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Saccharification of Native Sugar Cane Bagasse Pith by the Cross-Synergistic Action of Cellulases from *Penicillium* sp. CH-M-001 and *A. terreus* CH-M-013

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ABSTRACT

Filter paper, carboxymethylcellulase, β-glucosidase, and xylanase activities were determined and compared to cellulases originating from *Aureobasidium* sp. (Ab), *Penicillium* sp. (Pe), and *Aspergillus terreus* (At). The formation of total reducing sugar was measured as a function of time for the hydrolysis of the native sugar cane bagasse pith using culture filtrates with the same quantity of extracellular protein content from Ab, Pe, At, or from the following mixtures Ab:Pe/Ab:At and Pe:At at different volumetric ratios. The saccharification progress indicated a synergistic effect for the mixture of all the enzymes, the highest being in the 40:60 relation of Pe and At, respectively. The synergistic action has been assigned to a better balance of endo- and exoglucanases in this system and essentially to the addition of β-glucosidase and xylanases from At to Pe cellulases.

Index Entries: Cellulases; enzymatic hydrolysis; synergism; *Aureobasidium* sp; *Penicillium* sp; *Aspergillus terreus;* bagasse pith; mixtures.

INTRODUCTION

Lignocellulosic biomass includes such materials as agricultural and forestry waste, municipal solid waste, waste paper, and wood and herbaceous energy sources. These materials represent one of the most abundant renewable resources on earth in that both the cellulose and hemicellulose fractions of biomass can be converted to simple sugars that can subsequently be fermented.

Cellulose, the major constituent of plant cell walls, is recycled in nature essentially as a microbial energy source. This cellulosic biomass also represents a

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source potentially inexpensive for the bioproduction of a variety of chemicals *(1)*. There is currently great interest in the degradation of lignocellulose complexes to provide suitable raw materials for food, fuel, or chemical needs. Much of this interest is centered on the hydrolysis of cellulose by cellulase enzymes.

Acid and enzyme processes based on hydrolysis are the two most common methods of converting cellulosics to simple sugar. Various processes based on dilute and concentrated acids are used to hydrolyze the cellulose and hemicellulose to their respective sugars *(2,3)*. Acid hydrolysis reduces the degree of polymerization of cellulose and hemicellulose, and ultimately results in the formation of glucose. Acid treatments proceed with rapid rates of conversion, but suffer either high catalyst consumption in concentrated acid processes or low sugar yields in high-temperature, dilute-acid processes owing to sugar destruction.

Enzymatic hydrolysis is favored over these processes, since this catalyst is noncorrosive environmentally harmless, potentially reusable, and offers an efficiency above 90% *(4)*.

The inherent recalcitrance of the lignocellulosic feedstock to bioconversion requires a pretreatment of the biomass to render the cellulose fraction amenable to enzymatic conversion to glucose, which then can be utilized. Physical, chemical, and biological means, used alone or in combination, have been reported *(5,6)* to be effective pretreatment techniques. Enzymatic processes are based on the use of a pretreatment step to hydrolyze the hemicellulose or dissolve the lignin, thereby increasing the digestibility of the cellulose. Cellulolysis is complex, since it involves interacting enzymes and an insoluble substrate constituted by crystalline microfibrils, recalcitrant to enzymatic attachment.

Thus, the enzymatic hydrolysis of cellulose has been widely investigated *(7,8)*. Efficient hydrolysis is accomplished by the synergistic action of three types of enzymes: endoglucanase (endo-1,4-β-glucose 4-glucanohydrolase, EC 3.2.1.4), cellobiohydrolase (1,4-β-D-glucose cellobiohydrolase, EC 3.2.1.91), and cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21). It is important to note that during cellulolysis, the products glucose and cellobiose accumulate and act as end-product inhibitors.

Cellulases from several fungal and bacterial systems have been identified, characterized, and studied for this ability to react synergistically. There also have been several studies *(9–12)* investigating how various cellulases act synergistically to yield higher rates and extents of hydrolysis than would be predicted from the sum of the hydrolytic activities of the individual enzymes when the individual cellulolytic enzymes act alone on a crystalline cellulosic substrate, such as cotton. These components are unable to hydrolyze this substrate to any great extent, but in combination they cooperate to effect its solubilization *(13)*. Because native cellulose is both insoluble and structurally heterogeneous, comparisons of activity between different enzymes or enzyme mixtures are difficult. Most synergism experiments have utilized a constant time of hydrolysis and/or a constant amount of enzyme *(14)*, and a cross-synergism between cellulases from different microbial systems has been shown by several investigators *(15)*.

