measured after 72 h employing 25 times more enzyme per unit weight of substrate.

- Statistical procedures. Analysis of variance was performed on the IVDMED values using the F test. Those significant different from the control at the 0.95 level are indicated in the corresponding table.

Results and discussion

The IVDMED and saccharification rates, dry weight, lignin, cellulose and hemicellulose losses and production of soluble solids are given in Table 1. Individual component losses are expressed as relative values as % of the original value in the control and were obtained using the analytical data for each component, the solid yield and mass balance. The procedure will be exemplified for the first fungi in Table 1, **P. chrysosporium**: initial lignin content: (50 g) (0.125) = 6.25 g; initial cellulose content: (50 g) (0.433) = 21.65 g; initial hemicellulose content: (50 g) (.38) = 19.0 g; % lignin on residue after fungal growth according to analysis = 4.8% dry weight; % cellulose on residue after fungal growth according to analysis = 42.0% dry weight; % hemicellulose on residue after fungal growth according to analysis = 4.2% dry weight; final lignin content = (50 - 5.48) (0.084) = 3.74 g; relative amount of lignin lost = ((6.25 - 3.74)/6.75)100 = 40.16%; final cellulose content = (50 - 5.48) (0.42) = 18.7 g; relative amount of cellulose lost = ((21.65 - 18.7)/21.65)100 = 13.63%; final hemicellulose content = (50 - 5.48)(.042) = 1.87 g; relative amount of hemicellulose lost = ((19 - 1.87)/19)100 = 90.16%; ratio of degraded hemicellulose over degraded lignin = (19 - 1.87)/(6.25 - 3.74) = 6.82; ratio of degraded hemicellulose over degraded cellulose = (19 - 1.87)/(21.65 - 18.7) = 5.81. A column has been included that gives for each sample of fungi-degraded sugarcane chips, the difference between its NDF value and the sum of the analyzed values of lignin, cellulose and hemicellulose. These form the cell wall polymers and should be close then to the NDF value; the difference being possibly neutral detergent insoluble ash and other components. The average difference was 7.03 which is an 8% of the average NDF value for all samples. Note the following points: a) all fungi tested produced a solid residue which had a lower IVDMED than the original bagasse, with the exception of **Coriolus versicolor** which showed a slight increase of 0.65 units; b) there was no obvious correlation between IVDMED loss and change in soluble solids or between IVDMED and dry weight, cellulose, hemicellulose and/or lignin losses; c) all fungi tested produced a solid residue which had a lower saccharification rate than the control sample. The residue obtained after growth of **Sporotrichum pulverulentum** showed only (0.36/1.51) 100 = 23.8% of the saccharification susceptibility of the

original bagasse. This residue had the greatest dry weight and cellulose losses and was the second in hemicellulose loss. The relative dry weight loss was (8.13/50.00) 100 = 16.3%; d) the saccharification rates were also low for **Phanerochaete chrysosporium, Ganoderma applanatum** and **C. versicolor**; e) **G. applanatum** showed very little weight and cellulose losses. Hence no general patterns emerge. The enzymatic susceptibility of the solid residues cannot be predicted from the chemical composition of the residues only. Undoubtedly the type of bonds hydrolyzed, the new compounds synthesized by the fungi and the physical characteristics of the solid residues play a major role in determining the extent and rate of further enzymatic hydrolysis.

The lignin losses were similar for all fungi tested. An average lignin loss of 38.64% was calculated. An average hemicellulose loss of 32.22% was observed and only **P. chysosporium** showed almost total hemicellulose hydrolysis. In absolute terms, however, more hemicellulose was degraded than lignin, as their content ratio in the original material was: 38.0/12.5 = 3.04. Lignin is not only the glueing element of the lignocellulose matrix, but also part of the lignin-carbohydrate complex (LCC) stabilized by phenolic acids such as ferulic and p-coumaric acids and acetyl constituents of the cell walls (Hartley, 1972, 1973, 1981; Chesson et al, 1983; Gordon et al, 1983; Tanner and Morrison, 1983). The existance of LCCs in sugarcane bagasse has been reported (Nagaty et al, 1982; Crosthwaite et al, 1984; Atsushi et al, 1984; Molina et al, 1984; du Toit et al, 1984) and at least three different fractions have been identified. Hence it is not unexpected to have concurrent hemicellulose and lignin biodegradation. These LCCs are usually modified chemically during microbial attack (Gaillard and Richards, 1975) and are not recovered in the acid detergent residue.

In Table 2 we have calculated the hydrolysis ratios of hemicellulose:lignin and hemicellulose:cellulose components for the twelve fungal strains. The values for the first ratio are close to two with the exception of **P. chysosporium**, which showed a high hemicellulose loss. The values are also within a narrow range 1.30 to 2.91. On the other hand most fungi showed a preference for hemicellulose hydrolysis over cellulose. Very low cellulose degraders were **Agrocybe aergerita** and **C. versicolor**; the best cellulolytic fungi were **S. pulverulentum** and **Dichomitus squalens**. In the table we have included the results of a two-week solid substrate fermentation by two basidiomycetes isolated by Nigam and Prabhu (1985a,b) and designated BH1 and BW1. They showed very similar losses between themselves, so the average has been calculated. A lower hemicellulose to lignin loss ratio was observed. Both were very selective for cellulose hydrolysis, however, showing a rather active cellulase enzyme complex produced in sumerged culture (Nigan and Prabhu, 1985a).

Kirk and Moore (1972) first reported that lignin removal from

aspen and birch woods by white-rot fungi was always accompanied by removal of polysaccharides, although not necessarily correlated with removal of any particular fraction. In fact lignin degradation by **P.chrysosporium** was stimulated by addition of carbohydrates (Reid, 1979). Wood polysaccharides provide the energy required for lignin attack (Hattaka and Uusi-Rauva, 1983), so previous to or during lignolysis these fungi show holocellulose degradation. Blanchette (1984), employing scanning and transmission electron microscopy of wood decayed by **G. applanatum** and **Ischnoderma resinosum**, showed that in delignified areas the middle lamella was degraded causing an extensive cell defiberization.Wood sugar analysis by HPLC demonstrated that hemicelluloses were removed in preference to cellulose. This seems to coincide with our observations.

Haars and Huttermann (1980) reported lignin biodegradation by **Fomes annosus** to follow an exo-mechanism with small molecules having a molecular weight below 1000 being the first products cleaved. This fact was also observed by Leisola et al (1984) employing **P. chrysosporium** and wheat straw as substrate. The same fungi could degrade lignin monomers during primary and secondary metabolism, however dimers and more complex lignin-derived structures could only be degraded during the secondary phase (Ander et al, 1983). This suggests that lignin biodegradation is nitrogen inhibited (Keyser et al, 1978; Reid, 1979). Inorganic and organic nitrogen in the substrate or medium are quickly assimilated by the fungi and free-intracellular glutamate accumulates, repressing ligninolytic enzymes (Fenn and Kirk 1981; Fenn et al, 1981). Reducing the nitrogen concentration in the medium greatly enhanced degradation of 14-C lignin labelled wheat lignocellulose by **P. chrysosporium**, **S. pulverulentum** and **C. versicolor** (McCartly et al, 1984). A partial regulation has been observed with **Pholiota mutabilis**, and no regulation at all with **Pleurotus ostreatus** and **Lentinus edodes** (Leatham and Kirk, 1983). Sugarcane bagasse was relatively low in protein (1.2%) and probably this caused the average lignin loss to be around 40% of the original quantity present. Intermitent aeration also provided the adequate oxygen concentration needed for an effective ligninolytic activity (Reid and Seifert, 1980; Bar-Lev and Kirk, 1981).

Weight losses for four to six weeks shown in Table 1 are somewhat low. No appreciable weight loss could be detected with **A. aergerita** and **Flammulina velutipes**. Three more fungi were below a loss of 10% of initial dry weight and the rest were below 17%. To our knowledge no similar data have been published on weight losses of bagasse under solid state fermentation by these white rot fungi. Higher weight losses have been reported for cereal straws. In 17 weeks at 30C **Stropharia rugosoannulata** and **Pleurotus cornucopiae** degraded 60-65% of wheat straw; **Pleurotus florida** 45% and **A. aergerita** only 25% at 22-30C (Zadrazil, 1977). In 7 weeks at 22C, **S. rugosoannulata** degraded 26%, **P. florida** 26.3%, **Pleurotus eryngii** 10.6%, **L. edodes** 12.7%, **Kuehneromyces mutabilis** 11.8%,

G. applanatum 28.7%, **F. velutipes** 5.1%, **A. aergerita** 11.4% (Zadrazil and Brunnert, 1980). **S. pulverulentum** at 35C degraded 58 and 72% at 5 and 9 weeks and **D. squalens** 19.1 and 58.9% (Zadrazil and Brunnert, 1982). Streeter et al (1982) found that **P. ostreatus** in mixed culture with **Erwinia carotovora** degraded from 28.8 to 55.9% of wheat straw in 8 weeks. Hattaka (1983) tested 19 strains of white rots for 4 weeks at 28C; those that degraded more biomass were **Fomes ignarius** 51.8%, **Phlebia radiata** 45.2%, **Phanerochaete sordida** 42.3%, **Polyporus brumalis** 38.5%, **Pycnoporus cinnabarinus** 35.6%, **Lenzites betulina** 35.6% and **C. versicolor** 30.8%.

With oat straw Levonen-Munoz et al (1983) obtained with eleven white rots a rather wide range of results, from 3-28% of dry matter loss. In cotton straw, flavonoids present stimulated **P. florida** growth and in 3 weeks it degraded 17% of dry matter, compared to only 10.8% for wheat (Platt et al, 1983).

The nine white rots tested by Ibrahim and Pearce (1980) attacked very little the lignin from sugarcane bagasse, however the authors did not record dry matter losses . None of those white rots were tested by us. The two basidiomycetes isolated by Nigam and Prabhu (1985b) degraded up to 60% of cane bagasse lignin with high dry weight losses of up to 58% (Nigam and Prabhu, 1985a). In submerged culture Ibrahim and Pearce (1985 a) reported a 18.3% lignin degradation by **P. ostreatus** and 20.9% by **C. versicolor**. These figures were increased to 26 and 29.8% when peptone was added to the medium. As shown in Table 1 our data for **C. versicolor** is higher, i.e. 38.89% lignin loss. The difference is difficult to explain but different culture conditions, cane varieties and fungal strains might be reasons. **C. versicolor** effectively degrades lignin from grasses and hard and softwoods; Antai and Crawford (1982) in 10 weeks at 28C obtained values of 61.6, 58.8 and 63.3% respectively. These values are higher than ours in about twice the incubation time.

After 3 weeks four **Pleurotus** strains degraded from 10 up to 56% of lignin in cotton straw (Platt et al, 1984). With eight white rot strains, Zadrazil and Brunnert (1980) reported from 0% (**F. velutipes**) to 54.7% (**P. florida**) lignin degradation in wheat straw. With sunflower hulls and rice husks, **S. rugosoannulata** showed 46.8 and 42.1% degradation; **P. florida** 60.3 and 21.6%; **P. cornucopiae** 68.1 and 59.6% and **A. aergerita** 13.4 and 12.6% (Zadrazil, 1980). In nine weeks with wheat straw, Zadrazil and Brunnert (1982) reported very high lignin degradation values, 60-70% with **S. pulverulentum** and up to 80% with **D. squalens**. About the same values were obtained by Streeter et al (1982), 69% in eight weeks employing **P. ostreatus** with **E.carotovora**.

The response of various fungi in different lignocellulosic substrates is somwehat contradictory towards modifying susceptibility to enzymatic hydrolysis or **in vitro** dry matter enzymatic digestibility. Zadrazil (1977) was successful in

increasing by 75% the IVDMED of wheat straw after 17 weeks either at 25 or 30C with **S. rugosoannulata**, but only 50% at 22C with **P. cornucopiae**. In only 8 weeks Streeter et al (1982) did not improve the IVDMED of wheat straw employing **P. ostreatus**; however it was increased by 45.9% when a mixed culture of **P. ostreatus** and **E. carotovora** were employed.

Hattaka (1983) was very successful in increasing the amount of carbohydrates produced in 72 h with an enzyme from **Trichoderma reesei** from samples of wheat straw that had been incubated with **P. ostreatus** (an increase of 2.89 times the control), **Pleurotus sp** (an increase of 2.67 times), **P cinnabarinus** (an increase of 2.67 times) and **Ischnoderma benzoinum** (an increase of 2.94 times).

Zadrazil (1980) reported unsuccessful attempts of increasing the **in vitro** dry matter digestibility of rice husks; a 36, 64 and 53% decrease of the control with **S. rugosoannulata**, **P. florida** and **A. aergerita**, respectively.

These brief comments on results obtained with other fungi and substrates show in essence what we said before, that the major parameters influencing the extent and rate of further enzymatic hydrolysis of solid residues obtained after the solid state substrate growth of basidiomycetes, are as yet not completely determined. The production of more water soluble compounds or the extent of lignin removal do have an influence but are not the only causes. In fact contradictory results have been published in the past and our data with cane bagasse prove this point.

Some fungi prefer certain substrates. For example Sengupta et al (1984) showed that bagasse, enriched with minerals, was digested with **Termitomyces clypeatus** for one week at 55C and pH 5.0. This fungus saccharified 71% of the carbohydrate contents. However only 10% was saccharified for wheat bran and 3.3% for green coconut coir. No clues were given by the authors to explain these differences.

In the last few years both basic and applied research on microbial conversion and modification of lignocellulosic materials have vastly expanded as reviewed by Eriksson (1984), but certainly some new experimental approaches need to be developed in order to better understand how white-rot enzymes interact with surface components and how this activity is regulated.

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Table 1. Basidiomycetes used, substrate chemical composition changes, saccharification rate and digestibility results

No.	Fungi	Source	IVDMED (average of two values)	Saccharification rate at 8h mg reducing sugars h^{-1}	Dry weight loss g	Soluble solids % of dry weight	NDF-SUM[g]	Lignin loss % of control	Cellulose loss % of control	Hemicellulose loss % of control
1.	Phanerochaete chrysosporium (F 1107)[a]	USDA-FPL[b]	0.88*	0.58	5.84	6.14	10.3	40.16	13.63	90.16
2.	Agrocybe aergerita (F 1090)	CBS-BAARN[c]	0.42*	0.94	0.00	7.79	7.4	31.29	0.00	20.76
3.	Flammulina velutipes (F 1100)	CMI-KEW[d]	3.73*	0.69	0.00	3.50	7.3	37.46	4.41	16.13
4.	Coprinus fimentarius (F 1094)	NRC-SASK[e]	8.53*	0.82	2.07	3.93	1.6	37.89	2.82	24.83
5.	Ganoderma applanatum (F 1102)	CMI-KEW[d]	8.62*	0.58	1.14	1.93	6.7	40.58	1.37	20.02
6.	Sporotrichum pulverulentum (F 1113)	CMI-KEW[d]	10.07*	0.36	8.13	6.62	10.4	47.75	24.39	41.83
7.	Pycnoporus sanguineus (F 1112)	CMI-KEW[d]	10.78*	0.86	3.52	6.15	8.3	32.33	5.12	31.27
8.	Dichomitus squalens (F 1099)	CBS-BAARN[c]	12.44*	1.12	2.90	4.39	4.6	35.94	14.06	24.39
9.	Ischnoderma resinosum (F 1105)	USDA-FPL[b]	14.25*	0.79	6.34	3.27	7.6	42.72	10.26	33.13
10.	Pleurotus flabellatus (F 1011)	IARI-DELHI[f]	15.54*	0.75	5.35	0.34	8.5	40.70	12.55	27.14
11.	Bondarzewia berkeleyi (F 1093)	USDA-FPL[b]	15.80*	0.62	5.41	1.02	5.4	37.93	13.29	26.07
12.	Coriolus versicolor (F 1095)	USDA-FPL[b]	19.69	0.55	6.10	2.80	6.3	38.89	0.00	30.91

* Statistically different from control, significant 95%. Control was the analytical data for the EX-FERMented cane chips.

a ICAITI collection number

b Forest Products Laboratory, Madison, Ms. Frances F. Lombard, P. chrysosporium ME-446; I. resinosum (or Polyporus resinosus older name) L-13682-Sp; B. berkeleyi (or Polyporus berkeleyi older name) FP-105239-5; C. versicolor (or Polyporus versicolor older name) R-105-Sp

c Centraalbureau voor Schimmelcultures, Baarn

d Commonwealth Mycological Institute, Kew. F. velutipes 176670; G. applanatum 157818; S. pulverulentum 174727; P. sanguineus 75002

e National Research Council of Canada, Saskatoon, Dr. Ian D.Reid, C. fimentarius PRL 3001 (Source ATCC 36567)

f Indian Agricultural Research Institute, New Delhi, Dr. L.M. Joshi, P. flabellatus 1724

g Neutral detergent fiber minus the sum of the lignin, hemicellulose and cellulose contents. It should be close to cero for a balanced composition analytical data (see text)

Table 2. Biodegradation ratios of various bagasse components

Fungal strain	Ratio 1[a]	Ratio 2[b]
1	6.82	5.81
2	2.01	very high
3	1.30	3.19
4	1.99	7.74
5	1.50	12.67
6	2.67	1.51
7	2.91	5.30
8	2.06	1.52
9	2.36	2.83
10	2.03	1.90
11	2.09	1.72
12	2.42	very high
BH 1 } BH 1 }	0.97	0.43

a. Ratio of the amounts of hemicellulose degraded to lignin degraded
b. Ratio of the amounts of hemicellulose degraded to cellulose degraded

PAPER No. 4

"Biodelignification of lemon grass and citronella bagasse employing white-rot fungi"

(Accepted for publication by Applied and Environmental Microbiology)

BIODELIGNIFICATION OF LEMON GRASS AND CITRONELLA BAGASSE EMPLOYING WHITE-ROT FUNGI

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Twelve white-rot fungi were grown in solid state culture on lemon grass (**Cymbopogon citratus**) and citronella (**Cymbopogon winterianus**) bagasse. The two lignocellulosic substrates had 11% permanganate lignin and a holocellulose fraction of 58%. After five to six weeks at 20C, 9 fungi produced a solid residue from lemon grass with a higher IVDMED than the original bagasse; 7 did the same for citronella. The best fungus for both substrates was **Bondarzewia berkeleyi**, it increased the IVDMED (in vitro dry matter enzyme digestibility) for lemon grass to 22 percent and 24 percent for citronella. The increases were correlated with weight loss and lignin loss. All fungi decreased lignin contents, 36% of the original value for lemon grass and 28% for citronella. Practically all fungi showed a preference for hemicellulose over cellulose.

INTRODUCTION

In natural decomposition of lignocellulosic matter, both fungi and aerobic bacteria play an important role in degrading holocellulose and lignin to lower molecular weight products, some of which are then further metabolized by facultative and obligate anaerobic soil bacteria and actinomycetes (5, 28, 38). Fungi are usually faster lignin degraders, particularly the so-called white-rots which completely metabolize the complex polymer, exhibit the highest reported rates and have been the most studied (10,25). All of them have the enzymatic capacity to use the holocellulose components as a source of carbon and energy; hence total biomass breakdown usually occurs and lignin removal is accompanied by removal of polysaccharides (24). Under certain culture conditions white rots use lignin preferentially, producing soluble monomers, breaking up the cellulose-hemicellulose matrix, making the solid more susceptible to further enzymatic action by other microorganisms, rumen bacteria for example. These conditions, however, remain as yet specific for each fungus-substrate combination. Some of the fungi produce fruiting bodies when growth occurs on a solid substrate . These two points make the solid state fungal delignification process an attractive alternative for converting lignocellulose into human food (the fruiting bodies of edible fungi) and animal feed (the solid residue with increased digestibility).

Recent research reported on this subject has been briefly commented (35, 36). Most of the work has been done with cereal straws from temperate countries; very few tropical lignocellulosic residues have been studied. Although the effect of temperature, pH, carbon and energy sources, nitrogen and oxygen availabilities on the ligninolytic activity has been studied, no clear picture on how to enhance rates or optimize a production system has been presented. Moreover fungal response varies tremendously. Little lignin degradation associated with decreased substrate digestibility has been reported. There is still controversy if fruiting of the fungi benefits lignin degradation.

Lemon grass and citronella bagasse are the lignocellulosic residues of steam distillation of freshly cut lemon grass and citronella leaves for the recuperation of the respective essential oils. The plants belong to the Gramineae and have been classified as **Cymbopogon citratus** and **Cymbopogon winterianus**, respectively. The essential oil content is low, 0.5-1.3% by weight of fresh grass, and its recovery is not complete. After steam distillation, the bagasse is partially dried in the fields and a fraction is burned to generate steam for the stripping; the rest is left in the fields where natural biodegradation takes place. Its use as a ruminant feed is limited due to animal rejection because of the residual aroma and flavor. According to recent information in essential oil production (34) there is an estimated worldwide availability of about 200 000 t of dry bagasse per year that could be used as a source of lignocellulosic biomass.

In this contribution we report the results of solid state growth of twelve basidiomycetes on citronella and lemon grass bagasse and its effect on the chemical composition and **in vitro** dry matter enzymatic digestibilities (IVDMED).

MATERIALS AND METHODS

- Fungi employed. The twelve white-rot fungi are described in Table 1. They were kept on potato dextrose agar plus 3% yeast extract.

- Bagasse samples. These were obtained from plantations located in the Guatemalan Pacific lowlands at 420 meters above sea level, at the start of the rainy season and the harvest of the leaves. They were collected as they came out of the essential oil steam distillery as 2-3 cm pieces, sun dried to a final moisture around 6-7% and packed in plastic ventilated bags. A control sample was analyzed.

- Incubation procedure. A recently inoculated culture tube for each fungus was left for 5 to 6 days at ambient temperature, about 20C. This was transferred to a petri dish with the same medium. Usually it took 9-10 days at ambient temperature to

cover the whole surface. If the dish was free of any contaminant as seen macroscopically, one sixth of its contents were used to inoculate a 700 ml was mouth glass jar with a metal cap, in which 50 g of dried substrate plus 100 ml of water had been sterilized for 30 min at 121C. Duplicates for each fungal culture were left for five to six weeks at ambient temperature until fungal growth had completely covered the substrate surface. The jars were covered loosely with the metal caps and they were oxygenated every week for 15 min by introducing a flow of air through a tube connection installed in the metal cap. The entire jar contents were dried at 60C in a forced air laboratory dryer. Control units were uninoculated jars which had received the same sterilization and were incubated as described.

- Chemical analysis. After drying, all samples were milled in a laboratory Wiley mill employing a 0.84 mm sieve. The following analysis were made: Kjeldahl nitrogen and ash content by standard methods; neutral and acid detergent fiber, permanganate lignin and cellulose following the Van Soest techniques (15, 42). IVDMED was determined as suggested by Dowman and Collins (10). Briefly, 200 mg of the material were suspended in 20 ml of a 2 g l-1 pepsin solution (No. P-7125 Sigma Chemical Co. Ltd.) in 0.2M acetate buffer pH 4.8 and incubated at 40C for 48 h. Samples were centrifuged, washed once with distilled water, centrifuged and suspended in 20 ml of a 2.5% by weight solution of fungal Onozuka FA cellulase (Maruzen Chemical Co. Ltd.) in 0.2M acetate buffer pH 4.8 and incubated again at 40C for 48 h. The suspension was filtered in sintered glass crucibles, washed with water and weighed. Controls used distilled water (no enzyme). During incubation the tubes were agitated twice a day. The IVDMED values represent percent weight loss due to enzymatic action in terms of initial dry matter of the sample, taking into account the weight loss of the control sample. So it indicates net enzymatic action upon the substrate.