Studies about synergism in cellulose systems have shown that two broad classifications of cellulase synergism are possible: synergism between endocellulases and exocellulases, and synergism between different exocellulases. Synergism between endoglucanases and exoglucanases can be explained by the action of the EG component on cellulose (which catalyzes the hydrolysis of the glyco-

sidic bonds along the length of the cellulose chain), resulting in the formation of new cellulose chain ends through which the CBH component acts, releasing cellobiose. Wood and McCrae *(16)* have suggested that the formation of a complex of endoglucanase and cellobiohydrolase on the surface of the cellulose chain is essential for synergism.

The process development for the biological conversion of native cellulosic materials requires further investigation dealing with the mechanism of enzyme attachment. Native cellulose in many agricultural byproducts is a miscellaneous material, characterized by a sequence of amorphous and crystalline regions.

Considerable attention has been focused on the enzymatic hydrolysis of cellulosic substrates in recent years; for this reason, and taking into account the abundance of sugar cane bagasse annually available and regarded as an underutilized resource in Mexico (approx 13 million ton/yr, about 35% in this total was bagasse pith), the present study was aimed at reaching cellulose saccharification of native sugar cane bagasse pith, using simultaneously two different cellulase complexes from three filamentous fungi isolated in our laboratory. This way cellulases from three different strains, *Aureobasidium* sp., *Penicillium* sp. and *A. terreus*, mixed in different ratios, were tested under proper operational conditions; these cellulase complexes were selected mainly because of their different predominant activities, which allows the exploration a large variety of enzyme compositions. Therefore, our goal was to examine the saccharifying capacity of the enzymes of the three cellulolytic strains, acting separately or jointly on an agroindustrial byproduct like native sugar cane bagasse pith.

MATERIALS AND METHODS

Microorganisms

Aureobasidium sp. CH-ME-18, *Penicillium* sp. CH-ME-001, and *A. terreus* CH-ME-013 were isolated in our laboratory from soil of a sugar cane field of the state of Morelos, México as previously reported *(17,18)*. The fungi strains were grown at 29 ± 1°C for 5 d on potato dextrose slants and maintained on the same medium at 4°C. Subculturing was carried out at monthly intervals.

Cellulase and Xylanase Production

Four grams of cellulosic material (native sugar cane bagasse pith collected locally in the "Emiliano Zapata" sugar mill, Morelos, México) were placed in a 500-mL Erlenmeyer flask, pouring in it only 180 mL of fermentation medium. All chemicals used in this culture medium were of industrial grade, and tap water was according to the formulation indicated for *Aureobasidium* sp. *(19)*.

For *Penicillium* sp. and *A. terreus*, we developed a modified medium with the same composition of chemicals, but using corn syrup liquor (CSL) as nitrogen source instead of urea *(20)*. Inocula were prepared by harvesting spores from 1-wk-old PDA slants of the three strains in the proportion 3:1 in sterile distilled water containing a small amount of Tween-80.

Fermentation flasks were inoculated to give 10^6–10^7 spores/mL. Incubation was at 29 ± 1°C for *Penicillium*, but for *Aureobasidium* sp. and *A. terreus*, it was at 37°C on a rotary shaker (180 rpm). The pH value was initially adjusted to 4.5 for

Aureobasidium sp. and 5.5 for the other strains. Samples were removed aseptically and were centrifuged to remove solids and to obtain cell-free filtrates.

Enzymatic Assays and Soluble Protein Determination

These were carried out using the supernants after removing the cell growth and residual bagasse pith. The protein content was performed according to Lowry *(21)* in aliquots of the obtained mixture after a ±4°C dialysis overnight. The culture filtrates of *Aureobasidium* sp. and *Penicillium* sp. were concentrated by ultrafiltration to obtain the same protein content with respect to the *A. terreus*.