- Statistical procedures. Analysis of variance was performed, using the F test. Treatment effects, the deviation of the treatment mean from the general mean, were evaluated using the F statitic, and those significant at the 0.95 level are indicated in the tables.

RESULTS AND DISCUSSION

- Analytical data of original bagasse. The data are given in Table 2. The two Cymbopogon plants were similar. The holocellulose fraction, amenable to enzymatic hydrolysis to simple sugars, represented on dry basis 58.4% for lemon grass and 58.5% for citronella. The corresponding figures on ash

free basis were 65.2 and 64.1%, respectively. The crude protein content was lower than values representative of hardwood leaves, lucerne and ordinary grass hays, which usually are in the range of 8 to 23% (8, 41). However it was higher than that of common straws (8, 41) resembling instead the leaves (sheath and blade) of such materials (1). In terms of neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents they showed some similarity to the common straws and they were of intermediate lignin content, again being close to the straws and sugarcane bagasse (22, 30).

- Digestibility of treated samples. The IVDMED values obtained for the twelve solid residues from lemon grass bagasse after fungal growth are presented in Table 3, and those for lemon grass in Table 4. Note the following points: a) Most of the fungi significantly increased the IVDMED of the solid residues. For both substrates the fungus that modified the substrate to be more susceptible to enzymatic hydrolysis was Bondarzewia berkeleyi, which increased lemon grass digestibility to 22 percent and citronella digestibility to 24 percent. The five fungi to most improve the bagasse were the same for both substrates, although with some changes in individual ranking. Although all fungi increased the IVDMED for lemon grass, the differences for Ischnoderma resinosum, Dichomitus squalens and Agrocybe aegerita were not significant. In the case of citronella, five of the fungi, Pycnoporus sanguineus, D. squalens, A. aegerita, Flamulina velutipes and Ganoderma applanatum, did not produce IVDMED values that were statistically different from the control. b) There was a proportional correlation between the increase in IVDMED and the weight loss. For all significant data for lemon grass and citronella, the data could be correlated by:

IVDMED = -7.12 + 3.47 (weight loss) $r2 = 0.74$
weight loss > 13%
c) No direct relationship was found between IVDMED increase and soluble solids of the resulting residues. In practically all cases soluble solids decreased by one third to one half the original value. Hence the increase in IVDMED values could clearly involve a structural polymer modification and delignification.

- Biodelignification. With the analytical data of the solid samples from each treatment and the corresponding solid yields a mass balance of the lignocellulosic fraction was made. The resulting data is shown in Table 5 for lemon grass and Table 6 for citronella, and is expressed in percentage change relative to the original sample values. The following points can be observed: a) all fungi showed ligninolytic activity, an average of 36% for lemon grass and only 28% for citronella. In both substrates the fungi showing higher lignin losses were: B. berkeleyi, Coriolus versicolor, Pleurotus flabellatus and Phanerochaete chrysosporium (Sporotrichum pulverulentum). The ones showing less: D. squalens, A. aegerita, F. velutipes and

G. applanatum. b) Practically all fungi showed a preference to degrade hemicellulose over cellulose. The ratio of hemicellulose to lignin losses and hemicellulose to cellulose losses for the average values were: for lemon grass, 0.65 and 1.48; for citronella, 0.88 and 1.72.

Comparing the IVDMED values with respect to the extent of biodelignification, the following correlations for the twelve fungi were obtained:

Lemon grass:
IVDMED = -51.70 + 3.21 (lignin loss) r2 = 0.79

Citronella:
IVDMED = -55.84 + 3.47 (lignin loss) r2 = 0.55

These correlations show that in order to obtain an IVDMED increase a 16-17% lignin loss with respect to the original content is required.

Lignin is not only the gluing element of the lignocellulose matrix, but also forms a part of a lignin-carbohydrate complex (LCC) stabilized by phenolic acids such as ferulic and p-coumaric acids and acetyl constituents of the cell walls (7, 16, 18, 19, 39). Hence it is not unexpected to have the fungi degrade about the same amounts of hemicellulose and lignin. These LCCs are usually chemically modified during microbial attack (14) and are not recovered in the acid detergent residue.

For lemon grass the ratio of hemicellulose to lignin loss was below one, ranging from 0.41 to 0.81, with the exception of G. applanatum, which degraded more hemicellulose than lignin. In case of citronella only eight fungi showed a ratio below one, ranging from practically zero to 0.96. Four degraded more hemicellulose than lignin, B. berkeleyi, P. flabellatus, D. squalens and F. velutipes.

- Comparison with literature values. The range of experimental weight losses observed for lemon grass between 8 and 42%, and for citronella bagasse, between 1.6 to 37%, were in general slightly lower than what was expected. Antai and Crawford (3) have shown, for instance, that in 10 weeks at 28C, C. versicolor degraded more dry matter, 63.8%, in grasses than in hard or soft woods (about 40%). Somewhat higher weight losses have been reported for cereal straws and some specific white rots. For example with wheat straw the following data have been reported: a) 17 weeks at 30C, Stropharia rugosoannulata and Pleurotus cornucopiae degraded 60-65%, Pleurotus florida 45% and A. aegerita only 25% (43). b) In 7 weeks at 22C, S. rugosoannulata degraded 26%, P. florida 26.3%, Pleurotus eryngii 10.6%, Lentinus edodes 12.7%, Kuehneromyces mutabilis 11.8%, G. applanatum 28.7%, F. velutipes 5.1%, A. aegerita 11.4% (44). c) S. pulverulentum at 35C degraded 58 and 72% at 5 and 9 weeks and D. squalens

19.1 and 58.9% (46). In 4 weeks at 28C, **Fomes igniarius** degraded 51.8%, **Phlebia radiata** 45.2%, **Phanerochaete sordida** 42.3%, **Polyporus bromalis** 38.5%, **Pycnoporus cinnabarinus** 35.6%, **Lenzites betulina** 35.6% and **C. versicolor** 30.8% (20).

Lignin losses shown in Tables 5 and 6 fall within expected figures. After 3 weeks four **Pleurotus** strains degraded from 10 up to 56% of lignin present in cotton straw (31). Employing eight white-rot strains, Zadrazil and Brunnert (45) reported practically zero for **F. velutipes** and up to 54.7% for **P. florida** in wheat straw. With sunflower hulls and rice husks, **S. rugosoannulata** biodegraded 46.8% and 42.1%, **P. florida** 60.3 and 21.6%, **P. cornucopiae** 68.1 and 59.6% and **A. aegerita** 13.4 and 12.6%, respectively (44). Very high lignin losses have also been reported with wheat straw in nine weeks, 60-70% employing **S. pulverulentum** and up to 80% with **D. squalens** (46). About the same values were obtained by Streeter et al (39) 69% in eight weeks employing a mixed culture of **Pleurotus ostreatus** and **Erwinia carotovora.**

Kirk and Moore (24) first reported that lignin removal by white-rot fungi from aspen and birch woods was always accompanied by removal of polysaccharides, although not necessarily correlated with removal of any particular fraction. In fact lignin degradation by **P. chrysosporium** was stimulated by addition of carbohydrates (32). Wood polysaccharides provided the energy required for lignin attack (21) so during or previous to ligninolysis, a holocellulose degradation activity is shown. This phenomenon has been studied by Blanchette (6) who,employing scanning and transmission electron microscopy of wood decayed by **G. applanatum** and **I. resinosum**, reported that in delignified areas the middle lamella was degraded causing an extensive cell defiberization. Wood sugar analysis by HPLC demonstrated that hemicelluloses were removed in preference to cellulose. This coincides with our observations.

Lignin biodegradation by **Fomes annosus** was reported by Haars and Huttermann (17) to follow an exo-mechanism, small molecules having a molecular weight below 1000 being cleaved first. This fact was also observed by Leisola et al (27) employing **P. chrysosporium** and wheat straw as the substrate. The same fungus could degrade lignin monomers during primary and secondary metabolism, however dimers and more complex lignin-derived structures could only be degraded during the secondary phase (2). This suggests that lignin biodegradation is nitrogen inhibited (23, 32). Inorganic and organic nitrogen available in the substrate or medium are quickly assimilated by the fungus and free-intracellular glutamate accumulates, repressing ligninolytic enzymes (12, 13). Reducing the nitrogen concentration in the medium greatly enhanced degradation of 14-C lignin labelled wheat lignocellulose by **P. chrysosporium**, **S. pulverulentum** and **C. versicolor** (29). Although a partial regulation has been observed with **Pholiota motabilis**, no

regulation at all could be shown with **P. ostreatus** and **L. edodes** (26). Lemon grass and citronella, being higher in nitrogen than wheat straw, have shown in these tests somewhat lower delignification values. Some organic nitrogen inhibition could have been present.

Intermittent aeration also provided the adequate oxygen concentration needed for an effective ligninolytic activity (4, 33).

The activity of various fungi in different lignocellulosic substrates towards modifying the further extent of enzymatic hydrolysis or **in vitro** dry matter enzymatic digestibility is somewhat contradictory. Zadrazil (43) was successful in increasing by 75% the IVDMED of wheat straw after 17 weeks either at 25 or 30C with **S. rugosoannulata**, but only 50% at 22C with **P. cornucopiae**. In only 8 weeks, Streeter et al (39) did not improve the IVDMED of wheat straw employing **P. ostreatus**; however it was increased by 45.9% when a mixed culture of **P. ostreatus** and **E. carotovora** was employed. Hatakka (20) was very successful in increasing the saccharification of samples of wheat straw that had been incubated with **P. ostreatus** (189% increase), **Pleurotus sp.** (162%), **P. cinnabarinus** (162%) and **Ischnoderma benzoinum** (194%). Zadrazil (44) reported unsuccessful attempts with rice husks, a -36% decrease with **S. rugosoannulata**, -64% with **P. florida** and -53% with **A. aegerita**.

- Final comments. It is obvious from our data and from data found in the literature that the effects of white rot fungi upon the lignocellulose matrix is a complex phenomenon controlled by many variables and their interactions. The responses observed so far seem quite fungus-substrate specific and hence it is hard to define a general trend. The IVDMED cannot be predicted only by the chemical composition of the solid residue. Undoubtedly the type of bonds hydrolyzed, the type of new compounds synthesized by the fungi and the physical characteristics of the residue play a major role in determining the extent and rate of further enzymatic hydrolysis.

Some fungi prefer certain substances. For example Sengupta et al (37) showed that digestion of sugarcane bagasse enriched with minerals with **Termitomyces clypeatus** for one week at 55C and pH 5.0, resulted in saccharification of 71% of the carbohydrate contents. However only 10% for wheat bran and 3.3% for green coconut coir was observed. No clues were given by the authors to explain these differences.

In the last few years both basic and applied research on microbial conversion and modifications of lignocellulosic materials have vastly expanded, as reviewed by Eriksson (11), but certainly some new experimental approaches need to be developed in order to understand better how white-rot enzymes interact with surface components and how this interaction is regulated.

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TABLE 1. Fungal strains used in solid state experiments

Designation[a]	Strain	Source
F-1107	Phanerochaete chrysosporium	Forest Products Laboratory, Madison, ME-446
F-1105	Ischnoderma resinosum	Forest Products Laboratory, Madison, L-13682-Sp
F-1093	Bondarzewia berkeleyi	Forest Products Laboratory, Madison, FP-105839-5
F-1095	Coriolus versicolor	Forest Products Laboratory, Madison, R-105-Sp
F-1090	Agrocybe aegerita	Centraalbureau voor Schimmelcultures, Baarn
F-1099	Dichomitus squalens	Centraalbureau voor Schimmelcultures, Baarn
F-1100	Flammulina velutipes	Commonwealth Mycological Institute, Kew, 176670
F-1102	Ganoderma applanatum	Commonwealth Mycological Institute, Kew, 157818
F-1112	Pycnoporus sanguineus	Commonwealth Mycological Institute, Kew, 75002
F-1113	Sporotrichum pulverulentum	Commonwealth Mycological Institute, Kew, 174727
F-1094	Coprinus fimetarius	National Research Council of Canada, Saskatoon, PRL 3001 (ATCC 36567)
F-1011	Pleurotus flabellatus	Indian Agricultural Research Institute, New Delhi, 1724

a ICAITI Collection number

TABLE 2. Analytical data of original bagasse samples on % dry weight basis

Component	Lemon grass	Citronella
Neutral detergent fiber (NDF)	72.6	72.0
Acid detergent fiber (ADF)	44.1	42.0
Parmanganate lignin	11.0	11.1
Cellulose	29.9	28.5
Hemicellulose (NDF-ADF)	28.5	30.0
Ash	11.0	9.3
Crude protein (Kjeldahl nitrogen x 6.25)	5.1	4.5
Essential oil	Traces	Traces
Water soluble substances[a]	16.2	23.3

a Determined in the controls of the IVDMED (see text)

TABLE 3. *In vitro* dry matter enzymatic digestibilities for lemon grass

Strain	IVDMED	Weight loss, g	Soluble solids, g
B. berkeleyi	34.73*	21.02	13.64
C. versicolor	29.97*	16.40	12.00
P. flabellatus	28.72*	16.06	16.17
S. pulverulentum	26.05*	17.90	15.21
C. fimetarius	23.30*	10.47	10.20
P. sanguineus	18.72*	6.60	14.41
P. chrysosporium	16.51*	6.66	15.48
F. velutipes	16.51*	6.60	13.17
G. applanatum	15.72*	9.46	15.48
I. resinosum	13.92	3.98	13.74
D. squalens	13.40	6.60	16.81
A. aegerita	12.96	5.20	18.76
CONTROL**	12.73	0	16.24

* 95% statistically significant from control.

** Control values are for uninoculated material which received the same sterilization process, incubation and aeration.

TABLE 4. *In vitro* dry matter enzymatic digestibilities for citronella

Strain	IVDMED	Weight loss, g	Soluble solids, g
B. berkeleyi	43.25*	18.33	13.43
S. pulverulentum	39.73*	17.45	13.33
P. flabellatus	36.64*	17.37	15.13
C. fimetarius	34.12*	7.34	13.17
C. versicolor	33.71*	17.45	17.73
I. resinosum	28.30*	8.09	14.76
P. chrysosporium	26.31*	6.13	12.20
P. sanguineus	21.74	6.16	14.13
D. squalens	18.75	4.56	14.82
A. aegerita	16.95	3.23	13.49
F. velutipes	14.88	4.55	12.37
G. applanatum	13.03	0.78	12.45
CONTROL **	19.32	0	23.29

* 95% statistically significant from control.

** Control values are for uninoculated material which received the same sterilization process, incubation and aeration.

TABLE 5. Biochemical changes in the lignocellulose fraction of lemon grass. Data as % of original sample.

Strain	Lignin loss	Hemicellulose loss	Cellulose loss
B. berkeleyi	64.17	36.95	40.68
C. versicolor	55.40	27.84	28.97
P. flabellatus	42.60	32.35	22.58
S. pulverulentum	56.24	31.76	35.60
C. fimetarius	33.88	27.32	17.77
P. sanguineus	31.34	24.76	9.70
P. chrysosporium	40.90	15.76	1.73
F. velutipes	19.29	16.72	6.68
G. applanatum	18.19	38.55	21.37
I. resinosum	30.54	14.08	0.00
D. squalens	20.29	8.31	5.35
A. aegerita	19.37	7.90	0.00
Average values	36.02	23.53	15.87

TABLE 6. Biochemical changes in the lignocellulose fraction of citronella. Data as % of original sample.

Strain	Lignin loss	Hemicellulose loss	Cellulose loss
B. berkeleyi	37.23	42.57	29.76
S. pulverulentum	44.83	35.15	31.06
P. flabellatus	30.03	44.96	36.57
C. fimetarius	25.44	0.00	1.50
C. versicolor	45.45	32.29	31.47
I. resinosum	27.52	26.53	7.66
P. chrysosporium	32.02	18.11	15.65
P. sanguineus	27.34	20.22	13.87
D. squalens	5.04	26.70	1.80
A. aegerita	19.10	15.50	1.53
F. velutipes	19.75	22.73	2.08
G. applanatum	21.96	12.72	0.00
Average values	27.98	24.76	14.41

PAPER No. 5

"Biodegradation of pretreated EX-FERMented sugarcane chips by fungal enzymes and mixtures of anaerobic bacteria"

(Accepted for publication in Biotechnology Progress)

BIODEGRADATION OF PRETREATED EX-FERMENTED SUGARCANE CHIPS BY FUNGAL ENZYMES AND MIXTURES OF ANAEROBIC BACTERIA

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Sugarcane lignocellulosic residue left after sucrose conversion into ethanol by the EX-FERM process was pretreated employing eleven different techniques and process conditions. These substrates were then hydrolyzed in separate experiments by a commercial fungal enzyme and by six different inocula of mixtures of anaerobic bacteria. In vitro dry matter enzymatic digestibilities (IVDMED) and saccharification rates were measured in the first instance. Percent digestion, gas production rate, and volatile fatty acid (VFA) yields were determined in the experiments with bacteria. IVDMED values were linearly correlated with the inverse of lignin content of each substrate. This correlation was not valid, however, for the enzymatic saccharification rates and the three fermentation parameters measured with bacteria. These results are discussed in terms of what has been recently reported in the literature regarding enzyme adsorption and bacteria-surface interactions. Alkaline pretreatments with sodium and calcium hydroxide and two of the organosolv variants tested were the most susceptible to bacterial attack. Higher VFA yields were obtained when using the mesophilic pure culture mixture or the mixture of anaerobic bacteria from the digestor and the sodium hydroxide and some of the organosolv pretreatments. The organosolv pretreated substrates were the samples which showed the highest IVDMED values: in one of them it was almost 90%.

Introduction

Lignocellulosic biomass is relatively refractory to bioconversion, in which a succession of fungi and aerobic and anaerobic bacteria in complex interactions bring about the required biochemical changes in nature (1,2). Sugarcane bagasse is no exception (3,4).

In order to increase the rate and extent of holocellulose hydrolysis, a substrate pretreatment is advisable to favor close contact between microbe and fibers of the lignocellulosic matrix and to secure efficient enzyme action. There is limited understanding of how these pretreatments enhance biodegradation, although there is little doubt that they, alone or in combination, are effective. A recent review (5) listed 10 physical, 18 chemical and 1 biological treatment. Hence the selection of the most appropriate treatment for a particular

situation is in most cases an empirical endeavor.

The mode of holocellulolysis may be quite species-specific, both in terms of the substrate and the microorganism. Holocellulolysis being defined as the enzymatic hydrolysis of the cellulose and hemicellulose polymers and any complex existing between them or the lignin polymer. Being an heterogeneous reaction, the interactions between microbe and substrate surface and the complexity of the associated enzymatic system are of paramount importance. They have become obvious in recent studies dealing with the strong surface interactions of rumen bacteria (6-8) and of strict anaerobes like **Clostridium thermocellum** which possess multifunctional multienzyme complexes within particular bodies which interact closely with the fibers. The microbodies have been called cellulosomes by Lamed et al.(9), and cellular vesicles in rumen bacteria. An extracellular matrix has been observed in brown rot fungi (10), which probably contains cytoplasmatic organelles like enzyme bags. The same structure is also present in white rot fungi but its function here seems to be attachment, nutrition and protection (11). In any event, it seems that in natural ecosystems where lignocellulose is being hydrolyzed, the adsorption of true extracellular enzymes might occur, but probably the adsorption of cytoplasmatic microbodies containing naturally immobilized enzymes might be as, if not more, frequent and important. Any pretreatment to be effective must enhance the accessibility and adsorption of such cellular structures.

The rate and extent of hydrolysis might depend, then, on the amount of substrate surface exposed, the proper adsorption of the biocatalyst, the degree of crystallinity and polymerization of the heteropolysaccharide and the proportion of potential inhibitors like lignin, phenolic acid and acetyl contents (12-17).

In the present work we present the experimental results obtained for the biodegradation of EX-FERMented sugarcane chips pretreated by various techniques. First, biodegradation was measured by employing a **Trichoderma reesei** commercial enzyme to determine the **in vitro** dry matter enzymatic digestibilities and the saccharification rate. Then, employing a complex mixture of anaerobic bacteria isolated from four different ecosystems and two mixtures of known anaerobic bacteria, the percent dry matter digestion, the yield of volatile fatty acids, and gas production rates for gas and volatile fatty acids were determined. The results are discussed in terms of the possible changes caused during substrate pretreatments.

Sugarcane and its pretreatment

- Substrate characteristics

Sugarcane was sampled from sugar mills on the Pacific coast of Guatemala located around 300 meters above sea level, during the month of April, which is at the end of the harvest, during the dry season. The sample was chipped in a pilot wood chipper (Type 9-72, OY Santsalo), the chips were placed in 7000 l tanks, water was added to just cover them, and 2% of commercial baker's yeast was mixed. The EX-FERM cycle (18-20) to convert all sucrose present into ethanol took 48 h. The fermented chips were pressed in a continuous screwpress (Model VP-6F, Vincent Corp.), washed with water in a mechanically agitated tank, and screwpressed again. They were finally sun-dried to a final moisture around 6-7% and packed in ventilated plastic bags. The sample was denominated CAST-1.

The results of chemical analysis of CAST-1 are shown in Figure 1, compared with data from several cane bagasse samples obtained directly from Guatemalan sugar mills. Both are practically the same; hence in terms of lignocellulosic macrocomponents sugarcane bagasse and the residue from the EX-FERM process are equal. This fact was more or less expected, as the yeast employed in EX-FERM only utilizes sucrose from sugarcane. On the other hand the effects of crushing and heating in the sugar mill upon the lignocellulosic components seem to be negligible, as was also shown by Gartside et al. (23).

The lignin content of sugarcane bagasse (12-15%) is slightly higher than most cereal straws (24, 25). The in vitro enzymatic dry matter digestibility (IVDMED) of the CAST-1 sample determined by the two-enzyme procedure (26), employing pepsin (No. P-7125 Sigma Chemical Co.) followed by the fungal Onozuka FA cellulase (Maruzen Chemical Co. Ltd.), was 19.04%. The average IVDMED for the bagasse samples was 17.92%. These values show that the lignocellulosic components of sugarcane are quite resistant to enzymatic hydrolysis. They are much lower than those found for legumes, around 70%, and most common grasses and cereal straws, around 29% (27).

- Substrate pretreatments

In Table 1 a summary of the pretreatment conditions and the expected effects is given for each identified sample. Solid-state techniques were employed for the NaOH, Na_2CO_3+$Ca(OH)_2$, NH_3 and SO_2 pretreatments. Solid suspensions and higher pressures were used for the alkaline organosolv trials. The aqueous-phenolic treatment was done by Dr. J. P. Sachetto from BATTELLE-Geneve and the steam explosion by Dr. J.N. Saddler of the Eastern Laboratory of Forintek Canada Corp., Ottawa.

IVDMED and saccharification rates employing fungal enzymes

In Table 2 are found the IVDMED values obtained as described before for each pretreated sample. The lignin, cellulose, and hemicellulose contents and the saccharification rate for the samples are also given. This last parameter was obtained after 8 h of contact at 40°C of 100 g of material suspended in 9 ml of 0.2M acetate buffer,pH 4.8,with 1 ml of a 1% by weight solution of Onozuka FA cellulase in 0.2M acetate buffer, pH 4.8. The sealed tube was placed in a rotary shaker and the sugars determined by the dinitrosalicylic acid method (41).