Total cellulase activity (FPU) was determined on filter paper following the protocol of Mandels et al. *(22)*. The determination of carboxymethylcellulose saccharifying activity (CMCase) was applied according to the method developed by us. Briefly, the assay determining reducing groups on CMC is: 0.5 mL of diluted culture filtrate, 1 mL of CMC at 7.5% suspension in citrates buffer 0.075*M*, pH 4.8, 60 min of incubation at 50°C.

The β-glucosidase activity was determined as in ref. 23 as an aryl-β-glucosidase by mean of the hydrolysis of *p*-nitro-phenyl-β-D-glucoside measuring the amount of 1 μmol of *p*-nitrophenol liberated by the enzyme/min and xylanase activity was determined by quantifying the reducing sugars (as xylose) as in ref. *24*.

All reducing sugar determinations were performed by the 3,5-dinitrosalicylic (DNS) method *(25)*. The enzyme activities were calculated as indicated by IUPAC *(26)*, using the recommendations of Ghose for enzyme solutions for which the undiluted solution does not reach the target glucose-release value. For this reason, the activity is taken as directly and linearly proportional to whatever amount of reducing sugar is released. The filter paper and CMC hydrolyzing activity was defined as 0.185/(enzyme releasing 1 mg glucose) U/mL enzyme solution.

Enzymatic Hydrolysis

The saccharification of nonpretreated sugar cane bagasse pith initially was carried out in 22 × 175 mm test tubes, containing 50 mg of material, suspended in 1.0 mL citrate buffer, 0.075*M*, pH 4.8, and 0.5 mL of the respective filtrate culture. At the same time, during the experiment the combinations mentioned below were made, using the following volumetric proportions: 80:20 (v/v); 60:40 (v/v), and 40:60 (v/v), using the same 0.5 mL final volume. Citrate buffer was used as a control instead of the respective portion of culture filtrate.

The tubes were parafilm-covered and shaken in a reciprocal water bath at 50°C for 60 min, separating a group of test tubes with their content in every 15-min period until the 60 min were completed. The reaction was stopped by adding 3 mL of DNS reagent to determine the reducing sugars, using glucose as standard.

Saccharification of Sugar Cane Bagasse Pith in a Stirred Reactor

This was carried out in a 1000 mL Berzelius beaker containing 20 g of bagasse pith, suspended in 500 mL of citrate buffer, 0.075*M*, pH 4.8. The beakers were shaken in a reciprocal water bath at 50°C. The total volume was 600 mL, which included 100 mL of filtrate, or a mixture of *Penicillium* sp. and *A. terreus* in the proportions of 60:40 (v/v) and 40:60 (v/v). A control was run, containing the respective proportion of the filtrate and the rest, was completed with citrate buffer. A few merthiolate drops were added to each system and the beakers were covered with a rubber top.

The total incubation time at 50°C was 48 h, taking samples of 5 mL every 3 h during the first 12 h to determine reducing sugars; and after every 12 h until the 48-h period was fulfilled. The percentage of saccharification (% SACCH) was calculated according to the formula: % SACCH: = P/S (0.55) where P is the reducing sugar concentration (g/L) in the hydrolysate estimated and S is the concentration (g/L) of bagasse, which is multiplied by 0.55, since bagasse pith contains approx 55% cellulose. The contribution of the pentose reducing groups was not taken into account to calculate the conversion percentage.

RESULTS AND DISCUSSION

The first stage of our work was the production of the cellulolytic and xylanolytic activities, using three different strains of filamentous fungi, previously chosen for the following reasons: *Aureobasidium* sp. CH-M-18 with characteristics of a yeast-like fungus. Maximum cellulase activity produced by this strain from crystalline cellulose was reached in a shorter time than that required by other microorganisms *(24)*. The microorganism grew well at 37°C on different carbon sources. *Penicillium* sp. CH-M-001 and *A. terreus* CH-M-013 were chosen based on their characteristics found in prior investigations *(18)*, which mainly point out different degrees of relations in the cellulolytic and xylanolytic activities obtained from a byproduct like bagasse cane pith; *Penicillium* sp. is characterized by its capacity to degrade filter paper. It is considered a microorganism with great ability to take advantage of the crystalline portion of cellulosic materials and grows at 29°C.

A. terreus is capable of producing a high degrading activity of amorphous regions of cellulosic materials like xylans, since it produces a high CMCase activity from the first 2 d of fermentation, as well as xylanases and β-glucosidase, and it may grow at 37°C just as *Aureobasidium* sp.

Another important aspect to be mentioned is that there have been very few reports on the use of byproducts like sugar cane bagasse pith as starting material for the production of enzymes of industrial interest *(27,28)*. This aspect is important, since agroindustrial byproducts contain large amounts of compounds, such as cellulose and hemicellulose, which could serve as inducers for the production of extracellular enzymes, such as cellulases and xylanases. The polysaccharide content could therefore be hydrolyzed until soluble sugar or other products with different applications are obtained. For these reasons, it would be interesting to develop processes for the use of these byproducts.

In prior reports *(19)*, similar results were presented of simple and economical culture media and the optimal inoculum of *Aureobasidium* sp. for the production of cellulases and xylanases by submerged fermentation of untreated sugar cane bagasse pith. Most of the studies on microbiological enzyme production are performed with analytical-grade salts and distilled water. However, for industrial production, it would be advantageous to the costs involved to simplify the medium and use industrial-grade salts and tap water.

Figure 1 shows the enzyme activities after 5 d of inoculation with culture of *Aureobasidium* sp., *Penicillium* sp., and *A. terreus* under liquid fermentation on bagasse sugar cane pith in a medium formulated with industrial materials. This culture medium was initially designed for *Aureobasidium* sp. (however, the production of cellulolytic enzymes with urea for the other strains was not appropriate).

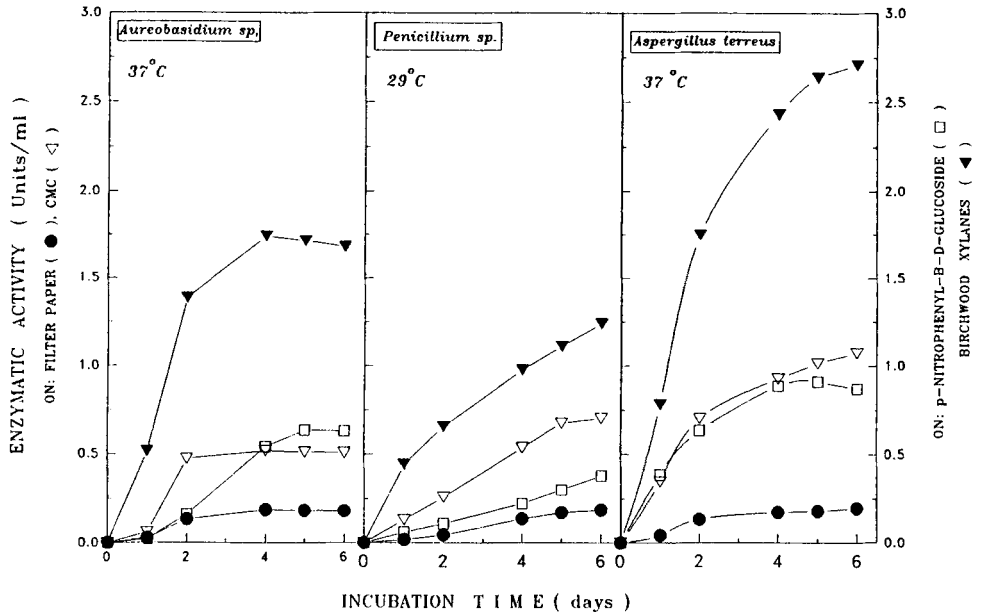

Fig. 1. Production of extracellular cellulolytic activity by fermentation of 2% (wt/vol) native sugar cane bagasse pith by *Aureobasidium* sp., *Pennicillium* sp., and *A. terreus* at the indicated temperature in shaking flask (180 rpm) in a culture medium containing industrial-grade components and tap water. For *Aureobasidium*, the nitrogen source was urea and $(NH_4)_2SO_4$; for the others strains, CSL replaced urea. Enzymatic activity on: (●) filter paper, (△) CMC, (□) *p*-nitrophenyl-β-D-glucoside, (▲) birchwood xylanes.

These fungi were initially tested to produce cellulases with a proteose peptone medium, originally reported by Mandels and Weber *(29)*. Because of economic considerations, we developed a modification that replaces this organic nitrogen source with a cheaper one, the corn syrup liquor (CSL). It was a good replacement, and it was no longer necessary to use urea.