First of all, it can be noted that the pretreatments were effective in increasing the IVDMED and saccharification rate values of the various samples compared with the untreated cane sample, CAST-1. The IVDMD increases found for CANA were within the same order of magnitude as those reported previously by other workers (42-45). For CACANA our values were lower than those by Matei and Playne (46), Playne (47), and Martin et al. (48). The one for CAAM compares well with others (46, 47, 49). Note that the result for CANA was better than those for CACANA or CAAM. The CASO data were in agreement with reported by scientists from the Volcani Center in Israel (40, 50, 51). Our data for CAEX was comparable to those by Hart et al. (52) in terms of experimental conditions used, but our results were about 50% better. Here the aspects of cane varieties might be partly responsible for the difference found. Secondly, practically all the IVDMED increases were higher for the organosolv samples, from 3.2 times over CAST-1 for CAOK-1 to 4.7-fold for CAOA-2. Thirdly, in terms of chemical composition all pretreatments did what was expected, in that they carried out mainly a partial or almost total delignification parallel to a partial or total hydrolysis of hemicellulose.

In terms of rates, CAOK-1 and CAEX gave values 4.0 and 3.3 times the value for CAST-1. This is about the same improvement reported for a steam treated sample by Rao et al. (55). For CANA a 1.8-fold increase can be calculated from the data of Table 2, a figure which is close to the one reported by Trivedi and Ray (53), but both are low compared to a close to 3-fold increase reported by Mukhopadhyay et al. (54) and Rao et al. (55). Again the differences might be due to the cane variety and the enzyme employed for the saccharification. This is reflected more if rates are calculated on the same basis as ours from their data. Values of 6.5, 2.2 and 0.6 mg h^{-1} of reducing sugars, analyzed after 8, 24 and 4 h, respectively, were obtained (53,55,54) in that order.

A more valid comparison can be obtained if the rates are divided by the filter paper units of the enzymatic preparation employed. Mishra et al. (56) give an initial rate (value extrapolated to zero time) between 100-180 mg reducing sugar FPU-1 h-1 and a final rate (after 48 h) between 0.8-1.3. These data were obtained with an enzyme complex from **Penicillum funiculosum** and sodium hydroxide treated bagasse. Our corresponding data for CANA is 19.2 mg reducing sugar FPU-1 h-1, well within the previous referenced figures.

It is interesting to confirm any correlation between IVDMED and lignin content of the pretreated samples. The data of Table 2 are plotted in Figure 2, where a linear inverse correlation is observed between these two values. The same does not hold for the initial saccharification rate, as in Table 2 it can be easily seen that there is no direct relationship between the two variables. Before attempting to explain this fact it is important to mention that in both enzymatic measurements the weight loss and reducing sugars of the control sample (no enzyme added) were taken into account when the IVDMED and saccharification rates were calculated for each pretreated sample, so both measurements reflect true enzymatic action upon the substrate. Also it should be pointed out that the existence of lignin-carbohydrate complexes (LCCs) has been previously reported in sugarcane bagasse (57-60), and Atsushi et al. (61) described in detail the isolation of three polymeric fractions different from each other in contents c. lignin, carbohydrates, phenolic acids, and molecular weight. Various authors have previously reported an increase in rate and extent of digestion in partially or totally delignified alfalfa and orchardgrass forages (62-64), and two (65, 66) have presented an inverse linear correlation between digestible dry matter and lignin content.

Recently it has been pointed out that the type and extent of lignin-polysaccharide bonding in LCCs after pretreatments could affect digestion more than the amount of lignin per se (15, 68-70). It was also reported for kraft-delignified hard- and softwoods, in general that the digestibility of softwoods increased more slowly as lignin was removed than did that of hardwoods (71). Some believe that lignin encrustation in the polymer matrix gives carbohydrate polymers a protective seal against enzymatic hydrolysis. This picture, however, does not take into account the existance of LCCs and their modification due to the action of different pretreatments. Some also assign lignin an inhibitory action upon enzymes, but the mechanism has not been fully elucidated. Recently Hidaka et al. (72) showed that lignin from soft- and hardwoods markedly depressed cellobiose activity of the enzymes and the accumulated cellobiose was causing a feedback inhibition. Lignin derived compounds also have a marked inhibition effect (73-75). Delignification, on the other hand, does indeed change the structural features of the lignocellulose. It might increase surface area and might reduce cellulose crystallinity (15) or degree of cellulose polymerization (38). Ford (76) has suggested from results with pangola grass stems that lignin presence is more important for hemicellulose degradation than structural features, while the opposite was true for cellulose.

So in a sense it seems logical that the IVDMED data should be inversely and linearly correlated with lignin content. It is the result of an extended contact between enzyme and substrate, which shows not only the inhibitory effect of lignin but also

the effect of lignin upon extent of enzyme penetration within the solid matrix and adsorption on effective and ineffective surface area segments. On the other hand, early saccharification rates (or the maximum-initial rates obtained extrapolating to zero time) show clearly that it is not only lignin **per se** that counts, but how lignin is complexed to hemicellulose and how these LCCs have been modified and translocated within the solid matrix by the various pretreatments. In our case, CAOK-1 and CAEX (which were not water- or alkali-washed after pretreatment) showed higher rates than almost completely delignified samples (BCSODA).

The individual enzymes comprising the **T. reesei** complex adsorb very fast to a lignocellulosic substrate like bagasse (77, 78). The extent of adsorption has been found proportional to the initial hydrolysis rate of pure cellulose of different structural parameters (79). Hence it could be said that those pretreated bagasse samples showing higher initial saccharification rates on Table 2 also displayed a more favorable adsorption affinity for the enzymes. Although Ghose and Bisaria (77) found less enzyme adsorption affinity on the previously hydrolyzed or spent bagasse which contained more lignin, this was not the case with by Deshpande and Eriksson (80), who clearly showed that there was the same amount of adsorption on steam-exploded wheat straw and on the lignin-rich residue. This fact confirms again that the presence of lignin per se is not as important as how lignin has been modified by the pretreatments and how it is complexed to the hemicellulose fraction. Why is it then, that those pretreated samples that showed the higher initial rates were not the ones showing also the higher **in vitro** dry matter enzymatic digestibilities? Unfortunately the adsorption phenomena is not as simple as originally thought. Recent findings show that with lignocellulosic substrates (81), the enzymes once adsorbed remained immobilized within the molecular and supramolecular organization of the substrate. Within the enzymatic complex there are degrees of adsorption, yielding both tightly and weakly adsorbed components (82). Synergism of hydrolyzing action was observed between the two groups of enzymes (83). This meant that the enzymes migrated on the surface of the solid while trying to saturate their adsorption and active centers (84). A dynamic adsorption-desorption mechanism plays a key role during the hydrolysis reaction. The type of substrate pretreatment determines how much enzyme actually desorbs at the end of the reaction and how much enzyme still is adsorbed but is no longer effective (85). There is a competitive adsorption model which includes a synergistic mechanism of action (86), which confirms more and more that the enzymes produced by microorganisms are serially aligned in complexes bound to cell carriers and which form a "lectin" type bond with the carbohydrate polymers (87).

Hence a rapid enzyme adsorption producing a high initial saccharification rate does not mean that there will be a higher extent of saccharification. Enzymes might reach non-effective and immobile adsorption sites, enzymes might desorb as reaction

proceeds, and/or they might be susceptible to inhibition by substrate components or reaction products.

Our IVDMED and initial saccharification rates were obtained employing a partially pure commercial cellulase and they should be taken within that context. For specific substrates there are various correlations between the in vitro digestibility determined by enzymes or by samples of rumen fluids. However they are not to be employed as general purpose correlations. Data taken with other microbial cellulases or a mixture of microbes and enzymes like they exist in the rumen fluid might give other results as it will be shown immediately even for the same substrates.

Degradation with mixtures of anaerobic bacteria

- Anaerobic pure cultures and mixed flora

Two mixtures of known anaerobic bacteria were used, one mesophilic, in which all tests were done at 37°C, and one thermophilic, which was incubated at 60°C. The mesophilic culture contained the following microorganisms: Ruminococcus albus NCDO 2250 (National Collection of Dairy Organisms, UK), Ruminococcus albus DSM 20455 (Deutsche Sammlung von Mikroorganismen, FRG), Bacteroides ruminicola subs. brevis NCDO 2206, and Bacteroides ruminicola subs. ruminicola NCDO 2202. The thermophilic culture was composed of Clostridium thermoaceticum var. Ljungdahl, C. thermoaceticum var Wood (both from the Center for Biological Resource Recovery, University of Georgia), C. thermocellum DSM 1237, and C. thermosaccharol ticum DSM 571.

In addition to the two synthetic bacterial mixtures described above, four microbial mixtures from natural sources were studied. They were kept in jars of 3.5 l, three of them at 35°C and one of 5 l at 60°C, and fed every week with 5% (w/v) of ground EX-FERMented sugarcane chips and 300 ml of rumen fluid. The inocula for the three mesophilic mixtures came from: a) rumen obtained from a slaughterhouse, b) sediments of a swamp close to Lake Amatitlan, and c) the effluent from an anaerobic acidogenic reactor operating with coffee pulp. The thermophilic inoculum came from sediments from a volcanic pond close to Lake Amatitlan and Pacaya volcano.

To standarize the inocula of the known cultures, each mesophilic was grown for 24 h and each thermophilic for 72 h in a recommended media (88), and then each was diluted to an absorbance at 600 nm between 0.5 and 0.6. The two mixtures contained equal volumes of each component. In each test, 0.25 g of substrate was added to a 50-ml vial, a flow of oxygen-free CO_2 was passed, and the vial was sealed. Some strict anaerobes are very susceptible to oxygen hence it was necessary to adsorb any residual oxygen over a hot copper packed column connected

in the CO_2 gas line. Each vial was then inoculated with 0.5 ml of the standardized inoculum and 30 ml of prereduced sterile basal medium (88), yielding a 0.83% w/v substrate suspension. The basal medium was reduced with 0.05% cysteine sulfide. The redox potential indicator was 0.033% resazurin. The initial pH was adjusted to 7 by adding an 8% sodium carbonate solution. The vials containing the mixtures of unknown microorganisms were prepared by placing 0.5 g of each substrate in a 125-ml vial together with 50 ml from each digestor passed through cheesecloth. A buffer solution (88) was added so that its final concentration in the vial was 0.025M. The initial pH was between 6.8-7.5. During all handling the vials were kept under an oxygen-free nitrogen atmosphere. All tests were done on triplicate. A fourth vial was used to check the initial solids content.

Gas production in each vial was measured by liquid displacement (88). Afterwards the vial was opened and the pH measured. The contents were centrifuged at 12 000 rpm for 15 min at 10°C. The solid residue was washed twice with distilled water and oven-dried at 60-65°C until constant weight was attained. Volatile fatty acids were determined by gas chromatography (88) employing a Hewlett-Packard 402 chromatograph with a 1.8-m column of Chromosorb W with 10% ethylene glycol succinate.

- Substrate digestion

The experimental results obtained are given in Table 3. They show the % digestion obtained for each substrate tested with the six mixtures of anaerobic bacteria after 5 days of incubation at 38°C or at 60°C. In some cases where the 5-day sample was lost, data are given for 15 or 30 days. In a few cases no data were obtained.
The digestion values were often much lower than the IVDMED data previously presented. Only CACANA showed more or less the same amount of digestion for all mixtures and very close to the IVDMED value of 33.43% shown in Table 2. The digestibility found with the mixtures of pure cultures was usually better than the one obtained with the four unknown mixtures. Pretreatments were effective in increasing digestibility. CANA, CACANA, CADA-2 and CADK-1 predominated in higher values and should be considered the best pretreatments in order to obtain maximum values. At the end of 5 days the pH of the cultures had increased slightly, 0.2 units for the two mixtures of known bacteria, 0.3 for the mud inoculum, 0.4 for the rumen inoculum and 0.6 for the other two. There was no digestibility inhibition due to any pH drop, which in rumen bacteria is extremely important (89, 90).

Previous workers (46, 91-92) have shown that about 40% digestion of lignocellulosic crop residues should be expected in about 7 days by mixed cultures of rumen microorganisms or pure cultures of rumen bacteria. Our results shown in Table 3 are much poorer, and it was only after extensive substrate pretreatment that those digestion values were obtained.

Differences in digestibility may, in part, be due to differences in the initial microbial population initially used to inoculate the fermentation. Hence it is difficult to compare results.
Although R. albus strains quite effectively degrade purified and mechanically pretreated cellulose samples (93, 94, 95), and B. ruminicola is also an efficient utilizer of purified xylans (96), their behaviour with untreated lignocellulosic residues has been more erratic and strain-dependent. For example, R. albus degraded 25% of dewaxed cotton linters according to Halliwell and Bryant (93), only 4.5% according to Morris and van Gylswyk (97), and practically zero according to Wood et al. (98). Also, when using mixtures of unknown rumen microorganisms, the sample preparation and culture enrichment techniques influence the hydrolyzing activity. Recently Amos and Akin (99) have shown that protozoa might contribute with 50% of the total degradation of orchard and coastal grass forages. This does not mean that in all substrates protozoa might be responsible for more than half of the polymer degradation. Also phycomycete fungi play a role in polymer breakdown (100, 101); hence it is not surprising to have different responses when unknown mixtures of rumen microorganisms are employed as inocula.

There was no obvious correlation between % digestion and lignin content of the pretreated substrates. Bauchop and Mountfort (101) reported recently that the digestion obtained by a mixture of a rumen fungi and two methanogenic bacteria on three substrates varied according to lignin content. The extent of digestion found was 98, 76.2 and 36% for ball-milled filter paper, sisal fiber and barley straw, respectively, which had 0, 3.2 and 7.9% lignin contents. It seems, however, that pretreatments on the same substrate as shown in our data in Table 3 affect differently the interactions between microbe and substrate surface (6-8). The lignin content per se is no longer as important; instead it seems that the receptivity of the specific surface for microbial adsorption is the key element. However we only offer it as a suggestion as we cannot substantiate this statement with experimental facts, but only with data from other authors on the pretreatment effects upon the surface characteristics of the substrate.
Our data for the thermophiles reported in Table 3 is lower than that found in the literature (102-106), were under optimum conditions and after microbe manipulation digestions, close to 90% have been reported.
Khan et al. (107) reported cellulose digestion values after 7 days at 35°C by a mixed culture of anaerobic bacteria of 4 to 19.5% on delignified wood derived substrates like newsprint, cardboard, and paper bags. For pretreated corn stover under mild alkaline conditions and employing an inoculum from a continuous anaerobic digestor, Datta (108, 109) found a 74% hemicellulose conversion but only a 36.9% cellulose conversion. Levy et al. (110) reported conversions of 10, 16, and 29% for untreated corn stover, cane bagasse, and alfalfa; 45% for

alkaline-pretreated corn stover and 40% for steam-exploded wood. Partos et al. (111) employed mixed cultures from anaerobic sediments or mud from temperate French and tropical Congo environments and found for untreated barley straw a degradation of 3.2 to 14%. After an alkaline pretreatment these figures increased to 17 to 26.5%. Bachman et al. (112) studied the biodegradation of oat straw by a mesophilic mixture of bacteria from an anaerobic digester and found a 34 to 41% conversion after 30 days. These values were not increased after pure cultures of **C. cellulolyticum** and/or **Methanosarcina barkeri** were added. These references briefly show the large variations found in the degradation of natural or pretreated substrates by mixed cultures of anaerobic bacteria coming from quite different origins.

- Gas production and volatile fatty acid yields

The data for the volume of gas produced per unit weight of substrate-day after 5 days for the six inocula and the eleven pretreated cane bagasse samples were in the range of 19 to 582 ml per g of digested dry matter-day. Table 4 gives the volatile fatty acid yields after 30 days. The two fermentation parameters did not show any trend with respect to the lignin content of the substrates analyzed individually for every inoculum or as an average figure for all of them. It is well known that in complex ecosystems, microbial interactions, specifically intermicrobial hydrogen transfer, have profound effects on the pattern of fermentation products (113, 114). Hence, in the inocula of mixtures of unknown anaerobic bacteria where methanogens were certainly present, more gas and acetate should have been produced. More gas was indeed produced but the rate of acid production was not necessarily higher. In fact, as shown in Table 4, the mesophilic mixture consisting of **R. albus** and **B. ruminicola** and the unknown mixture from the digestor gave the highest yields of volatile acids, especially for some of the organosolv and alkaline-pretreated samples. It can be assumed that the acetate was used for methane production by the mixed flora from the digestors, and therefore, accumulation of fatty acids was not observed within the time periods covered in the present study.

Most of the gas production data for the mixtures of unknown anaerobic bacteria were higher than those found by Khan et al. (115, 116) which were between 25 to 118 ml of biogas per g cellulose degraded-day. Also they were higher in comparison to those few selected references employing defined mixtures of microorganisms (117-119). For those samples where the substrate digestion was very low, a small error in substrate loss makes a large difference in the values obtained and thus contributes to aberrant results. In those cases where substrate loss was above 15-20%, the gas production rates were as expected.

Matei and Playne (46) obtained VFA yields of up to 0.74 g of organic matter digested of cane bagasse. This figure was quite constant for different pretreatments. However, for the combined sodium and ammonium hydroxide treatments, they

reported a low value of 0.46. As shown in Table 4 our CAAM sample gave also one of the lowest yields. Weimer and Zeikus (120) give a yield of 0.38 for **C. thermocellum** and 0.78 for a mixture of **C. thermocellum** and **M. thermoautotrophicum**, showing clearly the shift to acid formation by interspecies hydrogen transfer. Highest VFA yields were produced by the two mixtures of pure cultures and the flora from the acidogenic digestor with average yields expressed in g acid equivalents per g digested dry matter of 1.036, 0.506 and 0.745, respectively. The other units produced much lower yields, probably due to presence of methanogens and conversion of acids to gas. An average yield of 0.837 was reported by Datta (109) from alkaline-pretreated corn stover. Khan et al.(121) obtained yields of 0.68 and 0.61 from cardboard and newsprint. Nishio et al. (122) reported a yield of 0.46 from untreated citrus peel.

The interactions between anaerobic bacteria are quite complex, and hence it is still hard to predict the outcome in product yield (organic acids) and substrate consumption (cellulose and hemicellulose) of a typical anaerobic fermentation. For example, Khan and Murray (123) have shown that **A. cellulolyticus** in pure culture degraded 6691 g of pure cellulose equivalents and produced 1278 g of total acids for a yield of only 0.19; when grown in co-culture with a saccharolytic bacteria, **C. saccharolyticum**, only 5249 g of substrate were consumed, but 1809 g of total acids were produced, for a yield of 0.34. If homoacetogenic bacteria are present, the acetic acid yields will increase greatly. Le Ruyet et al. (124) have shown that co-cultures of **C. thermocellum** and **Acetogenium kivui** on pure cellulose (Whatman CC31) will produce a yield of 1.06 total volatile acids. This might explain the consistently high yields found for the unknown mixture from the acidogenic digestor.

Conclusions All the pretreatments tested were very effective in making the lignocellulosic fraction of sugarcane more susceptible to direct enzymatic hydrolysis and to more favorable microbe-fiber interactions with anaerobic bacteria. The untreated material was very resistant to biohydrolysis. Pretreatments were effective because there was a delignification reaction and possibly hydrolysis of the lignin-hemicellulose bond. In some cases swelling of the solid matrix or reduction in degree of polymerization was suspected according to previous references, which made the substrate more accessible for effective surface attachment by enzymes in solution or within the sphere of influence of anaerobic microorganisms.

IVDMEDs were linearly correlated with the inverse lignin content of the pretreated substrates. This was not so for the saccharification rates. Also, it was not valid for microbial digestion, gas production rates or VFA yields.

One of the organosolv samples, CAOA-2, gave an IVDMED close to 90%. The lignin content had decreased from 12.5 to 2.4%.

Two organosolv samples, CAOA-2 and CAOK-1, and two alkaline pretreated ones, CANA and CACANA, showed higher digestion values by mixtures of known or unknown anaerobic bacteria.

Gas production rates and VFA yields were comparable to literature values. CANA and CAOK-1 gave the higher average yields. It seems then that either of those pretreatments should be employed for anaerobic VFA production. The mesophilic-pure culture mixture of anaerobic bacteria and the unknown mixture from the digestor were probably the best inocula for acid production.

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TABLE 1

PRETREATED SAMPLES AND EXPECTED EFFECTS

Sample	Pretreatment	Expected effects
	Alkaline	Alkaline
CANA	80 g NaOH kg^{-1} dry matter in solid state for 10 days at room temperature (20°C)	Saponification of esters of uronic acids associated with the xylan chains, inducing extensive swelling of solid matrix. Hydrolysis of hemicellulose and alkali labile bonds between hemicellulose-lignin and hemicellulose-phenolic acids and acetyl constituents of cell walls (12, 28, 29).
CACANA	80 g (Na_2CO_3 + Ca $(OH)_2$) kg^{-1} dry matter as before	
CAAM	22 g NH_3 kg^{-1} dry matter as before	
BCSODA	Full soda pulp obtained employing 15% Na_2O for 3 h at 165°C	
	Solvent	Solvent
CAOK-1	Organosolv, ethanol:water (60:40), 1% Na_2O, 4 h at 175°C, water washed after treatment	Delignification and hydrolysis of hemicellulose-lignin bonds, solvation of breakdown products and/or redeposition of the breakdown products on the solids (30, 31). The alkaline conditions swell the lignocellulose matrix and induce higher lignin removal (32); however, there is no extensive modification of the crystalline cellulose structure.
CAOK-2	Organosolv, same ratio ethanol:water, 2% Na_2O, 4 h at 175°C, no washing	
CAOA-1	Organosolv, same ratio ethanol:water, 2% Na_2O, 0.05% anthroquinone, no washing	
CAOA-2	Organosolv, same ratio ethanol:water, 4% Na_2O, 0.05% anthroquinone, no washing	

TABLE 1

(continued)

CABG	Aqueous-phenolic solvent, BATTELLE-Geneve process conditions (33)	
	Steam explosion	Steam explosion
CAEX	Steam exploded at 240°C for 60 s (34)	Lignin depolymerization to low molecular weight phenol-related compounds with possibility of recondensation reactions. Hemicellulose hydrolysis caused by the acid medium generated; resulting mono- and oligosaccharides o low molecular weight could condense with lignin derivati Defiberization of cellular structure and increase in surface area. Cellulose crystallinity maintained but degree of polymerization reduced (31, 35-38).
	Gaseous	Gaseous
CASO	35 g SO_2 kg^{-1} dry matter in solid state for 72 h at 70°C	Delignification and hemicellulose hydrolysis with possi breakage of any bonds between them. Cellulose crystal-linity unchanged but a decrease in the degree of polymerization (39, 40).

TABLE 2

ENZYMATIC HYDROLYSIS

Pretreatment	IVDMED, %	Permanganate lignin, %	Cellulose %	Hemicellulose %	Initial saccharification rate mg reducing sugars h^{-1}
BCSODA	98.04	0.7	81.7	15.8	3.08
CAOA-2	89.80	2.4	72.8	15.5	4.55
CABG	69.02	3.5	89.7	0.0	4.14
CAOK-1	60.40	5.4	70.7	12.8	6.01
CAEX	56.68	5.7	46.1	2.0	5.00
CANA	72.28	5.8	36.9	16.4	2.74
CAOA-1	67.25	6.0	65.8	19.1	2.93
CAOK-2	64.88	6.4	68.8	20.2	2.31
CACANA	33.43	8.6	40.4	25.7	1.93
CAAM	29.58	9.3	44.0	12.5	1.48
CASO	26.19	9.6	55.2	16.9	3.19
CAST-1	19.04	12.5	43.3	38.0	1.51

TABLE 3

% DIGESTION (5 days)

Substrate	Undefined Cultures				Defined Cultures	
	Rumen	Mud	Thermophilic ponds	Digestor	Mesophilic mixture	Thermophilic mixture
BC SODA	10.3	4.0	-	26.8	63.7[b]	26.1
CAOA-2	2.9	11.4	22.8	32.9	37.4[a]	41.3
CABG	1.5	3.7	3.4[a]	7.3	-	-
CAOK-1	24.3	10.6	14.8	27.6	30.4[a]	30.4
CANA	19.2	16.9	-	11.6	43.4	58.2
CAEX	17.7	18.6	19.0	7.0	13.0	22.4
CAOA-1	6.8[a]	10.6[a]	30.9	11.5	13.8	24.0
CAOK-2	3.7	4.6	20.2	3.9	-	39.2
CACANA	34.0	35.4	34.5	32.6	21.4	17.0
CAAM	10.0	9.5	10.4	9.5	-	-
CASO	8.5	1.3	21.7[b]	8.1	1.5	8.6
CAST-1	19.0	0.2	9.4	2.3	-	-

(a) At 15 days

(b) At 30 days

TABLE 4

VOLATILE FATTY ACID YIELDS

g acetic acid equivalents per g of digested dry matter

Substrate	Undefined Cultures				Defined Cultures	
	Rumen	Mud	Thermophilic pond	Digestor	Mesophilic mixture	Thermophilic mixture
BC SODA	0.604[b]	0.155	-	0.483	0.744	0.323[b]
CAOA-2	0.104	0.110	0.228	0.567	0.876	0.329
CABG	0.444	0.130	0.566	0.365	-	0.519
CAOK-1	0.328	0.768	0.109	1.426	0.984	0.510
CANA	0.519	0.698	-	1.834	1.023	0.224
CAEX	0.427	0.476[a]	0.218	-	-	0.332
CAOA-1	0.088	0.135	0.230	0.658	-	0.685
CAOK-2	0.431	0.302	0.195	1.214	-	1.011
CACANA	0.197	-[c]	0.494	0.108	1.551	0.378
CAAM	-[c]	-[c]	0.246	-[c]	-	-
CASO	-[c]	0.018	0.327	0.050	-	0.749

(a) Average of dry weight loss at 5, 10, 30 days
(b) Data of dry weight loss at 5 days
(c) Traces

FIGURE 1

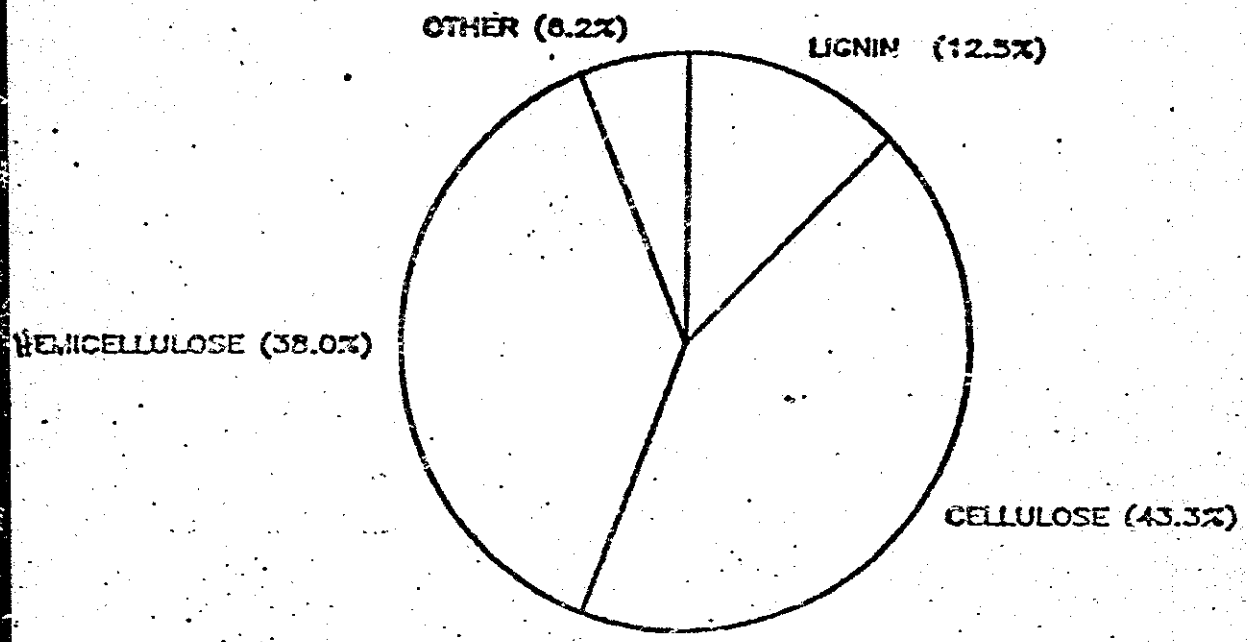
Macrocomponents of sugarcane lignocellulosic residues.

(A): EX-FERMented cane chips.

(B): Sugarcane bagasse.

Cellulose, hemicellulose and permanganate lignin obtained by the Van Soest techniques (21, 22)

A


OTHER (6.2%)
LIGNIN (12.5%)
HEMICELLULOSE (38.0%)
CELLULOSE (43.3%)

B

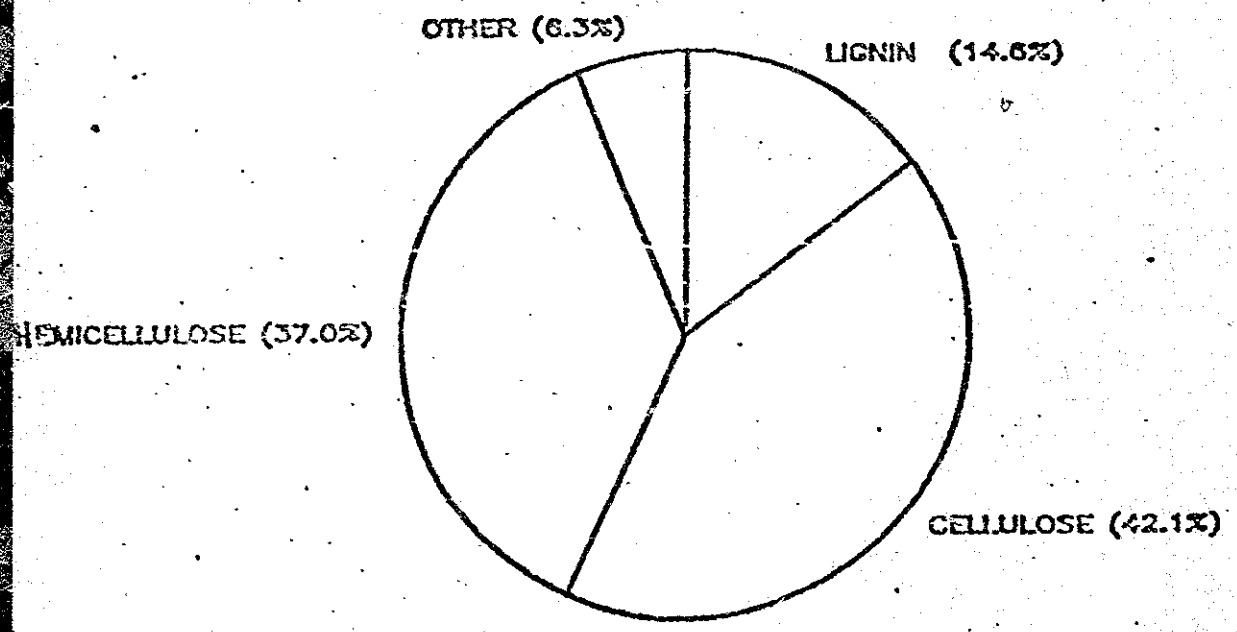
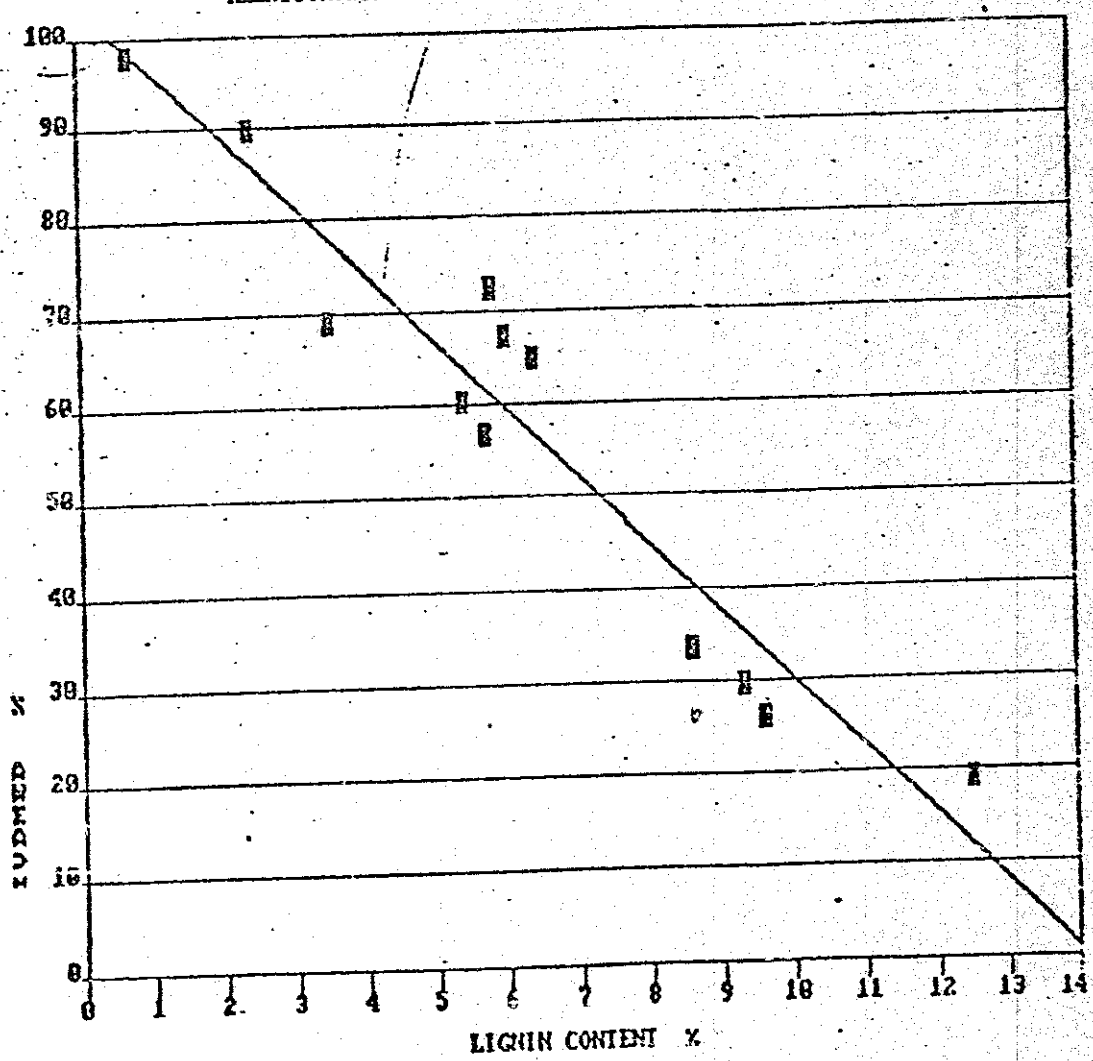

OTHER (6.3%)
LIGNIN (14.6%)
HEMICELLULOSE (37.0%)
CELLULOSE (42.1%)

FIGURE 2

Relationship between IVDMED and lignin content. IVDMED obtained by the two-enzyme method (26), employing pepsin followed by *T. reesei* cellulase.

RELATIONSHIP BETWEEN IVDMED AND LIGNIN CONTENT



PAPER No. 6

"Biodegradation of pretreated citronella and lemon grass bagasse by mixtures of anaerobic bacteria"

(Submitted for publication in Canadian Journal of Microbiology)

BIODEGRADATION OF PRETREATED CITRONELLA AND LEMON GRASS BAGASSE BY MIXTURES OF ANAEROBIC BACTERIA

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ASSTRACT

Bagasse from citronella (Cymbopogon winterianus) and lemon grass (Cymbopongon citratus), pretreated by six techniques and process conditions, was digested by six different mixtures of anaerobic bacteria. Percent digestion, gas production rate, and volatile fatty acid (VFA) yields were determined. A mixture of pure cultures of mesophilic anaerobic bacteria gave the highest yield of VFA. The source of inoculum did not have a statistically significant effect on digestion, but pretreatment of the substrates proved to have an important effect on digestion. The solid state pretreatment with sodium hydroxide or a mixture of sodium carbonate and calcium hydroxide fostered more complete digestion of the materials. The results are compared with those obtained from in vitro enzymatic digestion and with sugarcane bagasse.

INTRODUCTION

Lemon grass and citronella bagasse are the lignocellulosic residues from steam distillation of freshly cut lemon grass and citronella leaves for the recovery of the essential oils. The plants belong to the Gramineae and have been classified as Cymbopongon citratus and Cymbopogon winterianus respectively. Direct use as an animal feed is not possible due to rejection because of the residual aroma and flavor. Use as a source of fiber for paper and board has been taken only to the pilot plant level in Guatemala. Lemon grass oil production is around 1 000 t per year, being produced by India, Guatemala, the Republic of China and Sri Lanka. Citronella production is around 3 000 t per year, with Indonesia, Sri Lanka, the Republic of China, Taiwan, Guatemala and Brazil being the principal producers (Robbins, 1983). This represents about 200 000 of dry bagasse which could be available annually as a source of lignocellulosic biomass.

In the present work, the hydrolysis of the holocellulose material in the bagasse by anaerobic bacteria was studied. In order to increase the rate and extent of holocellulose hydrolysis, substrate pretreatment was used in order to modify the lignocellulosic matrix, favoring close contact between microbe and fibers, and efficient enzyme action. The mode of holocellulolysis may be quite species-specific both in terms of the substrate and the microorganisms, influenced by the structural features of the substrate, the interaction between microbe and substrate surface, and the complexity of the enzyme system. The effect of the pretreatments on the in vitro digestibility has been reported previously (Rolz et al, 1986a) for these two bagasses. At this time a comparison between the in vitro results, and the microbial digestion will be made, as well the acidogenisis by anaerobic bacteria. Use of lignocellulosic material as a substrate for

organic acid production by anaerobic bacteria has been reported (Khan et al, 1981; Taya et al, 1979; Datta, 1981a; Datta, 1981b; Nishio, 1982; Rolz et al, 1986b).

MATERIALS AND METHODS

Substrate

Gramineae bagasse was obtained from plantations located in the Guatemalan Pacific lowlands at 420 meters above sea level, in June at the start of the rainy season when the leaves are harvested. They were collected as they came out of the essential oil steam distillery, sun dried to a final moisture of around 6-7% and packed in plastic ventilated bags. The untreated samples were prepared by milling the dried raw material in a pilot Fitz Mill Comminutor (The Fitzpatrick Co.) with a .84 mm sieve. The final powders were packed in plastic bags, CIST being the identification for untreated citronella bagasse and TEST for untreated lemon grass bagasse. Chemical analysis for the substrates are found in Table 1.

Pretreatments

Details of the pretreatment procedures have been described previously (Rolz et al, 1986a). Briefly, the treatments and the respective identifications were solid state sodium hydroxide, CINA for citronella and TENA for lemon grass; solid state mixture of sodium carbonate and calcium hydroxide, CICANA for citronella and TECANA for lemon grass; gaseous ammonia, CIAM for citronella and TEAM for lemon grass; gaseous SO2, CISO for citronella and TESO for lemon grass; steam explosion, CIEX for citronella and TEEX for lemon grass; and modified alkali organosolv process, CIOA for citronella and TEOA for lemon grass. Composition and in vitro dry matter enzyme digestibilities (IVDMED) are given in Table 1. See Rolz et al (1986a) for analytical techniques.

Microbial Cultures

Two mixtures of known anaerobic bacteria were used. The mesophilic mixture, incubated at 37°C, contained the following microorganisms: Rumicococcus albus (strain NCDO 2250 from the National Collection of Dairy Organisms, UK), Ruminococcus albus (strain DSM 20455 from the Deutsche Sammlung von Mikdroorganisem, FRG), Bacteroides ruminicola subs. brevis (received as strain NCDO 2206), and Bacteroides ruminicola subs. ruminicola (received as strain NCDO 2202). The thermophilic mixture was kept at 60°C, and was composed of Clostridium thermoaceticum var. Liungdahl, C. thermoaceticum var. Wood (both from the Center for Biological Resource Recovery, University of Georgia), Clostridium thermocellum (received as strain DSM 1237) and Clostridium thermosaccharolyticum (received as DSM 571). The cultures were maintained as described by Valdez (1985). Four microbial mixtures from natural sources were also used. The inocula for the three mesophilic mixtures came from local sources: rumen contents from a slaughterhouse, sediments of a swamp close to Amatitlan Lake, and effluent from an anaerobic acidogenic reactor operating with coffee pulp juice. The flora was maintained in reactors of 3.5 l at 35°C and fed weekly with 5% ground EX-FERMented sugarcane chips and 200 ml of rumen extract. EX-FERMented sugarcane chips are defined as whole cane chips, in which sucrose originally present was transformed into ethanol by yeasts according to the

EX-FERM technique (Rolz et al, 1979). A fourth reactor, which had been charged with an inoculum from sediments from a volcanic pond close to Amatitlan Lake and Pacaya Volcano, was kept at 60°C and fed weekly with ground sugarcane and 300 ml of rumen extract. In all cases, tap water was added as necessary to make up volume. The reactors had been operating continually for over a year at the time this work was done.

Methodology for acidification and digestion tests

To standarize the inocula of the known cultures, each mesophilic strain was grown separately for 24 h and each thermophile for 72 h in its respective medium (Valdez, 1985). Each culture was diluted to an absorbance at 600 nm between 0.5 and 0.6. Then the cultures were mixed to contain equal volumes of each component. In each test, 0.25g of substrate was added to a 50 ml vial, a flow of oxygen-free CO_2 was passed to displace air, and the vial was sealed. Residual oxygen was trapped in a hot copper packed column placed in the CO_2 gas line. Each vial was then inoculated with 0.5 ml of the standardized inoculum and 30 ml sterile basal medium pre-reduced with 0.05% cysteine. Resazurin at 0.033% was used as redox potential indicator. Initial pH was adjusted to 7 by adding 8% sodium carbonate solution.

The test vials containing the microbial flora from natural sources were prepared by placing 0.5 g of substrate in a 125 ml vial together with 50 ml of digestor contents which has been filtered through cheesecloth. An appropriate buffer solution was added so that its final concentration in the vial was 0.025M, and the initial pH between 6.8-7.5. Buffer salt mixtures included KH_2PO_4, NH_4OH and $NaHCO_3$; KH_2PO_4, NH_4OH and Na_2CO_3; and KH_2PO_4 and $NaHCO_3$. During all handling the vials were kept under an oxygen-free nitrogen atomosphere.

Gas production in each vial was measured by liquid displacement. Then the vial was opened and pH measured with a corning 10 pH meter. The contents of the vial were centrifuged at 12 000 rpm for 15 min at 10°C. Volatile fatty acids (VFA) were determined in the supernatant by GLC using a Hewlett-Packard 402 Chromatograph with a 1.8 m column packed with Chromosorb W with 10% ethylene glycol succinate (Holdeman et al, 1977). The solid residue was washed twice with distilled water and then dried at 60°C to constant weight. Per cent digestion was calculated as per cent weight loss correcting for moisture content of the original substrates.

Statistical Methods

Analysis of variance was carried out on blocks of data, grouping the citronella and lemon grass substrates. The mixtures of pure cultures were analyzed separately from the natural flora for the digestion data, but grouped for VFA production. Significance was determined by calculating the F statistic and comparing it with the respective table values at 95%. The treatment means were compared using Tuckey's method (Li, 1964).

RESULTS AND DISCUSSION

Digestion was evaluated at 5, 15 and 30 days of incubation. Digestion by the mixtures of pure cultures for citronella bagasse

are found in Table 2. Table 3 presents the results for lemon grass. The conditions which led to highest degradation were the mixture of thermophilic bacteria on CIEX at 30d of incubation. Analysis of variance showed that substrate (citronella versus lemon grass) is not significant, nor was length of incubation. CINA and TENA proved to be the most digested; however, there was no statistically significant difference between them and the following pairs: CICANA and TECANA, CISO and TESO, and CIEX and TEEX. The lowest values were given by CIST and TEST, the untreated bagasse.

When the same substrates were fermented with the natural flora, the digestion was greater than that observed earlier. Results are in Table 4 for citronella and Table 5 for lemon grass. Again species of Cymbopogon is not significantly different; however, length of incubation and substrate pretreatment are significant. The substrate which provided the greatest digestion was CICANA and the least, TEOA. Statistically, the following show no difference: CICANA and TECANA at 15, 30 and 5 days, CISO and TESO at 30 and 15 days, CIAM and TEAM at 30 and 15 days and CIEX and TEEX at 30 days.

VFA production for the citronella substrates is given in Table 6 and for lemon grass in Table 7. Acid production is expressed as g acetic acid equivalents/g initial dry ash-free substrate. Correction for ash was made because the solid state sodium hydroxide and the calcium hydroxide-sodium carbonate samples had high ash contents due to the pretreatment (Table 1). The samples were analysed for VFA at 30 days, only. The mesophilic co-culture gave the highest values for VFA in both substrates. Statistically there was no difference at the 95% level between substrates, nor was there a difference among pretreatments. The mesophilic co-culture was statistically different from the rest of the inocula.

The microbial digestion of the substrates can be compared with the in vitro digestibility of the same substrates. TEOA and CIOA at 74 and 70% respectively, have the highest IVDMED (Table 1) and were also those which gave high digestion values at 30 days with the mesophilic mixtures of pure cultures, 53 and 57%. TENA and CINA with IVDMEDs of 66 and 67%, have high microbial digestions also, 53% and 51%, respectively. With the nature flora, these results are not repeated. Here TECANA and CICANA with TESO and CISO give the highest digestion values, with the sodium carbonate-calcium hydroxide treated bagasses giving good digestion values at even 5 days.

The solid state alkaline treatment with sodium hydroxide increased soluble solids considerably, 100% when compared to the untreated substrates. A pronounced increase was also shown by the steam explosion pretreatment. This would foster microbial growth and facilitate hydrolysis of holocellulose. And the reduction in lignin which occurs with the alkaline organosolv treatment should make the holocellulose substrate more available to microbial attack. Steam explosion opens the holocellulose matrix, facilitating contact between enzyme and substrate. For a more complete discussion of pretreatment effects, see Rolz et al (1986a).