The use of tap water has no negative effects on the production of cellulolytic and xylanolytic activity, or on β-glucosidase activity. Table 1 shows the gross composition of the culture medium mentioned before.

Since our main goal was the utilization of agroindustrial byproducts, such as sugar cane bagasse pith, in a saccharification reaction to produce soluble sugars useful for other purposes, we initially evaluated the capacity of hydrolysis of the cellulases and mixtures obtained by fermentation on a cellulosic material, such as filter paper, which is characterized as having amorphous as well as crystal regions, similar to native lignocellulosic materials. Table 2 shows the results of these considerations.

In these experiments, the existence of cross-synergesis between the cellulases of different origins became apparent. Because of this, we decided to evaluate these systems on native sugar cane bagasse pith, as indicated in Figs. 2, 3, and 4. We conclude from these experiments that the synergistic effect is similar to the one obtained with filter paper on all the combinations carried out.

According to Woodward et al. *(30)*, we calculated the degrees of synergistic effect (DSE). The results obtained are indicated in Table 3. DSE is defined as the

Table 1
Culture Medium Composition Utilized
for the Production of Cellulolytic and Xylanolytic Activities

Mandels Medium	Industrial grade medium, original	Industrial grade medium, modified
Sugar cane bagasse pith	Sugar cane bagasse pith	Sugar cane bagasse pith
KH_2PO_4	KH_2PO_4	KH_2PO_4
$(NH_4)_2SO_4$	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$
$CaCl_2$		
Trace elements		
$FeSO_4 \cdot 7H_2O$		
$MnSO_4 \cdot 7H_2O$		
$ZnCl_2$		
$CoCl_2$		
Urea	Urea	
Proteose peptone		CSL
Distilled water and analytical grade chemicals	Tap water and Industrial-grade chemicals	Tap water and Industrial-grade chemicals
Tween-80		Mazu $_{6000}$
pH 5.5	pH 5.5	pH 5.0

product concentration over time in the hydrolysis of a cellulose mixture divided by the sum of the product concentration of the individual cellulases at the concentration in the mixture. DSEs are comparable to those reported in the literature by other investigators *(10,12)*.

Since the combination of *Penicillium* and *A. terreus* was higher in DSE value than the others, mainly with volumetric relations 40:60 (v/v) and 60:40 (v/v), we considered it worthwhile to evaluate them in a stirred reactor. Figure 5 shows the behavior obtained with the individual culture filtrates and with the combinations mentioned. Table 4 shows the saccharification percentage obtained with this combination compared to each one of the culture filtrates individually. A maximum increase of 42% in the extent of saccharification was obtained when culture fluids with these cellulolytic strains were mixed in a 40:60 volumetric proportion higher than that obtained separately with the most active filtrate and gives a 12% greater extent of saccharification than would result from the theoretical sum of the action of each individual cellulase. The observed synergism in reducing sugar formation suggests a cooperative interaction between cellulases, which opens up the cellulose structure for increased degradation; the synergistic effect shown by the cellulases from *Penicillium* sp. and *A. terreus* could be assigned to a better balance of endo- and exoglucanase action, as well as to the high level of β-glucosidase and xylanase activities in the latter strain, acting on the native sugar cane bagasse pith. The synergism between cellulase components also appears to be independent of the ratio in a reaction mixture and dependent only on their concentration. This is important because a practical goal of cellulase research is to define both the optimal types and proportions of the enzymes necessary to gain efficient hydrolysis. The latter is especially difficult to define, in that the ratio of enzymes depends on the changing status of the substrate throughout cellulolysis.