Acid production at 30 days depended on inoculum source rather than substrate or pretreatment. With such long times, rates can not be calculated, which could have indicated differences between substrates. Statistical analysis of pH change and gas production were indeed highly significant with respect to time in all of the

units studied.
The differences among inocula can be explained by the following comments. In the digestors inoculated with natural flora, methanogens are present. These convert the organic acids to methane, and, indeed, analysis of the accumulated gas did show methane production, even under thermophilic conditions. By 30 days conditions of methane and acidity toxicity had developed which arrested further biodegradation. Datta (1981b) observed methane production at 16 days of fermentation of corn stover, with a corresponding decrease in VFA. Rolz et al (1986b) found that solid state soidum hydroxide pretreatment of sugarcane bagasse resulted in highest levels of VFA production. Different alkaline organosolv treatments were also effective.
When the mixtures of pure cultures were used, the mesophilic co-culture proved to be a better acid producer, in absence of methogenic flora. This may be due to increase acid tolerance of the cultures or CO_2 utilization as discussed by Taya et al (1979). CO_2 fixation could account for some of the acid production.
From the present study, it can be seen that the anaerobic fermentation of citronella and lemon grass bagasse is feasible for VFA production. Future work will be directed at control of methane production and a kinetic study of the process.

ACKNOWLEDGEMENT

Support for the project came from contract No. 936-5542-G-00-2041-00 from the U.S. Agency for International Development Program in Science and Technology Cooperation (PSTC). We sincerely appreciate their encouragement and support.
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Table 1. Chemical analysis of the *Cympopogon* substrates [a]

Substrate	IVDMED, % [b]	Permanganate Lignin, %	Holocellulose, %	Ash, %	Soluble Substances, %	Moisture,
CIST	18.24	11.1	58.5	9.8	28.0	6.25
CINA	67.10	5.7	35.1	18.9	55.3	17.23
CICANA	37.87	7.8	54.2	18.8	36.2	14.73
CIAM	45.86	8.5	52.9	8.7	33.3	9.02
CISO	56.86	7.8	39.6	9.6	49.6	8.30
CIEX	47.12	11.8	28.0	9.8	54.0	6.11
CIOA	70.44	9.7	62.7	10.1	16.4	5.75
TEST	13.80	11.0	58.4	1.5	27.4	7.45
TENA	66.14	4.7	44.5	19.3	51.8	17.49
TECANA	40.80	6.9	51.2	19.7	36.5	14.03
TEAM	41.12	9.7	49.7	11.2	33.6	9.22
TESO	49.49	6.8	43.3	11.8	45.5	7.62
TEEX	47.76	9.5	28.7	11.4	55.0	5.27
TEOA	73.57	7.7	68.0	12.3	14.6	4.60

a) IVDMED, lignin, holocellulose, ash and solubles are expressed on dry basis. Holocellulose is the sum of cellulose and hemicellulose. Soluble substances is calculated by (100 - neutral detergent fiber).

b) *In vitro* dry matter enzyme digestibilities.

Table 2. Digestion of citronella bagasse by mixtures of pure cultures of anaerobic bacteria [a, b]

Substrate	Source of inoculum: Mesophilic Mixture 5d	Mesophilic Mixture 15d	Mesophilic Mixture 30d	Thermophilic Mixture 5d	Thermophilic Mixture 15d	Thermophilic Mixture 30d
CIST	10.2	- 0.3	21.7	- 13.9	- 2.4	- 6.5
CINA	39.3	41.7	51.1	40.8	34.3	19.5
CICANA	30.6	45.6	43.0	29.6	14.4	15.8
CIAM	18.9	15.6	33.6	6.6	6.4	2.0
CISO	24.8	6.9	32.6	3.2	15.4	24.8
CIEX	11.6	- 5.1	30.6	19.1	18.0	60.8
CIOA	43.6	26.4	56.9	4.1	8.5	-15.9

a) The values are average of duplicate samples.
b) Digestion expressed as per cent weight loss.

Table 3. Digestion of lemon grass bagasse by mixtures of pure cultures of anaerobic bacteria [a, b]

Substrate	Source of inoculum					
	Mesophilic Mixture			Thermophilic Mixture		
	5d	15d	30d	5d	15d	30d
TEST	1.5	- 5.9	20.5	- 1.8	3.2	1.5
TENA	40.7	30.7	52.7	40.2	22.2	48.8
TECANA	34.2	35.1	52.1	30.2	21.1	21.1
TEAM	29.9	9.9	30.6	28.0	10.6	-17.0
TESO	21.0	22.5	48.5	13.6	16.2	20.1
TEEX	21.9	15.5	35.6	11.0	18.9	11.7
TEOA	18.9	44.9	52.8	-19.9	1.9	4.0

a) The values are averages of duplicate samples.
b) Digestion expressed as per cent weight loss.

Table 4. Digestion of citronella bagasse by microbial flora from natural sources [a]

Substrate	Rumen			Swamp Mud			Thermophilic pond			Acidogenic digestor			Average		
	5d	15d	30d	5d	15d	30d	5d	15d	30d	5d	15d	30d	5d	15d	30d
CIST	11.7	22.3	26.2	4.9	24.9	28.3	- 1.5	30.0	31.3	- 6.7	16.8	40.7	2.1	23.5	31.6
CINA	28.5	31.9	16.4	24.1	33.3	49.4	30.9	28.0	10.6	30.9	38.1	24.1	28.6	32.8	25.1
CICANA	50.3	64.3	55.0	41.4	64.8	72.8	52.2	48.9	57.3	46.1	69.0	50.7	47.5	61.8	59.0
CIAM	36.7	44.6	41.1	9.0	40.2	55.6	26.1	43.7	21.3	12.9	33.6	35.8	21.2	40.5	38.4
CISO	10.7	51.5	57.6	21.2	45.5	50.3	17.6	53.6	55.6	24.1	49.1	50.3	18.4	49.9	53.4
CIEX	15.2	27.1	28.9	8.8	34.8	37.4	0.3	4.1	24.2	5.8	25.9	28.8	7.5	23.0	29.8
CIDA	-10.8	30.4	38.5	- 2.7	31.7	50.8	-15.0	4.6	16.0	2.4	36.0	53.7	- 6.5	25.7	39.8
													17.0	36.7	39.6

a) Digestion expressed as per cent weight loss.

Table 5. Digestion of lemon grass bagasse by microbial flora from natural sources [a]

Substrate	Rumen			Swamp			Thermophilic pond			Acidogenic digestor			Average		
	5d	15d	30d	5d	15d	30d	5d	15d	30d	5d	15d	30d	5d	15d	30d
TEST	1.5	23.5	27.0	4.5	24.4	24.8	9.7	10.1	32.6	- 8.9	7.9	21.8	1.7	16.5	26.6
TENA	20.0	30.2	47.6	16.6	32.1	26.3	29.7	12.7	32.6	26.8	37.0	32.1	23.3	28.0	34.7
TECANA	41.8	52.5	58.1	36.3	67.9	61.8	32.5	48.8	41.8	47.0	65.6	53.9	39.4	58.7	53.9
TEAM	15.4	48.0	53.3	31.3	44.9	49.8	33.5	37.4	31.3	19.8	31.3	41.4	25.0	40.4	44.0
TESO	29.5	51.3	57.1	26.6	56.3	58.3	4.3	19.6	31.5	16.3	40.2	53.9	19.2	41.9	50.2
TEEX	3.6	14.7	63.7	15.9	19.8	44.1	6.1	17.2	30.0	4.0	9.5	15.5	7.4	15.3	38.3
TEOA	12.8	- 3.6	-19.9	-30.8	-15.7	-27.0	-14.5	10.3	45.5	0.2	18.7	45.1	- 8.1	2.4	10.9

a) Digestion expressed as per cent weight loss.

Table 6. Volatile fatty acid production by anaerobic bacteria on citronella bagasse at 30 d (g acetic acid equivalents/g initial dry ash - free substrate)

ubstrate	Inoculum						Avera
	Mesophilic Co-culture [a]	Thermophilic Co-culture [a]	Rumen	Swamp mud	Thermophilic pond	Acidogenic digestor	
CIST	.69	.12	.41	.23	.28	.13	.31
CINA	.58	.07	.20	.35	.41	.06	.28
CICANA	.29	.10	.25	.34	.25	.00	.20
CIAM	.50	.20	.24	.10	.19	.00	.20
CISO	.40	.10	.08	.12	.44	.02	.19
CIEX	.45	.16	.15	.12	.05	.19	.19
CIOA	.58	.10	.13	.14	.09	.03	.18
Average	.50	.12	.21	.20	.24	.06	.22

a) The values are the average of duplicate samples.

Table 7. Volatile fatty acid production by anaerobic bacteria on lemon grass bagasse at 30 d (g acetic acid equivalents/g initial dry ash - free substrate)

Substrate	Inoculum						
	Mesophilic Co-culture [a]	Thermophilic Co-culture [a]	Rumen	Swamp mud	Thermophilic pond	Acidogenic digestor	Ave
TEST	1.03	0.43	0.24	0.00	0.17	0.08	
TENA	0.43	0.22	0.00	0.04	0.00	0.06	
TECANA	0.50	0.15	0.00	0.00	0.00	0.00	
TEAM	0.67	0.09	0.00	0.00	0.05	0.00	
TESO	0.61	0.12	0.01	0.03	0.37	0.03	
TEEX	0.80	0.09	0.10	0.21	0.09	0.15	
TEOA	0.96	0.16	0.50	0.36	0.09	0.48	
Average	**0.71**	**0.18**	**0.12**	**0.09**	**0.11**	**0.11**	

a) The values are the average of duplicate samples.

PAPER No. 7

"Effect of monensin on acidogenesis of lignocellulosic biomass by mixed anaerobic cultures"

(Submitted for publication in Enzyme and Microbiol Technology)

EFFECT OF MONENSIN ON ACIDOGENESIS OF LIGNOCELLULOSIC BIOMASS BY MIXED ANAEROBIC CULTURES

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Keywords: methane, lignocellulose, pretreatment, acidogenesis, lactic acid, volatile fatty acids, monensin, fermentation, digestion, propionic acid.

ABSTRACT

The effect of the addition of monensin on acidogenesis by anaerobic cultures on pretreated bagasse samples was studied. Four inocula were used: a mesophillic **Ruminococcus** and **Bacteroides** co-culture, a thermophillic **Clostridium** co-culture, mesophilic flora from swamp mud, and thermophilic volcanic pond sediment. The bagasse (sugarcane, lemon grass and citronella) had been treated with alkali cook, alkali solid state, steam explosion, gaseous ammonia, gaseous sodium dioxide or organosolv. Substrate did not affect degree of digestion or acid production. Volatile fatty acid (VFA) and lactic acid production were higher when the mesophilic co-culture was used. Monensin increased acid production only in the case of the swamp mud inoculum, but this increase was not correlated with inhibition of methanogenesis.

INTRODUCTION

Acidogenesis from lignocellulosic biomass by anaerobic microorganisms poses an interesting alternative for the utilization of these materials. In our laboratory, acid production from sugarcane bagasse, lemon grass bagasse and citronella bagasse has been studied [1, 2, 3]. Sugarcane bagasse has been studied by Matei and Playne [4], while Datta [5, 6] reported work on corn stover, Levy et al [7] on water lily, Nishio et al [8] on mandarin orange peel, and Khan et al [9] on newspaper and other processed cellulosic material, to mention a few.

Pretreatment of the lignocellulosic substrate improves digestibility, as Cabello et al [10], Cabello and Conde [11], Playne [12] and Rolz et al [1] reported previously for sugarcane bagasse, and Rolz et al [13] recently showed for lemon grass and citronella bagasse. The mechanism for improvement varies with each pretreatment, but all foster enzyme-substrate contact, either by removal of interfering lignin or by modification of the crystalline cellulose structure. Pretreatment may also improve volatile fatty acid production as has

been shown by Rolz et al [1] for sugarcane bagasse, and Datta [5] for corn stover. Cabrera et al [2] did not observe any improvement with pretreated citronella and lemon grass bagasses, nor did Matei and Playne [4] with ammonia or sodium hydroxide treated sugarcane bagasse.

Degradation of lignocellulose and acidogenesis by co-cultures or mixed natural flora generally tends to be more complete than that realized by pure cultures alone, probably due to the complex enzyme system intervening in the hydrolysis of the holocellulose and posterior formation of a variety of volatile and fixed acids such as acetic, propionic, butyric and lactic acids. In nature, the process continues eventually to methane and carbon dioxide, and a non-degradable residue.

In order to maximize acid production and biomass degradation, several strategies have been offered: pH control or increased buffering capacity [8, 14, 15], thermophilic temperatures [16], use of compatible co-cultures which do not include methanogens [17-20], and addition of an inhibitor of methanogenesis, such as monensin [21-26]. Monensin is commonly used as an additive for the rations of feedlot cattle, with a capacity to improve feed efficiency. It is an antibiotic derived from Streptomyces cinnamonensis. Monensin acts selectively on different members of the rumen flora, retarding Ruminicoccus, for example. VFA accumulation is observed as a result of the blocking formation of formate and thus methane.

In the present study, eight selected substrates were fermented by four sets of inocula, two thermophilic and two mesophilic, with and without monensin. Acid production, weight loss of insoluble substrate, gas production and presence of methane were determined at seven and fifteen days.

MATERIALS AND METHODS

The bagasse samples were processed according to the information in Table 1. Further details can be found in Rolz et al [1] for sugarcane and in Rolz et al [13] for lemon grass and citronella bagasse. All were ground to 0.84 mm before utilization as substrate for microbial attack. Composition of the substrates is given in Table 2.

The mixture of anaerobic mesophilic pure cultures included Ruminococcus albus ICAITI 083, received as NDCO 2250, Bacteroides ruminicola subsp. brevis ICAITI 002, received as NCDO 2206, and Bacteroides ruminicola subsp. ruminicola ICAITI 003, received as NCDO 2202, all from the National Collection of Dairy Organisms, UK, and Ruminicoccus albus ICAITI 084, originally received as DSM 20455 from the Deutsche Sammlung von Mikroorganismen, FRG. The mixture of anaerobic thermophilic pure cultures included Clostridium thermoacticum var Ljungdahl, ICAITI 008, Clostridium thermoacticum var Wood ICAITI 009, both from the Center for Biological Resource Recovery, University of Georgia, and Clostridium thermocellum ICAITI 010, received originally as DSM 1237 and Clostridium thermosaccharolyticum ICAITI 011, received originally as DSM 571 from the Deutche Sammlung von Mikroorganismen, FRG.

In addition to the two co-cultures described above, two groups of

flora from natural sources were also used, one coming from sediment of a swamp close to Amatitlan Lake in Guatemala, and another from sediments from a volcanic pond close to Pacaya Volcano and Amatitlan Lake in Guatemala. The mesophilic swamp mixture was kept in a 3.5 l reactor at 35°C and fed weekly with 5% ground EX-FERMented sugarcane chips and 200 ml rumen extract. The thermophilic mixture was kept at 60°C in a 5 l reactor and fed weekly with 5% ground EX-FERMented sugarcane chips and 300 ml rumen extract. Tap water was added as needed to replace water which had evaporated. At the time this research began, the reactors had been functioning for over two years in a stable fashion.

Details for inocula preparation and standarization are found in Cabrera et al [2]. Briefly, the pure cultures were standardized to the same optical density, and equal volumes were used to make up the inoculum for the reaction vials. The reactor contents were filtered through cheesecloth before adding to the reaction vials. Precautions to maintain anaerobic conditions were used throughout.

Tha vials where the co-cultures were incubated with the test substrate were prepared by first adding 0.25 g of substrate to each 50 ml vial under a stream of oxygen-free carbon dioxide. Then the vials were sealed, and 30 ml of sterile prereduced basal medium were added aseptically inside an anaerobic chamber, followed by 0.5 ml of the co-culture. The basal medium has the following composition per liter: tryptic digestic of casein, 5 g; yeast extract, 5 g; $(NH_4)_2SO_4$, 0.9 g; KH_2PO_4, 0.9 g; NaCl, 0.9 g; $CaCl_2$, 0.02 g; $MgCl_2.6H_2O$, 0.02 g; $MnCl_2.4H_2O$, 0.01 g; $CoCl_2.6H_2O$, 0.01 g; $FeSO_4.7H_2O$, 0.01 g; resazurin, 0.33 g; rumen fluid, 0.1 ml; acetic acid, 1.7 ml; cysteine, 2.5% solution, 20 ml; Na_2CO_3, 8% solution, 50 ml, pH was adjusted to 6.8.

The test vials where the natural flora was used were prepared by first adding 0.25 g of the appropriate substrate, then 25 ml of the digestor filtrate and 25 ml of a buffer composed of a mixture of KH_2PO_4, NH_4Cl and Na_2CO_3, resulting in an initial pH of approximately 8 and a concentration of 0.025M. All work was done under a flow of oxygen-free nitrogen.

Monensin (prepared as the sodium salt from SIGMA) was added on the third day of incubation so that the concentration in the vials would be 10 ug ml^{-1}.

At the end of the respective incubation period, 7 or 15 days, gas production was measured by liquid displacement using an aqueous acidified saturated NaCl solution. Gas composition was determined by GLC using a Varian Aerograph Chromatograph, Model 3100, equiped with a thermal conductivity detector. The stainless steel column (6 ft x 1/8 in ID) was packed with Porapak N(80-100 mesh), and helium was used as carrier gas at 30 ml min^{-1}. Detector temperature was set at 190°C, column temperature at 50°C, and filament temperature at 20°C. A mixture of carbon dioxide and methane, 51.9 and 48.1% respectively, was used as standard.

The vials were opened and the contents centrifuged at 10 000 rpm,

10°C for 20 min. The supernatant was used for pH and VFA determinations. The residue was washed and centrifuged twice with sterile distilled water, and then dried to constant weight at 60°C. Digestion was calculated as the percent reduction in weight. VFAs were determined by GLC according to Holdeman et al [20].

Statistical evaluation of the data was carried out using analysis of variance with the appropriate F statistic at 95%. Treatment means were compared using Tuckey's method. Paired comparison technique was used to evaluate the effect of monensin [29].

RESULTS AND DISCUSSION

The digestion of the substrates by the different anaerobic inocula is presented in Table 3. The mixed natural flora digested the substrates more completely than did the pure cultures, with overall average values being 16.8% for the mesophilic co-cultures, 15% for the thermophilic co-cultures. 42.0% for the swamp mud digestors, and 37.6% for the thermophilic digestor. The difference between the co-culture and the mixed flora digestors was statistically significant. In the case of the co-cultures, there was a slightly better digestion at 7 days than at 15 days, while with the mixed flora digestors the opposite was true. However, the difference between 7 and 15 days was not significant. Cabrera et al [2] had observed increased digestion by the mixed flora compared with co-cultures on lemon grass and citronella substrates. However, with pretreated sugarcane substrates, Rolz et al [1] observed better performance by the known co-cultures.

No difference was found among the eight different substrates. This was indeed unexpected, since the in vitro dry matter enzymatic digestibilities (IVDMED) are very diverse as can be seen in Table 2. The BCSODA substrate had been included because of its almost complete delignification and high IVDMED. Only in the case of the swamp mud inoculum at 15d with monensin did the digestion values near those that had been expected.

The addition of monensin did not have a significant effect on digestion. This had been reported by Van Nevel and Demeyer [21] who indicated that the amount of substrate digested remained unchanged in the presence of monensin. Only fermentation products and animal growth efficiency were altered. Henderson et al [26] found that the effect of monensin on rumen flora was species dependent, with **Bacteroides ruminicola** not affected by the levels used in the present study. **Ruminicoccus albus** activity on cellobiose decreased. **In vitro** digestibility trials with rumen contents showed complete inhibition of digestion of cotton fibers and barley straw in the presence of monensin; digestion of dried grasses and powdered filter paper was reduced.

Values for volatile fatty acid (VFA) production are given in Table 4. The data has been expressed as acetic acid equivalents of VFA per g of original dry ash-free (DAF) substrate in order to take into account the different ash levels of the pretreated substrates. See Datta [6] for further discussion on this technique for handling data. There was no significant difference between the values for 7 days

and for 15 days, as had been observed earlier for the digestion data. And again, no significant difference was found among substrates. A difference does exist among inocula. The mesophilic co-culture proved to be the superior acid producer. Since digestion was poor for this inoculum, the fermentables in the basal medium were thought to be the preferred substrate. There is no significant difference between the behaviour of the mesophilic co-culture and the swamp mud digestor, but a difference does exist between the mesophilic co-culture and the two thermophilic inocula.

The addition of monensin did have an effect on VFA production. Increased VFA production was observed with the swamp mud digestor inoculum in the presence of monensin, a response similar to that reported in the literature for rumen fluid [21, 23, 24] where the production of H2 and formate and thus methane is inhibited. The thermophilic co-culture was a significantly better VFA producer in the absence of monensin. No report on the effect of monensin on the thermophilic **Clostridium** cultures which composed the co-culture could be found. The only report of use of monensin at thermophilic temperatures was that of Varel and Hashimoto [22] who observed monensin effects at 55°C similar to those at 35°C, that is to say increase in VFA and pH drop, coupled with severe inhibition of methane production.

When the presence of lactic acid in the fermented broth was studied, the co-cultures showed considerable production as can be seen in Table 5. In fact, lactic acid production is greater than VFA production for the co-cultures, representing 61% and 55% of the total acid production for mesophilic and thermophilic co-cultures respectively, when all data are expressed as acetic acid equivalents. In the case of the inocula from natural flora, the proportion decreased considerably, to 4% and 15% for the swamp mud and thermophilic digestor inocula, respectively. Lactic acid is a known end product of the fermentation of celulose and sugars of the bacteria which compose the two co-cultures [30] and probably serves as a final electron acceptor. In the mixed flora inocula, the diversity of microflora leads to multiple end products, including methane, as electron acceptor. See Table 7, also.

The mesophilic co-culture of **Ruminococcus** and **Bacteroides** produced significantly more lactic acid than the other three inocula, and lactic acid production by this co-culture was significantly higher in the absence of monensin. The mesophilic swamp mud inoculum produced higher levels of lactic acid when monensin was present. The two thermophilic inocula showed no significant difference in lactic acid production with or without monensin. The substrate did not influence significantly lactic acid production. The effect of monensin on lactic acid production and the prevention of lactic acidosis was studied by Dennis et al [24], who found the antibiotic effective in lowering lactate production by rumen fluid on sugars and starch. They attributed the reduction to inhibition of lactic acid producing members of the rumen flora, such as **Streptococcus bovis**. Henderson et al [26] observed reduced production of lactic acid by **Ruminococcus albus** on cellubiose in the presence of monensin. When Jouany and Senaud [28] studied the use of monensin in

ovine diets, they observed higher levels of lactic acid production, with a change in the rumen ecosystem. Here, the difference in substrate and inoculum must account for the different response.

When lactic acid was converted to acetic acid equivalents and added to the VFA values, the mesophilic co-culture proved to be an excellent acid producer, significantly better than the other three inocula. There is no difference among substrates. There is a significant effect towards greater acid production in the swamp mud digestor when monensin is added. The difference is not significant with the rest of the inocula.

The effect of monensin on the ratio of propionic acid to the other VFAs was evaluated, since the literature [21, 23] had shown a tendency towards accumulation of propionic acid from the decarboxylation of succinic acid. Propionic acid production was greatest in the trials with the swamp-mud inoculum and the difference from the other inocula was significant. However, the ratio of propionic acid to total VFA is significantly greater without monensin. Kaspar et al [31] reported that monensin did not increase the yield of propionic acid by Propionibacterium, and in fact was quite inhibitory to the cultures. Adaptation of the cultures did not improve yield either. Substrates did have an effect on propionic acid production, with TESO being significantly better than CIAM, CICANA, CIEX, TECANA, BCSODA, and CACANA. There was no significant difference between 7 and 15 days. The ratio of propionic acid to total VFA is given in Table 6.

Since the principal benefit of monensin appears to be repression of methane production, the presence of methane in vial headspace was determined. Results are given in Table 7. No methane was detected at 7 days in any vial. At 15 days, methane was detected in the mixed culture digestors at relatively low levels. Monensin has no apparent effect on methane production in the present study. Chen and Wolin [23] had reported earlier that monensin-resistant strains developed, and Varel and Hashimoto [22] had shown a delay in the onset of methane production under batch conditions, but no change in total methane production.

In conclusion, the mixed flora from the digestors were more active in digesting the pretreated lignocellulosic substrates. There was no difference among substrates, nor did the addition of monensin appear to affect digestion. VFA production was higher when the known mesophilic co-culture was used. The addition of monensin increased VFA production in the case of the inoculum from the swamp mud digestor, but decreased VFA production by the thermophilic co-culture. The amount of lactic acid produced by the co-cultures was unexpectedly high, and when the total acid production was taken into account, VFA plus lactic acid, the mesophilic co-culture proved to be significantly better than the other three inocula. Substrate did not have an effect. Monensin again improved total acid production in the case of the swamp mud inoculum. However, this increase was not correlated with inhibition of methanogenesis.

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Table 1. Pretreatments used on bagasse samples

Sample	Bagasse	Pretreatment
CACANA	Sugarcane which had been EX-FERMented	80g $(Na_2CO_3+Ca(OH)_2)Kg^{-1}$ dry matter in solid state for 10 days at 20°C
CICANA	Citronella bagasse	as above
TECANA	Lemon grass bagasse	as above
CIAM	Citronella bagasse	22gNH_3Kg^{-1} dry matter in solid state for 10 days at 20°C
CIEX	Citronella bagasse	Steam exploded at 240°C for 60s (Saddler, et al (27))
TESO	Lemon grass bagasse	36gSO_2Kg^{-1} dry matter in solid state for 72 h at 70°C
TEOA	Lemon grass bagasse	Organosolv, same ratio ethanol: water, [illegible] Na_2O, 0.05% anthraquinone, no washing
BCSODA	Sugarcane bagasse	Full soda pulp obtained employing 15% Na_2O for 3 h at 165°C

Table 2. Chemical analysis of substrates

Substrate	IVDMED, %	Permanganate lignin, %	Holocellulose, %	Soluble substances, %	Moisture, %	Ash, %
CACANA	33.43	8.6	66.1	22.6	15.58	16.64
CICANA	37.87	7.8	54.2	36.2	14.26	18.75
TECANA	40.80	6.9	51.2	36.5	14.45	19.69
CIAM	45.86	8.5	52.9	33.3	6.80	8.70
CIEX	47.12	11.8	28.0	54.0	6.11	9.75
TESO	49.49	6.8	43.3	45.5	5.15	11.84
TEOA	73.57	7.7	68.0	14.6	4.60	12.24
BCSODA	98.84	0.7	97.5	0.0	8.07	0.49

Table 3. Anaerobic digestion expressed as per cent weight loss

Substrate	Monensin	Inoculum: Mesophilic co-culture 7 d	Mesophilic co-culture 15 d	Thermophilic co-culture 7 d	Thermophilic co-culture 15 d	Swamp mud digestor 7 d	Swamp mud digestor 15 d	Thermophilic digesto 7 d	Thermophilic digesto 15 d
CACANA	-	17.8	13.1	10.1	3.0	47.3	29.9	28.8	49.5
	+	21.1	14.2	18.3	23.1	37.5	45.1	9.6	12.4
CICANA	-	23.1	18.6	28.0	0.0	50.5	57.7	21.6	29.3
	+	15.2	17.8	32.5	19.4	45.0	53.2	38.9	29.4
TECANA	-	25.3	7.9	28.2	24.5	51.4	62.9	27.8	54.6
	+	28.0	27.0	36.8	33.3	55.8	52.8	28.8	34.4
BCSQDA	-	11.9	1.7	4.6	55.9	43.6	47.1	43.2	13.2
	+	- 3.3	-13.0	- 9.6	43.5	25.0	71.0	38.8	15.0
CIAM	-	17.9	21.4	22.7	31.0	22.3	42.3	41.4	42.7
	+	28.5	17.2	-18.1	14.7	39.5	40.4	35.8	50.0
CIEX	-	16.9	10.0	37.9	32.0	6.3	31.2	8.4	55.6
	+	10.9	1.0	18.3	3.7	8.1	8.1	26.3	46.1
TESO	-	25.0	17.6	24.5	-30.0	35.6	47.3	51.2	58.3
	+	23.3	10.7	27.9	-59.7	39.9	52.3	54.7	66.1
TEOA	-	25.8	16.2	6.5	24.0	42.4	63.2	45.8	74.4
	+	37.6	30.5	11.5	-19.0	40.0	49.4	6.5	64.6

Table 4. Effect of monensin volatile fatty acid production by anaerobic inocula on lignocellulose
g VFA in acetic acid equivalents/g DAF substrate [a]

Substrate	Monensin	Inocula							
		Mesophilic co-culture		Thermophilic co-culture		Swamp mud digestor		Thermophilic digestor	
		7 d	15 d	7 d	15 d	7 d	15 d	7 d	15 d
CACANA	-	0.52	0.25	0.09	0.13	0.36	0.23	0.16	0.34
	+	0.49	0.26	0.09	0.11	0.27	0.51	0.17	0.21
CICANA	-	0.50	0.61	0.19	0.23	0.31	0.32	0.07	0.15
	+	0.40	0.38	0.09	0.08	0.29	0.65	0.13	0.15
TECANA	-	0.51	0.50	0.23	0.21	0.25	0.36	0.06	0.34
	+	0.58	0.61	0.10	0.14	0.31	0.81	0.14	0.17
BCSODA	-	0.41	0.74	0.04	0.01	0.21	0.12	0.16	0.02
	+	0.41	0.29	0.02	0.07	0.33	0.44	0.10	0.02
CIAM	-	0.40	0.53	0.06	0.02	0.28	0.33	0.13	0.29
	+	0.30	0.27	0.02	0.10	0.25	0.44	0.11	0.18
CIEX	-	0.43	0.40	0.05	0.12	0.17	0.15	0.05	0.22
	+	0.33	0.50	0.02	0.02	0.17	0.46	0.06	0.18
TESO	-	0.52	0.50	0.25	0.23	0.14	0.15	0.31	0.40
	+	0.46	0.99	0.23	0.02	0.27	0.26	0.30	0.34
TEOA	-	0.54	0.26	0.02	0.24	0.20	0.48	0.16	0.17
	+	0.44	0.41	0.03	0.16	0.20	0.45	0.01	0.23

a. In the case of the co-cultures, 0.21 g of fermentable substances per vial of the basal medium were taken into account.

Table 5. Effect of monensin on lactic acid and total acid production by anaerobic inocula at 15 d

Substrate	Monensin	Lactic acid production g/g DAF substrate				Total acid[a] production g/g DAF substrate			
		Mesophilic co-culture	Thermophilic co-culture	Swamp mud digestor	Thermophilic digestor	Mesophilic co-culture	Thermophilic co-culture	Swamp mud digestor	Thermophilic digestor
CACANA	-	0.62	0.15	0.00	0.00	1.01	0.31	0.23	0.34
	+	0.42	0.01	0.06	0.00	0.078	0.12	0.58	0.21
CICANA	-	0.72	0.32	0.00	0.00	1.49	0.62	0.32	0.15
	+	0.67	0.03	0.00	0.06	1.19	0.11	0.65	0.22
TECANA	-	0.59	0.00	0.00	0.00	1.22	0.21	0.36	0.34
	+	0.56	0.00	0.06	0.06	1.29	0.14	0.88	0.24
BCSODA	-	0.58	0.00	0.00	0.00	1.44	0.01	0.12	0.02
	+	0.57	0.08	0.02	0.00	0.99	0.17	0.46	0.02
CIAM	-	0.65	0.00	0.00	0.00	1.32	0.02	0.33	0.29
	+	0.57	0.14	0.00	0.05	0.97	0.26	0.44	0.23
CIEX	-	0.67	0.01	0.00	0.00	1.21	0.13	0.15	0.22
	+	0.66	0.00	0.02	0.05	1.30	0.02	0.49	0.24
TESO	-	0.70	0.51	0.00	0.02	1.36	0.84	0.15	0.43
	+	0.65	0.00	0.00	0.02	1.78	0.02	0.26	0.37
TEOA	-	0.57	0.47	0.00	0.14	0.96	0.81	0.48	0.35
	+	0.50	0.13	0.05	0.10	1.02	0.32	0.51	0.35

a. Total acid production is the sum of volatile fatty acids and lactic acid, all converted to acetic acid equivalents.

Table 6. Effect of monensin on propionic acid production[a]

Substrate	Monensin	Inocula							
		Mesophilic co-culture		Thermophilic co-culture		Swamp mud digestor		Thermophilic digesto	
		7 d	15 d	7 d	15 d	7 d	15 d	7 d	15 d
CACANA	-	6.72	8.59	12.28	3.48	6.52	15.43	0.01	5.85
	+	9.06	4.13	8.31	9.96	12.94	7.71	4.54	3.71
CICANA	-	6.91	0.01	6.66	8.83	21.80	26.20	6.23	5.50
	+	8.79	0.01	11.65	15.21	20.33	4.52	6.57	2.73
TECANA	-	8.09	0.01	7.41	9.53	15.25	34.75	0.01	5.02
	+	8.36	0.01	11.01	10.09	15.93	5.18	5.89	4.85
BCSODA	-	8.43	12.74	5.39	0.01	13.20	26.20	0.01	0.01
	+	9.75	3.32	0.00	10.39	9.27	35.32	0.01	0.01
CI/M	-	6.39	21.07	0.00	0.01	31.24	41.52	5.34	9.45
	+	10.83	7.48	0.01	5.25	22.86	1.55	3.03	3.84
CIEX	-	8.19	20.80	0.01	2.49	29.42	22.12	0.01	7.72
	+	8.71	24.34	0.01	0.01	20.29	1.47	0.01	0.01
TESO	-	10.44	24.13	4.92	6.72	44.67	68.05	5.45	5.97
	+	12.42	58.23	4.42	0.01	36.12	63.96	5.56	4.96
TEOA	-	8.67	3.89	0.01	4.66	37.10	55.85	4.29	5.84
	+	7.15	0.01	0.00	11.09	40.09	15.07	0.00	8.80

a. Results are given as propionic acid calculated as acetic acid equivalents/total volatile fatty acids as acetic acid equivalents x 100.

Table 7. Per cent methane in headspace at 15 d [a]

Substrate	Monensin	Inocula			
		Mesophilic co-culture	Thermophilic co-culture	Swamp mud digestor	Thermophilic digestor
CACANA	-	0	0	8	7
	+	0	0	8	0
CICANA	-	0	0	8	0
	+	0	0	9	0
TECANA	-	0	0	11	0
	+	0	0	17	0
BCSODA	-	0	0	0	0
	+	0	0	7	0
CIAM	-	0	0	0	13
	+	0	0	0	0
CIEX	-	0	0	0	0
	+	0	0	0	0
TESO	-	0	0	14	10
	+	0	0	12	9
TEOA	-	0	0	7	0
	+	0	0	10	9

a. No methane detected at 7 days.

PAPER No. 8

"Kinetics of lignocellulosic degradation and acidogenesis by mesophilic anaerobic bacteria on alkali treated sugarcane bagasse and lemon grass bagasse"

(Submitted for publication in
Journal of Chemical Technology and Biotechnology)

KINETICS OF LIGNOCELLULOSIC DEGRADATION AND ACIDOGENESIS BY MESOPHILIC ANAEROBIC BACTERIA ON ALKALI TREATED SUGARCANE BAGASSE AND LEMON GRASS BAGASSE

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Key words: Acidogenesis, volatile fatty acids, anaerobic bacteria, lignocellulose, alkali pretreatments, digestion, kinetics, atmosphere, sugarcane bagasse.

SYNOPSIS

Acid production and lignocellulose digestion rates were calculated and compared when a full soda cook sugarcane bagasse and citronella bagasse pretreated in the solid state with $Ca(OH)_2$ and Na_2CO_3 were fermented with two sources of anaerobic microorganisms, a mixture of pure cultures including 2 strains of Ruminicoccus albus and two strains of Ruminicoccus ruminicola, and a natural mixed culture from swamp mud. Either CO_2 or N_2 was used as atmosphere, and the experimental vials were shaken or left stationary. Inoculum composition and substrate influenced digestion, with average digestibility of 79.5% by the swamp mud inoculum on the treated sugarcane bagasse. The digestion rate constant, K_D, was 0.28 day^{-1}. Neither substrate, inoculum, atmosphere nor agitation exercised a significant effect on acidogenisis. The acidogenesis rate constant, K_A, for the mesophilic co-culture was 0.23 day^{-1}, with values of 0.14 and 0.15 day^{-1} for the other substrate-inoculum combinations.

INTRODUCTION

The conversion of lignocellulosic wastes to higher value organic compounds through fermentation by anaerobic bacteria has been a subject of considerable interest in the last decade. In Central America, sugarcane bagasse is of course a readily available substrate. Citronella bagasse is the residue remaining from steam distillation of citronella (Cymbopogen winterianus) for the obtention of essential oil.

Kinetic studies on acidogenesis have been carried out using sugars (De la Torre and Goma,[1] among others). However, due to the difficulties encountered in handling insoluble substrates, little is found in the literature regarding kinetics for pretreated lignocellulosic fermentations. In a particularly detailed study, Taya et al[2] estimated acid production and substrate degradation by measuring gas production over a relatively short period of time. Baccay and Hashimoto[3] developed simple first order equations to describe the acidogenic fermentation of alkaline treated straw.

In the present study we have attempted to compare acid production rates and lignocellulose digestion when two differen substrates are used, a full soda cook sugarcane bagasse, and citronella bagasse pretreated in the solid state with $Ca(OH)_2$ and Na_2CO_3, with two sources of mesophilic anaerobic flora, and under either carbon dioxide or nitrogen atmospheres with and without agitation. In

previous work[4,5] both substrates and inocula provided more complete digestion and greater volatile fatty acid production. Interest in defining the relevant parameters necessary for scale up of the process led to the present study.

The kinetic parameters for the results obtained in this study are compared with those calculated from data from the literature.

MATERIALS AND METHODS

Experimental Procedure: Sugarcane bagasse was prepared with a traditional full soda cook, 15% Na_2O for 3 h at 165°C as described by Rolz et al[5]. Citronella bagasse was pretreated by another alkaline process, a solid state mixture of Na_2CO_3+$Ca(OH)_2$ previously reported in Rolz et al.[6] Composition is shown in Table 1. Both were ground to 0.84 mm before utilization as substrate for microbial attack.

The mixture of anaerobic mesophilic pure cultures included **Ruminococcus albus** ICAITI 083, received as NCDO 2250, **Bacteroides ruminicola** subsp. **brevis** ICAITI 002, received as NCDO 2206, and **Bacteroides ruminicola** subsp. **ruminicola** ICAITI 003, received as NCDO 2202, all from the National Collection of Dairy Organisms, UK, and **Ruminicoccus albus** ICAITI 084, originally received as DSM 20455 from the Deutsche Sammlung von Mikroorganismen, FRG.

Preparation of inocula has been described previously by Cabrera et al.[4] The maintenance media were those recomended by the National Collection of Dairy Organisms.[7] The basal medium for the degradation tests had the following composition per liter: tryptic digest of casein, 5 g; yeast extract, 5 g; $(NH_4)_2SO_4$, 0.9 g; KH_2PO_4, 0.9 g; NaCl, 0.9 g; $CaCl_2$, 0.02 g; $MgCl_2.6H_2O$, 0.02 g; $MnCl_2.4H_2O$, 0.01 g; $CoCl_2.6H_2O$, 0.001 g; $FeSO_4.7H_2O$, 0.01 g; resazurin, 0.33 g; rumen fluid, 0.1 ml; acetic acid, 1.7 ml; cysteine, 20 ml of 2.5% solution; Na_2CO_3, 50 ml of an 8% solution. pH was adjusted to 6.8. The culture vials where the mixture of equal portions of each of the four microorganisms were incubated with the test substrate were prepared by first adding 0.25 g of substrate to each 50 ml vial under a stream of the appropriate gas, CO_2 or N_2. Then the vials were sealed, and 30 ml of sterile prereduced basal medium was added aseptically inside an anaerobic chamber, followed by 0.5 ml of the co-culture.

The mixed natural flora came from a digestor which had been inoculated initially with swamp mud, and had been fed weekly for over two years with milled EX-FERMented sugarcane bagasse and rumen extract.[4] Inoculum for the test vials was prepared by straining digestor contents through cheesecloth under a flow of inert gas. Then 25 ml of the filtrate was added to each vial which contained 0.25 g of the appropriate substrate and required atomosphere of carbon dioxide or nitrogen. Then 25 ml of a buffer composed of a mixture of KH_2PO_4, NH_4Cl and Na_2CO_3 was added, resulting in an initial pH of approximately 8 and a concentration of 0.025 M. The gases for all of the experiments had been rendered oxygen-free by using a hot reduced copper packed column in the gas line.

The vials were incubated at 35°C, with the agitation provided in a rotatory incubator shaker at 100 rpm. Vials were removed at 0, 2, 4, 7, 9, 11, 14 and 16 days for analysis. Gas production was measured by liquid displacement using an aqueous acidified saturated NaCl solution. Gas composition was determined by GLC using a Varian Aerograph Chromatograph, Model 3100, equiped with a thermal conductivity detector. The stainless steel column (6 ft x 1/8" I D) was packed with Porapak-N, 80-100 mesh, and helium at 30 ml min^{-1} was used as carrier gas. Detector temperature was set at 190°C, column temperature at 50°C and filament temperature at 210°C. A mixture of CO_2 and methane, 51.9% and 48.1% respectively, was used as standard.

The vials were opened and the contents centrifuged at 10,000 rpm, 4°C, for 20 min. The supernatant was used for pH and VFA determinations. The residue was washed and centrifuged twice with sterile distilled water and then dried to constant weight at 60°C. Digestion was calculated as the percent reduction in weight, with the original weight corrected for moisture. VFAs were determined by GLC according to Holdeman et al.[8]

Statistical Evaluation: The maximum values indicated in the tables were compared as a factorial design, with separation of the treatment sum of squares for each treatment. Analysis of variance of the data compared calculated F values with the F statistic at 0.05.

Rates and Yields of Acidogenesis and Digestion: From Baccay and Hashimoto,[3] the following equations for rate and yield of acidogenesis were obtained:

$$\frac{-d(V_o-V)}{dt} = \frac{dV}{DT} = k_A(V_o-V) \qquad (1)$$

where dV/dt is the rate increase of VFA yield (g/g day); V_o is the apparent ultimate VFA yield (g/g); V is the VFA yield at any time; and K_A is the acidogenic rate constant (day^{-1}). Integration of the equation, again from Baccay and Hashimoto,[3] yielded.

$$\ln\ (V_o-V) = \ln\ (V_o-a)-k_A t \qquad (2)$$

Similarly, the following equation was developed for rate of digestion:

$$\frac{-d(D_o-D)}{dt} = \frac{dD}{dt} = k_D\ (D_o-D) \qquad (3)$$

where dD/dt is the rate of increase of percent digestion (day^{-1}), D_o is the apparent ultimate percent digestion, D is the percent digestion at any time, and k_D is the digestion rate constant

(day^{-1}).

The digestion percentage was calculated as:

$$D = \frac{\text{g digested cellulose}}{\text{g initial cellulose}} * 100$$

Again, integration of equation (3) yielded

$$\ln (D_e - D) - \ln D_o = - k_D t \qquad (4)$$

RESULTS

Digestion

The results for digestion of pretreated bagasse are found in Table 2 for BCSODA and Table 3 for CICANA. The values used as highest are underlined. Highest digestion values were obtained using swamp mud as the inoculum source and BCSODA as substrate, with an average digestibility of 79.5% for this inoculum in sugarcane bagasse. These results approach the IVDMED reported in Table 1 for BCSODA. Digestion by the mesophilic co-culture was much less complete, reaching an average of only 43.5%. Greater digestion was observed when the CO_2 atmosphere was used. Agitation or no agitation have no effect.

Lower digestion values were observed with CICANA, with an overall average of 43.3% (Table 3). However, this was higher than the IVDMED reported in Table 1. The calculations for microbial digestion were not corrected for the non-digestible ash component, but only for solubles at 0 days. It can be assumed that a part of this ash is soluble NaOH; however, a portion is composed of minerals in insoluble forms. Highest digestion was again observed when the swamp mud digestor contents were used as inoculum. There is no apparent effect due to agitation or atmosphere.

Statistical evaluation comparing the highest digestion values in both Tables 2 and 3 indicated that inocula and substrate were significant at 95%. F values for agitation and gas environment were not significant.

Acidogenesis

Volatile fatty acid production data are presented in Table 4 for BCSODA and Table 5 for CICANA. Maximum values for each treatment are indicated. The average values for BCSODA, 0.566 g/g DAF substrate, with acid expressed as acetic acid equivalents, is very similar to that for CICANA, 0.529 g/g. Highest values were 0.72 g/g with swamp mud inoculum in nitrogen atmosphere, not agitated, using CICANA as substrate, and 0.74 g/g with the mesophilic co-culture in carbon dioxide atmosphere, not agitated using BCSODA as substrate. Satistical analysis comparing the highest values from Tables 4 and 5, those underlined, showed no significant differences attributable to inoculum, substrate, gas atmosphere or agitation.

The [illegible] yields are quite low, with values near 1 g/g expected. The conversion of volatile acids to methane was investigated, and in Tables 4 and 5, the samples where methane was detected in vial headspace at levels greater than 1% are indicated. Methane production is more frequent in the presence of the swamp mud inoculum, a natural source of methanogens.

Kinetic Study

Table 6 gives the kinetic parameters for the data described. Values for K_D and K_A were calculated as described in the methods section employing equations 2 and 4 and a linear least squares fit criterion. The digestion data were grouped with respect to substrate and inoculum, since agitation and gas atmosphere did not prove to have a significant effect on the digestion. The acidogenesis data were calculated in the same manner. Apparent maximum values, V_o and D_o, were calculated from the maximum values indicated in Tables 2 and 3, and weighted when necessary to reduce the standard deviation. Maximum digestion times did not coincide with maximum VFA production times. The lower K_A values for the swamp mud inoculum coincide with diversion of the VFA to methane in these cultures.

DISCUSSION

Effect of Agitation

From the work of other investigators, agitation was considered to be beneficial to digestion and acidogenisis. Khan,[13] studying anaerobic degradation of cellulose by sewage sludge, observed that in 3 l fermentors agitation was needed to keep the material dispersed, and to achieve degradation. However, stirring rates above 300 rpm appreciably lowered degradation of cellulose, probable by interfering with the formation of fiber-bacteria complex necessary for attack. Bhadra et al [16] also found that mechanical re-dispersion of the large substrate aggregates enhanced acid production. Blending in N_2 atmosphere was compared with blending in air, and a certain inhibition of their culture by air was observed. These authors carried out the redispersion on a weekly basis. The small-scale of the experimental units described in this paper as well as relatively low substrate concentration may have reduced the need for mixing.

Effect of CO_2 Environment

Since there are indications of CO_2 fixation by some anaerobic mesophiles[9] and that a carbon dioxide-carbonate buffer system is recommended for the pure culture of anaerobes,[6] a difference was expected between the two gas atmospheres, CO_2 and N_2. The fact that this did not prove to be significant indicated that the systems used provide sufficient buffer capacity, and that the mixed cultures were capable of using the organic carbon present.

Digestion

Greater digestion of BCSODA over CICANA could be expected from IVDMSD data in Table 1. Previous work with both substrates [4, 6, 7, 10] showed a similar behavior by both with maximum digestion of BCSODA at 78% with the mesophilic co-culture,[7] and 71% with swamp

mud inoculum[4]; and 59% digestion of CICANA with the mesophilic co-culture, and 73% with the swamp mud inoculum.[4] Both treatments are alkaline, similar to those used by Datta[11] for corn stover, where digestion values were not given, and by Matei and Playne[12] on $Ca(OH)_2$+Na_2CO_3 treated begasse who observed 76% digestion of insoluble DAF substrate in 66 h by rumen fluid.

The effect of inoculum indicating greater digestion by the swamp mud inoculum is probably due to the complex interactions between the different members of the consortium. Cabrera et al[10] reported % digestion values 2.5 times greater using swamp mud inoculum compared with the mesophilic co-culture when the same two substrates are compared. Valdez[7] obtained superior digestion with swamp mud inoculum on CICANA, 73% versus 59% for the mesophilic co-culture; however the reverse was obtained with BCSODA, 79% for the mesophilic co-culture against 39% for the swamp mud.

The kinetic evaluation of the data found in Table 7 showed similar rates for the four combinations, with the highest being the K_D=0.27 d^{-1} for BCSODA with the mesophilic co-culture. Values for K_D calculated from the data available in the literature range from 0.18 h^{-1} for digestion of tissue paper by an anaerobic digestor inoculum as reported by Khan[13] to an extremely rapid digestion of a 4.5% slurry of pure cellulose by a mixed culture of **Ruminococcus albus** and **Ruminococcus flavefaciens** reported by Taya et al,[9] which gave 93% digestion in 3.8 days with a K_D of 0.90 d^{-1}. Matei and Playne[12] also obtained rapid digestion of ammoniated sugarcane bagasse by rumen contents, with a K_D of 0.76 d^{-1}. The activity of the pure cultures of Guiliano et al[14] should be pointed out since the substrate used was untreated cotton fiber, a crystalline form of cellulose. The data reported in this paper are lower than the majority listed in Table 7; improvement could be investigated through increasing levels of inoculum used or substrate as suggested by Baccay and Hashimoto.[5]

Acidogenesis

Acid production was not found to be significantly affected by any of the treatments used in this study. Previous work with both substrates reported by Cabrera et al,[10] gave similar acid production data, with averages of 0.47 g/g for the mesophilic co-culture in CICANA, 0.46 g/g for the same inoculum in BCSODA, 0.39 g/g for the swamp mud inoculum in CICANA and 0.28 g/g in BCSODA. In that study, considerable amounts of lactic acid were produced. Only VFAs were studied in the present work. Initially higher pH and improved buffer system could account for the acidogenesis leading preferencially to lactic acid. Previous work reported by Valdez[7] gave .5 g acetic acid equivalents/g with the mesophilic co-culture on BCSODA. However the same inoculum gave values of 1.0 g/g for other substrates, such as untreated lemon grass bagasse. In general, the swamp mud inoculum gave poor acid production due to conversion to methane. Loss of carbon through methane had been observed by other investigators.[3, 11, 13]

When kinetics of acid production are compared with literature data, the values in Table 8 are obtained. The data in the present study

are similar to those obtained by others. Two general comments should be made. First of all, low K_A's are associated with methane production as commented earlier.[3, 11] Second, the V_∞ values for the work by Matei and Playne[12] are not comparable because their data is expressed as mM VFA, and the exact acid profile was not given which would have made conversion to acetic acid equivalents possible. Also, these authors did not take into account the fermentable compounds contributed by the inocula and the medium which contained yeast extract.

CONCLUSIONS

Digestion and acidogenesis by anaerobic microorganisms is influenced by inoculum composition and substrate. Future work should investigate concentration of substrate and inoculum to improve rates. Particularly interesting should be the research in area of inoculum composition, either eliminating methanogens from the swamp mud digestor or adding more efficient celulolytic microorganisms to the co-culture.

NOMENCLATURE

KA	Acidogenic rate constant (day^{-1})
t	Time in days
V	VFA yield at any time (g/g)
V_∞	Apparent ultimate (or maximum) VFA yield
a	V at t = 0
D_∞	Apparent ultimate digestion percentage
D	Digestion percentage at any time, calculated as (g digested cellulose/g initial cellulose)*100
K_D	Digestion rate constant (day^{-1})
BCSODA	Sugarcane bagasse treated with a full soda cook
CICANA	Citronella bagasse treated with $Ca(OH)_2$ and Na_2CO_3 in the solid state
VFA	Volatile fatty acid
IVDMED	In vitro dry matter enzymatic digestibility
DAF	Dry ash free

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Table 1. Composition of substrates used

Substrate	BCSODA	CICANA
IVDMED, %	98.7	37.9
Permanganate lignin, %	0.7	7.8
Cellulose, %	81.7	30.0
Hemicellulose, %	15.8	24.3
Soluble substances, %	0.0	36.2
Ash, %	0.4	15.9
Protein	2.7	7.6
Moisture, %	8.1	14.3

Composition expressed on a dry weight basis.

IVDMED is *in vitro* dry matter enzymatic digestibility.

Table 2. Digestion of treated sugarcane bagasse

Atmosphere Inoculum Time, days	Digestion[a], %							
	Nitrogen				Carbon dioxide			
	Mesophilic co-culture		Swamp mud digestor		Mesophilic co-culture		Swamp mud digestor	
	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated
2	11.3	9.0	9.6	-3.4	8.5	6.9	-67.3	-30.0
4	13.1	15.1	17.9	22.0	23.5	17.3	9.3	35.5
7	32.6	23.2	19.9	25.1	21.9	21.4	84.3	53.6
9	17.2	22.6	21.2	54.0	21.3	19.2	67.3	54.6
11	29.1	28.7	26.6	62.9	24.0	26.1	75.4	71.4
14	25.9	28.1	79.8	31.6	30.1	23.6	50.5	56.9
16	19.8	24.3	84.6	57.7	22.9	81.6	64.1	86.1

a. $\frac{\text{weight, day o} - \text{weight, day n}}{\text{weight, day o}} \times 100$

Table 3. Digestion of treated citronella bagasse

Atmosphere Inoculum Time, days	Digestion[a], %							
	Nitrogen				Carbon dioxide			
	Mesophilic co-culture		Swamp mud digestor		Mesophilic co-culture		Swamp mud digestor	
	agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated
2	9.9	2.2	13.4	10.1	18.5	- 2.0	- 0.9	- 30.4
4	24.6	19.5	38.9	43.6	22.9	1.0	25.4	23.5
7	18.2	8.6	36.4	46.3	32.2	28.1	40.0	32.4
9	28.6	22.7	52.2	37.2	32.2	b	30.9	27.0
11	29.6	23.8	56.7	52.3	38.3	21.6	40.0	30.4
14	25.6	37.8	57.5	50.0	41.4	35.7	37.3	44.1
16	22.7	25.9	54.7	51.8	33.9	43.7	37.3	40.2

a. $\frac{\text{weight, day o} - \text{weight, day n}}{\text{weight, day o}} \times 100$

b. lost sample

Table 4. Volatile fatty acid production by anaerobic flora on treated sugarcane bagasse

Acid production, g acetic acid/g DAF substrate

mosphere noculum ime, days	Nitrogen				Carbon dioxide			
	Mesophilic co-culture a		Swamp mud digestor		Mesophilic co-culture a		Swamp mud digestor	
	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated
0	0.17	0.09	0.00	0.00	0.16	0.12	0.00	0.00
2	0.18	0.17	0.01	0.01	0.34	0.34	0.00	0.00
4	0.36	0.17	0.05	0.05	0.43	0.38	0.00	0.19
7	0.31	0.17	0.07	0.09	0.21	0.40	$\boxed{0.55^{m}}$	0.31^{m}
9	$\boxed{0.41}$	0.37	0.07	0.43^{m}	$\boxed{0.59}$	0.53	0.23^{m}	0.44^{m}
11	0.37	0.46	0.06	0.33^{m}	0.42	0.41	0.47^{m}	0.25^{m}
14	0.40	0.30	$\boxed{0.70^{m}}$	0.17^{m}	0.46	0.39	0.40^{m}	$\boxed{0.61^{m}}$
16	$\boxed{0.41}$	$\boxed{0.49}$	0.54^{m}	$\boxed{0.44^{m}}$	0.44	$\boxed{0.74}$	0.46^{m}	0.52^{m}

a. For these calculations, the carbohydrate and protein content of the medium (0.21 g/30 ml) was taken into account as potential substrate.

m. Methane was detected in headspace at levels greater than 1% 1/100 l.

Table 5. Volatile fatty acid production by anaerobic flora on treated citronella bagasse (g acetic acid/g DAF substrate)

Atmosphere	Nitrogen				Carbon dioxide			
Inoculum	Mesophilic co-culture[a]		Swamp mud digestor		Mesophilic co-culture[a]		Swamp mud digestor	
Time, days	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated	Agitated	Not agitated
0	0.09	0.07	0.03	0.03	0.16	0.12	0.02	0.01
2	0.41	0.28	0.11	0.07	0.48	0.33	0.05	0.04
4	0.37	0.39	0.27	0.22	0.51	0.47	0.16	0.14
7	0.20	0.31	0.17	0.27	0.33	0.39	0.29	0.25
9	0.51	0.54	0.41	0.36	0.50	0.39	0.17	0.31
11	0.38	0.41	0.42	0.46	0.59	0.34	0.24[m]	0.14
14	0.48	0.58	0.15[m]	0.15	0.51	0.69	0.15[m]	0.23[m]
16	0.63	0.39	0.42[m]	0.72[m]	0.49	0.24[m]	0.05[m]	0.22[m]

a. For these calculations, the carbohydrate and protein content of the medium (0.21 gr/30 ml) was taken into account as potentia substrate.

m. Methane was detected in headspace at levels greater than 1% 1/100 l.

Table 6. Kinetic parameters

Digestion	K_D day^{-1}	Sd	D_0 % digestion	t days
CICANA				
- mesophilic co-culture	.21	.0475	38	14.0
- swamp mud	.24	.1597	48	11.5
BCSODA				
- mesophilic co-culture	.27	.1137	43	12.0
- swamp mud	.28	.1502	79	12.5

Acidogenisis	K_A day^{-1}	Sd	V_o g VFA/g DAF substrate fed	t days
CICANA				
- mesophilic co-culture	.23	.1477	.52	16.0
- swamp mud	.15	.0754	.49	16.0
BCSODA				
- mesophilic co-culture	.15	.0702	.57	14.0
- swamp mud	.14	.0767	.35	16.0

Table 7. Kinetic parameters calculated from the literature for digestion of lignocellulose by anaerobes

Substrate	Microorganism	K_D day^{-1}	D_0 % digestion	t days	Reference
Cellulose (cotton) 5% (w/v)	*Acetivibrio cellulotyticus*	0.38	36.7	11	Guiliano et al [14]
Cellulose (cotton) 5% (w/v)	new isolate, strain BA5	0.45	40.2	10	Guiliano et al [14]
Cellulose (cotton) 5% (w/v)	new isolate, strain SPB-25	0.33	42.1	12	Guiliano et al [14]
Cellulose (cotton) 5% (w/v)	*Clostridium thermocellum*	0.36	34.1	11	Guiliano et al [14]
Tissue paper 1.5% (w/v)	mixed culture	0.19	41.7	21	Khan [13]
Cellulose CF11; Whatman 0.5% (w/v)	mixed culture	0.33	92.0	14	Khan et al [15]
KC flock W-300 (Sanyo Ko Kusaku Pulp Co.) 4.5% (w/v)	*Ruminicoccus albus* and *Ruminicoccus flavefaciens*	0.90	93.0	3.8	Taya et al [9]
Cellulose (α-floc) 1% (w/v)	Cellulotytic culture	0.30	39.0	20	Bhadra et al [16]
Ammoniated sugarcane bagasse 5% (w/v)	Rumen contents	0.76	60.0	6.4	Matei and Playne [12]

Table 8. Kinetic parameters for VFA production by anaerobes on lignocellulosic substrates calculated from the literature

Substrate	Microorganism	K_A	V_0 g VFA/g cellulose fed	t days	Reference
Cellulose (cotton)	*Acetivibrio cellulolyticus*	0.30	.43	8	Bhadra et al [16]
Corn stover NaOH treated 25°C, 1% (w/v)	50:50 mixture of fresh cow manure and sewage sludge	0.27	.40	15.8	Datta [11]
Corn stover NaOH treated 35°C, 1% (w/v)	50:50 mixture of fresh cow manure and sewage sludge	0.16	.43	24	Datta [11]
Corn stover $Ca(OH)_2$ $+Na_2CO_3$ treated, 25°C, 5% (w/v)	50:50 mixture of fresh cow manure and sewage sludge	0.13	.32	24	Datta [11]
Ammoniated sugarcane bagasse, 2.5% (w/v)	rumen bacteria	0.69	4.57[a]	7	Matei and Playne [12]
Ammoniated sugarcane bagasse, 5% (w/v)	rumen bacteria	0.67	5.71[a]	7	Matei and Playne [12]
Wheat straw, ball-milled and pretreated with NaOH, 8% (w/v)	digestor feed with cow manure	0.24	.18		Baccay and Hashimoto

a. Expressed as mM VFA/g cellulose.

PAPER No. 9

"Conversion of organic acids to biomass by yeasts"

(Submitted for publication in Applied Environmental Microbiology)

CONVERSION OF ORGANIC ACIDS TO BIOMASS BY YEASTS

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ABSTRACT

Continuous fermentation techniques were used in a preliminary study on the use of organic acids such as acetic, butyric, propionic and lactic acids by yeasts as carbon source with the conversion of these acids to biomass. **Saccharomyces cerevisiae** L-181 and **Candida utilis** L-283 were studied. Neither was capable of producing ethanol under the conditions used in this study; however, both were capable of producing biomass.

Mixed cultures of anaerobic bacteria are capable of transforming lignocellulosic material to volatile organic acids through acidogenic fermentation. The conversion of sugarcane, citronella, and lemon grass bagasse has been studied locally (C. Rolz, S. de Cabrera, M. J. Valdez, M. C. de Arriola and J. Valladares. Manuscript submitted to Biotechnology Progress). The purpose of the present study was to transform the organic acids produced to ethanol and/or biomass. As a preliminary step, we studied the ability of yeasts **Saccharomyces cerevisiae** and **Candida utilis** to carry out this transformation, using synthetic substrates with one or a mixture of these acids.

The yeast cultures used in this study were maintained on YM slants. **Saccharomyces cerevisiae** ICAITI L-181 had been received as CBS 1242 and **Candida utilis** ICAITI L-283 as DSM 2361. The fermentor was inoculated with a 24 hour culture from the YM slants.

The basal medium for the fermentation studies contained in grams per liter: yeast extract, 15; $(NH_4)_2SO_4$, 40; KH_2PO_4, 1; $CaCl_2$, 1; and $MgSO_4$, 5. The levels of glucose or organic acid added as carbon source are given with the results in Table 1. The pH was adjusted to 5.0 with NaOH before autoclaving.

The experiments were carried out in a one-liter modular bench-top fermentor (Gallenkamp, U. K) containing 300 ml of medium, at 30 C, with magnetic stirring and a dilution rate of $D = 0.1\ h^{-1}$. For the aerobic conditions and aeration rate of 400 ml min^{-1} was employed.

The fermentation was started with glucose, then the strains were adapted to acetic acid using the mixture of glucose and acetic acid for a period of 24 hours, then acetic acid was used as carbon source. Then a mixture of acetic acid and propionic acid was employed to adapt the strains to propionic acid. For the adaptation with butyric and lactic acids, the same technique was used.

Viable yeast cell counts were performed according to Lee et al (5). The dry weight of the culture was obtained by centrifuging an aliquot, washing the pellet twice with sterile isotonic saline solution and drying at 60 C to constant weight. For glucose determination, the colorimetric glucose oxidase/peroxidase kit from Sigma was used. Ethanol was determined by the gas chromatographic method of Lie et al (6). The organic acids were determined using

the gas chromatographic method of Holdeman et al (3).
The utilization of the organic acids by **S. cerevisiae** and **C. utilis** was evaluated in terms of disappearance of the substrate, pH change, biomass production and ethanol production. Ethanol was produced only in the presence of glucose by **S. cerevisiae** under aerobic and anaerobic conditions, as was expected. Thermodynamic restraints prevented its production from other substrates by this yeast. The **Candida utilis** strain used in this study is not a known ethanol producer.
In Table 1, the aerobic behavior of **S. cerevisiae** is given. Butyric acid alone is not utilized. Propionic acid alone is consumed; however, in mixtures with acetic acid or lactic acid, the other acids are preferred. Biomass production as well as cell count were highest for the glucose-containing media, although lactic acid permitted a higher level of cells in the fermentor. With acid consumption the pH also rose as was expected.
The results of anaerobic growth for the same yeast are given in Table 2. Although tendences are similar to aerobic growth, substrate utilization and biomass concentration have both decreased. Table 3 provides the data for aerobic growth of **C. utilis**. This culture proved to be capable of reducing the concentration of each of the acids to less than 15% of the original level except for butyric acid in the presence of lactic acid where preference was given to the lactic acid. Here the highest levels of biomass and pH were observed also.
Anaerobic conditions greatly reduced acid utilization by this yeast also as can be seen in Table 4. Particularly noteworthy is the inhibition of cell numbers by butyric acid and, to lesser extent, by propionic acid. Utilization of lactic acid was also greatly reduced; biomass production was quite low in all cases, also.
It must be assumed in these studies that the yeast extract in the medium provided carbon for cell maintenance and growth, either from the carbohydrate or amino acid components. The concentration must have been limiting, because under aerobic conditions, the added carbon sources were utilized effectively by the yeasts. Under anaerobic conditions the yeast were not capable of using butyric acid, and use of others was reduced, perhaps due to a lack of ready electron acceptors, thermodynamic considerations or absence of an active transport system for entering the anions into the cell. Organic acids in undissociated form penetrate by diffussion into an intact cell and acitve transport of anions may take place under aerobic conditions (8). In the rumen, butyrate and propionate are metabolized during passage through the rumen wall, the first being converted to B-hydroxybutyrate and the later to succinic acid (4). Datta and Zeikus (2), have demonstrated that **Clostridium acetobutylicum** is capable of taking up butyric acid. Uptake is increased when the metabolism of this microorganism is modulated with carbon monoxide; this effect is observed during the early part of the fermentation. The acid apparently acts as an electron sink. Ohyama and Hara (7) showed that butyric acid inhibited yeast growth on silage under aerobic conditions, while propionic acid was less effective in retarding yeast growth at the same molar concentrations. Lower pH was also effective in inhibiting growth. Interesting work recently reported by Bruinenberg et al (1) on the use of formate as energy source under aerobiosis by **C. utilis** and **S.**

cerevisiae shows that both were capable of oxidizing formate under glucose limited conditions. However, only in C. utilis was the cell yield on glucose enhanced by the utilization of formate.

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TABLE 1

AEROBIC GROWTH OF SACCHAROMYCES CEREVISIAE (ICAITI L-181) ON ORGANIC ACIDS AS CARBON SOURCE IN CONTINUOUS CULTURE

CARBON SOURCE	INITIAL SUBSTRATE CONCENTRATION G/100 ML	SUBSTRATE UTILIZED PER CENT OF INITIAL CONCENTRATION	pH FERMENTOR	DRY WEIGHT OF BIOMASS PRODUCED G/100 ML	YEAST COUNT X 10^7 CC^{-1}
Glucose	0.46	99	4.70	0.41	35.3
Glucose	0.11	100	6.60	0.43	26.5
Acetic Acid	0.30	91			
Acetic Acid	0.29	54	6.40	0.08	3.8
Acetic Acid	0.05	100	6.20	0.13	3.6
Propionic Acid	0.10	51			
Propionic Acid	0.10	83	6.15	0.14	3.8
Propionic Acid	0.05	28	5.50	0.08	4.9
Lactic Acid	0.77	66			
Lactic Acid	0.71	66	7.00	0.23	8.8
Lactic Acid	0.40	43	6.35	0.16	5.8
Butyric Acid	0.10	8			
Butyric Acid	0.11	0	6.25	0.12	4.6

TABLE 2

GROWTH OF SACCHAROMYCES CEREVISIAE ON ORGANIC ACIDS AS CARBON SOURCE. THE FERMENTATION WAS CARRIED OUT ANAEROBICALLY IN CONTINUOUS CULTURE AT 30°C AT A DILUTION RATE OF 0.1 H^{-1}. pH OF MEDIA WAS ADJUSTED TO 5.0

CARBON SOURCE	INITIAL SUBSTRATE CONCENTRATION G/100 ML	SUBSTRATE UTILIZED PER CENT OF INITIAL CONCENTRATION	pH IN FERMENTOR	DRY WEIGHT OF BIOMASS PRODUCED G/100 ML	YEAST COUNT $\times 10^7$ CC $^{-1}$
Glucose	0.46	99	5.0	0.24	25.7
Glucose	0.11	100	5.7	0.17	9.4
Acetic Acid	0.30	12			
Acetic Acid	0.29	54	6.3	0.02	3.8
Acetic Acid	0.05	100	6.3	0.09	2.4
Propionic Acid	0.10	37			
Propionic Acid	0.10	44	6.0	0.04	1.6
Propionic Acid	0.05	20	5.0	0.02	3.2
Lactic Acid	0.77	33			
Lactic Acid	0.71	44	6.3	0.13	4.1
Butyric Acid	0.10	0			
Butyric Acid	0.11	1	5.6	0.11	1.6

TABLE 3

GROWTH OF CANDIDA UTILIS ON ORGANIC ACIDS AS CARBON SOURCE. THE FERMENTATION WAS PERFORMED AEROBICALLY IN CONTINUOUS CULTURE AT 30°C AND A DILUTION RATE OF 0.1^{-1}. pH OF MEDIA WAS ADJUSTED TO 5.0

CARBON SOURCE	INITIAL SUBSTRATE CONCENTRATION G/100 ML	SUBSTRATE UTILIZED PER CENT OF INITIAL CONCENTRATION	pH IN FERMENTOR	DRY WEIGHT OF BIOMASS PRODUCED G/100 ML	YEAST COUNT $\times 10^7$ CC^{-1}
Glucose	0.40	98	6.20	0.82	139.0
Glucose	0.10	76	7.20	0.20	21.4
Acetic Acid	0.28	89			
Acetic Acid	0.33	95	7.35	0.25	24.7
Acetic Acid	0.06	100	6.70	0.89	23.4
Propionic Acid	0.09	97			
Propionic Acid	0.10	100	6.75	0.30	8.0
Propionic Acid	0.05	100	7.20	0.41	12.9
Lactic Acid	0.78	97			
Lactic Acid	0.68	88	7.20	0.35	23.6
Lactic Acid	0.40	86	7.20	0.17	21.0
Butyric Acid	0.10	45			
Butyric Acid	0.11	95	7.05	0.18	8.9

TABLE 4

GROWTH OF CANDIDA UTILIS ON ORGANIC ACIDS AS CARBON SOURCE. THE FERMENTATION WAS CARRIED OUT ANAEROBICALLY IN CONTINUOUS CULTURE AT 30°C AT A DILUTION RATE OF 0.1 H^{-1}. pH OF MEDIA WAS ADJUSTED TO 5.0

CARBON SOURCE	INITIAL SUBSTRATE CONCENTRATION G/100 ML	SUBSTRATE UTILIZED PER CENT OF INITIAL CONCENTRATION	pH IN FERMENTOR	DRY WEIGHT OF BIOMASS PRODUCED G/100 ML	YEAST COUNT $X\ 10^7\ CC^{-1}$
Glucose	0.1	76	5.60	0.10	12.3
Acetic Acid	0.28	14			
Acetic Acid	0.33	54	6.30	0.17	14.7
Acetic Acid	0.06	71	6.40	0.11	8.8
Propionic Acid	0.09	33			
Propionic Acid	0.10	63	6.30	0.07	5.9
Lactic Acid	0.68	17	6.00	0.08	8.3
Lactic Acid	0.40	46	6.20	0.11	9.1
Butyric Acid	0.10	2			
Butyric Acid	0.11	7	5.60	0.09	4.7

PAPER No. 10

"Regulation of adsorption-desorption mechanisms during holocellulosis biomass"

(Trends in Biotechnology 4 (6) 135-136 (1986))

TRENDS IN BIOTECHNOLOGY 4 (6) 135-136 (1986)

Regulation of adsorption-desorption mechanisms during holocellulosis of biomass

Lignocellulosic biomass as it exists in nature is relatively refractory to bioconversion. In order to increase the rate and extent of holocellulose hydrolysis an effective pretreatment is required which would enhance the accessibility and adsorption of either true extracellular microbial enzymes or of enzymatic complexes associated within cellular structures (which have been shown demonstrated in several microbes) e.g., Refs 1–4. A dynamic adsorption–desorption mechanism plays a key role during the hydrolysis reaction and the physico-chemical surface characteristics usually determine how much biocatalyst is bound to the substrate, how much desorbs during, and at the end of the reaction and how much remains inactive but still tightly bound to the residual substrate[5–6].

Many believe that the understanding of the mechanisms of cellulose biodegradation has remained rather superficial despite considerable research, largely on the filamentous fungi *Trichoderma reesei* and its mutants growing in suspension cultures of processed cellulose fractions. Others believe that, although the enzymatic system is indeed complex, it is by no means beyond explanation based on current knowledge and experimental techniques. What is certainly true, however, is that little is known about how enzymes are employed by microbes in natural ecosystems: how are they transported to the appropriate cell site, how are they excreted, what regulates and controls their adsorption into the substrate, and how do enzyme components interact while adsorbed in an heterogeneous surface? Knowledge along these lines would help in designing more productive applied systems. The situation in natural systems is obviously more complex than that in suspension cultures: relevant data can be obtained from alternative experiments on enzyme production in moist solids or solid substrate fermentations[7–8]. The reactions taking place are heterogeneous in nature and the properties of the substrate surface are changing constantly due to modifications in the polymer chain structure, degree of polymerization and crystallinity. Hence interpretation of experimental results from a particular microbe–substrate system must be taken in perspective before general conclusions are postulated.

Recently[9] it was found that the adsorption on tissue paper of the exo-1,4-β-glucanase component of a commercial *T. viride (reesei)* preparation was depressed by the addition of ethanol to the medium; a beautiful example of metabolites produced by microbes during holocellulose hydrolysis affecting the enzyme–surface interaction. It was also observed recently that application of an ultrasound field during enzymatic saccharification of sugar cane bagasse by a commercial *T. viride (reesei)* preparation enhanced the rate and extent of reaction, but only when the substrate had been previously pretreated[10]. Why did ultrasound affect enzyme catalysis? It is believed that ultrasound influenced the dynamic adsorption–desorption mechanism by the creation of small-scale acoustic streaming and local microturbulence near the surface. This phenomena increased the rate of reaction by avoiding unproductive enzyme–substrate contact and by stimulating enzyme–surface migration or displacement in solution to more productive surface sites. The enhancement was only seen in those pretreated substrates which had a favorable surface: an open or swollen matrix and/or a partial delignification. This observation might help in the design of improved enzymatic reactors or it might be used as a control to regulate enzyme action. (It is also conceivable that microorganisms might themselves use some form of vibration to regulate enzyme adsorption into solid surfaces.)

Cell-associated enzyme complexes

The only review dealing specifically with enzyme adsorption mechanisms[11] summarized work up to 1982, mainly done with purified preparations of *T. reesei*. Although preferential adsorption of individual enzyme components was discussed no mention was made of adsorption of enzymatic complexes associated within cellular structures. In the rumen, anaerobic cellulolytic bacteria adhere closely to the fibrous substrate[12]. Some bacteria produce copious amounts of

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Glossary

Glycocalyx – bacterial polysaccharide-containing structures lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells[22]. They have been divided into two types: (1) layers composed of a regular array of glycoprotein subunits at the cell surface, and (2) capsules composed of a fibrous matrix at the cell surface which may vary in thickness and can be rigid, flexible, integral or peripheral. Within glycocalyx a number of carbohydrases (and maybe other type of enzymes) appear to be organized into high molecular weight multienzyme complexes associated with cell surface structures or natural supports.

ous glycocalyx (see Glossary) ch serves to separate these cells n the substrates: this glycocalyx is ciated with enzymes. In extreme omorphic cells of *Bacteroides cinogenes*, subcellular vesicles essing holocellulolytic activities luced from blebs on the cell ace were observed[1–4]. In *Ruminococcus albus*, a polysaccharide capsule surrounding the cell wall, as well small vesicular structures that egated into larger spherical units contained or had associated celluiolytic enzymes. In the anaerobic thermophile, *Clostridium thermocellum*, a cellulose-binding factor is onsible for adherence, but it also ains a group of closely related plexes that show multiple cellu- activities and which has been ed a 'cellulosome'[13–16]. An extracellular matrix of sheaths have been rved encasing hyphae of brown white rot fungi growing on fibers. e former, cytoplasmic organelles icrobodies were associated with sheaths and were probably involved in cellulose degradation[17,18]. In *reesei* cellulases seemed to be ciated to pleiomorphic subcellular es or vesicles[19]. It seems, then, here is evidence that holocellulolytic enzymes exist in multiple forms associated with cellular structures, which in some cases serve to enhance the contact between the microbe and the surface. These structures also probably give enzymes stability and protection against catabolite repression and they could also provide natural entrapment in order to avoid excessive losses of enzymes by diffusion.

It is interesting to note that two structural genes coding for two different endo-1,4,β-glucanases were identified in *C. thermocellum* and were characterized and expressed in *Escherichia coli*[20,21]. Unfortunately the authors recovered the periplasmic and cell wall associated enzymes from osmotically shocked and sonicated cells and no evidence was sought for the formation of 'cellulosomes' in *E. coli*.

Therefore, one can speculate that microbes use subcellular structures or 'cellulosomes' to pack and stabilize holocellulolytic (and maybe other hydrolytic) enzymes. These then adsorb into fiber surfaces and act as biocatalysts if the site is productive. The microbe would employ various mechanisms to control and regulate the adsorption–desorption mechanism. One of them could be vibration-induced localized streaming and microturbulence. We believe that studies in moist solid systems employing fixed cells or fixed active subcellular structures will confirm, reject or modify this picture.

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PAPER No. 11

"Ultrasound effect on enzymatic saccharification"

(Biotechnology Letters 8 (2) 131-136 (1986))

Biotechnology Letters Vol 8 No 2 131-136 (1986)

ULTRASOUND EFFECT ON ENZYMATIC SACCHARIFICATION

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SUMMARY

An ultrasound field during enzymatic saccharification ehanced the rate and extent of reaction of sugarcane bagasse that had been previously pretreated inducing a matrix swelling and partial delignification.

INTRODUCTION

Lignocellulosic biomass is relatively refractory to bioconversion. In order to increase the rate and extent of holocellulose hydrolysis, substrate pretreatment is required to induce the structural alterations of the lignocellulosic matrix and favor the close contact between microbe and fibers to secure an efficient enzyme action. In a recent review (Fan et al, 1982) there was a list of 10 physical, 18 chemical and one biological pretreatments. Any pretreatment to be effective must enhance the accesibility and adsorption of true extracellular enzyme or enzymatic complexes associated within cellular structures (Dinsdale et al, 1978; Akin, 1980; Forsberg et al, 1981; Lamed et al, 1983; Highley et al, 1983, 1984).

A dynamic adsorption-desorption mechanism plays a key role during the hydrolysis reaction and the type of substrate pretreatment determines how much biocatalyst actually desorbs at the end of the reaction and how much remains adsorbed tightly bound to the substrate, but usually no longer effective (Lee et al, 1982; Ryu et al, 1984).

In the present work we provide experimental evidence that an ultrasound field during enzymatic hydrolysis favors the rate of reaction when the substrate surface has been pretreated.

EXPERIMENTAL METHODS

Substrate. Sugarcane was sampled from sugarmills in the Pacific coast of Guatemala. The sample was chipped in a pilot wood chipper (Type 9-72, OY Santsalo) and the chips were EX-FERMented in order to transform all sucrose present into ethanol in a 48 h cycle (Rolz et al, 1979). The fermented chips were pressed in a continuous screwpress (Model VP-GF, Vincent Corp), washed with water in a mechanically agitated tank and screwpressed again. They were sundried to a final moisture of around 6-7% and milled in a pilot Fitz Mill Comminutor (The Fitzpatrick Co.) employing a 0.84 mm sieve. The chemical composition on dry weight basis of this sample was: permanganate

lignin 12.5%, cellulose 43.3%, hemicellulose 38.0%, ash 2.4% and protein 1.2%. Its in vitro dry matter enzymatic digestibility was only 19.04% determined following the recommendations of Dowman and Collins (1982).

Pretreatments. One sample was pretreated employing the organosolv procedure using a mixture of ethanol:water, 60:40 and 1% of Na_2O for 4 h at 175C. The solids were water washed extensively afterwards. Another sample was treated with 80 g NaOH kg^{-1} dry matter in solid state for 10 days at room temperature, 20C.

Hydrolysis. 0.5 g of substrate were suspended in 45 ml of acetate buffer 0.2M at pH 4.8 and 40C. A sample was taken to measure sugars in solution by the dinitrosalicylic acid method (Miller, 1959). At zero time 5 ml of a 1% by weight solution of Onozuka FA cellulase (Maruzen Chemical Co.) were added. The contents were agitated constantly and every 5 min until one half hour 1 ml samples were taken for sugar analysis. Each substrate was tested at least 3 times.

A series of tests were done with a substrate that had been treated with ultrasound for 30 min at 40C in the set up described before. After such treatment the enzyme was added and the same procedure as before was followed.

Simultaneous hydrolysis and ultrasound treatment. Another series of tests were done maintaining an ultrasound field in the reactor described above during the 30 min of enzymatic reaction.

The ultrasound was generated with a Branson Sonifier Model 200 (Branson Sonic Power Co.) adapted with a tapered microtip, operated in a continuous mode and providing 32 watts of output power.

RESULTS AND DISCUSSION

The experimental data obtained is given in Tables 1, 2 and 3. In the tables the second column gives the total sugars produced employing the enzyme. The sugars produced by enzymatic hydrolysis of the substrate treated previously with ultrasound are given in the third column. The last column gives the amount of sugars produced in a simultaneous use of ultrasound during enzymatic action.

Note that for the untreated sugarcane bagasse (Table 1) the sugars produced in the three different tests were not statistically different. After 30 min the amount of holocellulose hydrolyzed was low, barely above 2%. The equilibrium value or IVDMED for this material (after 72 h contact time at 40C) was only 19% as said before.

In Table 2, the hydrolysis data is presented for the alkaline-organosolv pretreated sample. Note that there is no statistically significant difference between the data for only enzyme and the substrate treated previously with ultrasound. However both were different compared to the data resulting from the enzyme action within an ultrasound field. The highest saccharification extent was 13% of the holocellulose present. This sample had an IVDMED of 64.88%.

The data for the alkaline pretreated bagasse is presented in Table 3. The same results were obtained, however the differences here had a statistically lower probability. The highest saccharification extent was almost 25% of the holocellulose present. This sample had an IVDMED of 72.28%.

Why does ultrasound affect enzyme catalysis? The positive effects of ultrasound on the rate of chemical reactions has been well documented in a variety of situations. For example Kristol et al (1984) have found that acid hydrolysis of dextran was improved by 36% under ultrasound. It is also

well known that the mechanical perturbations caused by ultrasound act on the physical structure and integrity of the cells and, depending on the ultrasound intensity, can reversibly disrupt and kill, or completely desintegrate and homogenize cellular contents (Miller, 1983). Under controlled conditions however, it can induce small-scale acoustic streaming causing cavitation which might affect local surface phenomena.

Prevously, Clemente et al (1984) had pretreated rice husks with ultrasound at different amplitudes and its effect on the resulting IVDMED was similar to the one obtained with steam, but lower than the effect of NaOH.

Our explanation is that ultrasound influenced the dynamic adsorption-desorption enzyme mechanism due to acoustic streaming and local microturbulence near the surface. Hence this increased the rate of reaction by avoiding unproductive enzyme-substrate contact and stimulating enzyme-surface migration or displacement in solution to more productive surface sites. It was only seen in those substrates which had a favorable surface: an open or swollen matrix and a partial delignification.

This observation might help in the design of improved enzymatic reactors or also it might be used to control externally enzyme action. It is also possible that microorganisms might use it themselves to regulate enzyme-complexes adsorption into solid surfaces.

ACKNOWLEDGEMENTS

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Table 1

Saccharification of EX-FERMented sugarcane chips

Time, min	Carbohydrates, mg [a]		
	Only enzyme	Substrate pretreated by ultrasound	Simultaneous ultrasound + enzyme
5	5.20	4.40	0.98
10	7.91	10.21	5.59
15	9.02	9.60	9.34
20	8.67	9.49	10.71
25	10.26	11.72	9.36
30	9.16[b]	10.17[c]	8.79[d]

(a) Average value of triplicate runs
(b) Substrate holocellulose saccharified 2.11%
(c) Substrate holocellulose saccharified 2.35%
(d) Substrate holocellulose saccharified 2.03%
Data at 30 min not statistically different a = 0.01

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Table 2

Saccharification of EX-FERMented sugarcane chips pretreated by an alkaline organosolv procedure

Time, min	Carbohydrates, mg[a]		
	Only enzyme	Substrate pretreated by ultrasound	Simultaneous ultrasound + enzyme
5	5.88	2.47	11.48
10	13.43	13.97	26.81
15	17.22	18.11	40.30
20	20.37	21.09	47.93
25	18.37	24.38	54.34
30	30.85[b]	22.53[c]	61.17

(a) Average value of triplicate runs with exception of second column which is an average of four experiments
(b) Substrate holocellulose saccharified 6.56%
(c) Substrate holocellulose saccharified 4.79%
(d) Substrate holocellulose saccharified 13.01%
Data of first two columns at 30 min not statistically different at a = 0.01
Data of third column at 30 min statistically different at a = 0.01 from combined data of first two columns

Table 3

Saccharification of EX-FERMented sugarcane chips pretreated by a solid state sodium hydroxide procedure

Time, min	Carbohydrates, mg[a]		
	Only enzyme	Substrate pretreated by ultrasound	Simultaneous ultrasound + enzyme
5	4.26	9.85	- (b)
10	16.96	16.01	24.85
15	27.88	19.39	43.83
20	25.41	17.94	57.17
25	35.62	19.48	52.09
30	29.35[c]	27.16[d]	61.01[e]

(a) Average value of triplicate runs
(b) At 5 min in all three experiments negative values were obtained
(c) Substrate holocellulose saccharified 11.94%
(d) Substrate holocellulose saccharified 11.05%
(e) Substrate holocellulose saccharified 24.82%
Data of first two columns at 30 min not statistically different at a = 0.01
Data of third column at 30 min not statistically different at a = 0.01 (F calculated = 10.41 vs F table = 12.25). However statistically different at a = 0.05

LIST OF

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS IN ENGLISH

1. Effects of some physical and chemical pretreatments on the composition, enzymatic hydrolysis and digestibility of lignocellulosic sugarcane residue.
   C Rolz, MC de Arriola, J Valladares and S de Cabrera
   Submitted for publication in Process Biochemistry

2. Effects of some physical and chemical pretreatments on the composition and enzymatic hydrolysis and digestibility of lemon grass and citronella bagasse.
   C Rolz, MC de Arriola, J Valladares and S de Cabrera
   Accepted for publication in Agricultural Wastes

3. White-rot fungal growth on EX-FERMented sugarcane
   C Rolz, R de León, MC de Arriola and S de Cabrera
   Accepted for publication by Applied Microbiology and Biotechnology

4. Biodelignification of lemon grass and citronella bagasse employing white-rot fungi
   C Rolz, R de León, MC de Arriola and S de Cabrera
   Accepted for publication by Applied and Environmental Microbiology

5. Biodegradation of pretreated EX-FERMented sugarcane chips by fungal enzymes and mixtures of anaerobic bacteria
   C Rolz, S de Cabrera, MJ Valdez, MC de Arriola and J Valladares
   Accepted for publication in Biotechnology Progress

6. Biodegradation of pretreated citronella and lemon grass by mixtures of anaerobic bacteria
   S de Cabrera, C Rolz, MJ Valdez, MC de Arriola and J Valladares
   Submitted for publication in Canadian Journal of Microbiology

7. Effect of monensin on acidogenesis of lignocellulosic biomass by mixed anaerobic cultures
   S de Cabrera, C Rolz, MJ Valdez, SM Saravia, F de Micheo and J Valladares
   Submitted for publication in Enzyme and Microbiol Technology

8. Kinetics of lignocellulosic degradation and acidogenesis by mesophilic anaerobic bacteria on alkali treated sugarcane bagasse and lemon grass bagasse
   S de Cabrera, C Rolz, SM Saravia, MJ Valdez and F de Micheo
   Submitted for publication in Journal of Chemical Technology and Biotechnology

9. Conversion of organic acids to biomass by yeasts
   S Ortiz-Jaramillo, S de Cabrera, F de Micheo and C Rolz
   Submitted for publication in Applied Environmental Microbiology

10. Regulation of adsorption-desorption mechanism during holocellulosis of biomass
    C Rolz
    Trends in Biotechnology 4 (6) 135-136 (1986)

11. Ultrasound effect on enzymatic saccharification
    C Rolz
    Biotechnology Letters 8 (2) 131-136 (1986)

PRESENTATIONS IN ENGLISH

1. Fungal solid substrate growth on cane, citronella and lemon grass bagasse.

   C. Rolz, R. de León, S. Ortíz, MC de Arriola

   Presented at GIAM VII, Helsinki, Finland, August, 1985

2. Anaerobic conversion of pretreated lignocellulosic residues to acids.

   S. Ortiz

   Presented at Biomass Conversion Technology: Principles and Practice, Waterloo, Canada, July, 1984

PUBLICATIONS AND PRESENTATIONS IN SPANISH

Mayra Janeth Valdez Marroquín

Producción de Acidos Volátiles por Fermentación Anaeróbica de Substratos Lignocellulósicos. Asociación Guatemalteca de Microbiología, 31 agosto, 1985.

Carlos Rolz, María del Carmen de Arriola y Jaime Valladares

Digestibilidad de los Componentes Fibrosos de la Caña de Azúcar

VI Congreso de Tecnología Azucarera ATAGUA-ATACA, 2 - 6 septiembre, 1985

Guatemala, Guatemala

Sheryl de Cabrera

Pretratamiento de Residuos Lignocelulósicos

V Seminario Internacional: Hidrólisis Enzimática de Residuos Lignocelulósicos para Obtener Etanol como Sustituto de Gasolina. 18 - 22 noviembre, 1985,

Lima, Perú

Sheryl de Cabrera

Bagazo de Caña: Fermentación Anaeróbica para Producción de Acidos Orgánicos

VI Congreso de Tecnología Azucarera ATAGUA-ATACA, 2 - 6 septiembre, 1985

Guatemala, Guatemala

Sheryl de Cabrera

Mesa Redonda: Técnicas y Problemas Relacionados con el Pretratamiento de los Residuos Lignocelulósicos

V Seminario Internacional: Hidrólisis Enzimática de Residuos Lignocelulósicos

para Obtener Etanol como Sustituto de Gasolina. 18 - 22 noviembre, 1985
Lima, Perú

Mayra Janeth Valdez Marroquín
Degradación de Residuos Lignocelulósicos por Bacterias Anaeróbicas.
Informe de tesis, Universidad de San Carlos de Guatemala, Facultad de Ciencias Químicas y Farmacia, Guatemala, octubre, 1985

Producción de Basidiomicetos usando Desechos Agroindustriales
I Exposición Nacional de Hongos. Guatemala, agosto, 1985

Sheryl de Cabrera
Producción de Otros Compuestos Químicos por Fermentaciones Anaeróbicas.
Simposio Sobre Digestión Anaeróbica. 2 julio, 1986
San Salvador, El Salvador

FINANCIAL STATEMENT

INSTITUTO CENTROAMERICANO DE INVESTIGACION Y TECNOLOGIA INDUSTRIAL
DIVISION DE CONTROL DE PRESUPUESTOS Y COSTOS

PROJECT: INNOVATIVE SCIENTIFIC RESEARCH (LIGNOCELLULOSICS)
ICAITI 82/4.6.1777 AID 936-5542

EXPENSE REPORT IN C.A. PESOS (1 $CA=1) PERIOD: NOV/DEC-85

	BUDGET	PRIOR PERIOD ADJUSTED	THIS PERIOD NOV/DEC-85	ACCUMULATED TO DATE	AVAILABLE BALANCE
STAFF					
Salaries	118 000.00	107 519.97	6 264.70	113 784.67	4 215.33
TOTAL	118 000.00	107 519.97	6 264.70	113 784.67	4 215.33
TRAVEL & PERDIEM	5 760.00	6 090.42	-	6 090.42	(330.42)
TOTAL	5 760.00	6 090.42	-	6 090.42	(330.42)
MATERIALS & SUPPLIES					
Reagents & Mat.	5 500.00	3 318.07	2 243.76	5 561.83	(61.83)
Glassware & Sup.	12 000.00	11 847.24	1 884.53	13 731.77	(1 731.77)
Photocopies & Stat.	700.00	348.69	13.65	362.34	337.66
Assorted & Sup.	1 800.00	1 547.46	12.82	1 560.28	239.72
Communications	1 000.00	277.44	2.56	280.00	720.00
Subscriptions	2 000.00	1 166.52	152.00	1 318.52	681.48
Books	2 000.00	2 939.27	165.35	3 104.62	(1 104.62)
Equipment	20 000.00	21 795.26	1 326.00	23 121.26	(3 121.26)
Assorted O.D.C.	700.00	29.11	370.00	399.11	300.89
TOTAL	45 700.00	43 269.06	6 170.67	49 439.73	(3 739.73)
TOTAL COST	169 460.00	156 879.45	12 435.37	169 314.82	145.18

"The undersigned hereby certifies" (1) that payment of the sum claimed on the voucher is proper and due under the terms of the Agreement; and (2) that the information on the fiscal report is correct and such detailed supporting information as ROCAP may require will be furnished promptly on request."

COST CONTROL & BUDGET OFFICER

PROJECT PRINCIPAL

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FINAL REPORT

The research was performed by the scientific personnel of the Applied Research Division of ICAITI. This report was prepared by Sheryl de Cabrera.

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