Table 2
Reducing Sugar Concentration at Short Time
of Hydrolysis on Filter Paper for Cellulase Mixtures

Mixture of culture filtrates	Volumetric relation V/V	Reducing sugars, mg/mL[a] time, min 0	15	30	45	60
Aureobasidium sp.	100	0	0.30	0.56	0.74	0.90
Penicillium sp.	100	0	0.23	0.44	0.60	0.78
A. terreus	100	0	0.30	0.56	0.74	0.92
Aureobasidium sp.	80:20	0	0.50	0.76	0.96	1.14
+ *Penicillium* sp.	Control (80)	0	0.30	0.46	0.58	0.70
	Control (20)	0	0.12	0.18	0.26	0.30
	Theoretical sum	0	0.42	0.64	0.84	1.00
	60:40	0	0.48	0.74	0.94	1.10
	Control (60)	0	0.24	0.42	0.56	0.64
	Control (40)	0	0.16	0.26	0.32	0.36
	Theoretical sum	0	0.40	0.68	0.88	1.00
Aureobasidium sp.	80:20	0	0.48	0.74	1.00	1.18
+ *A. terreus*	Control (80)	0	0.22	0.44	0.60	0.78
	Control (20)	0	0.10	0.16	0.22	0.26
	Theoretical sum	0	0.32	0.60	0.82	1.04
	60:40	0	0.40	0.72	1.00	1.24
	Control (60)	0	0.20	0.38	0.54	0.68
	Control(40)	0	0.14	0.24	0.32	0.40
	Theoretical sum	0	0.34	0.62	0.86	1.08
Penicillium sp.	80:20	0	0.44	0.70	0.90	0.98
+ *A. terreus*	Control (80)	0	0.24	0.40	0.52	0.54
	Control (20)	0	0.12	0.20	0.30	0.34
	Theoretical sum	0	0.36	0.60	0.82	0.88
	60:40	0	0.48	0.72	0.92	1.04
	Control (60)	0	0.14	0.24	0.32	0.38
	Control (40)	0	0.26	0.38	0.46	0.52
	Theoretical sum	0	0.40	0.62	0.78	0.90
	40:60	0	0.56	0.74	0.86	0.94
	Control (40)	0	0.20	0.26	0.30	0.32
	Control (60)	0	0.16	0.30	0.38	0.44
	Theoretical sum	0	0.36	0.56	0.68	0.76

[a]The values of reducing sugars are the mean by triplicate.

Controls: Samples of filtrates were run at the same time as controls, and its volume was completed with citrates buffer 0.075*M*, pH 4.8.

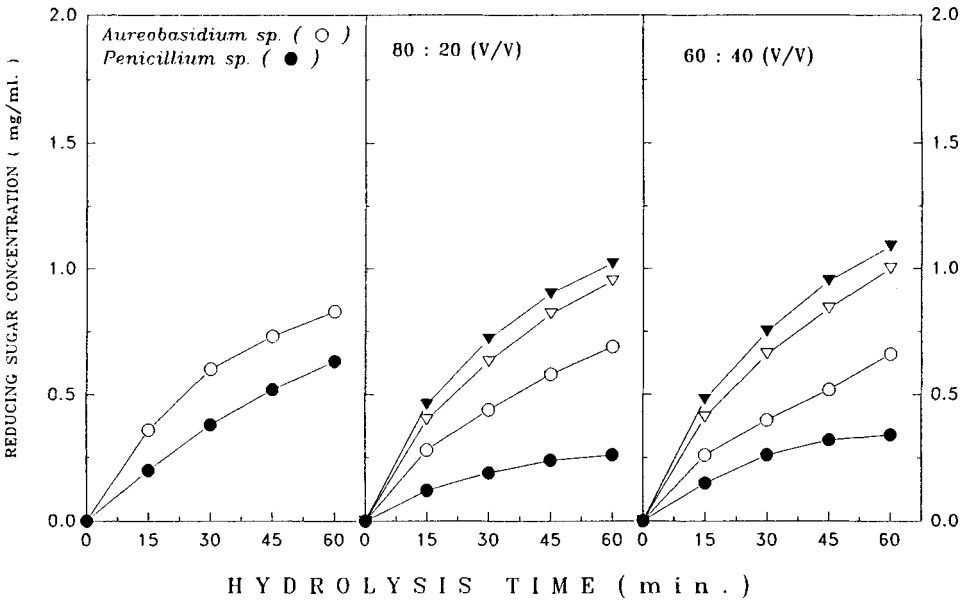

Fig. 2. Saccharification of native sugar cane bagasse pith (50 mg) by culture filtrates of *Aureobasidium* sp. (○) and *Penicillium* sp. (●) alone and with the indicated volumetric combination (▼), pH 4.8, 50°C, and shaking of 120 rpm. Theoretical sum of the action of each filtrate, acting separately (▽).

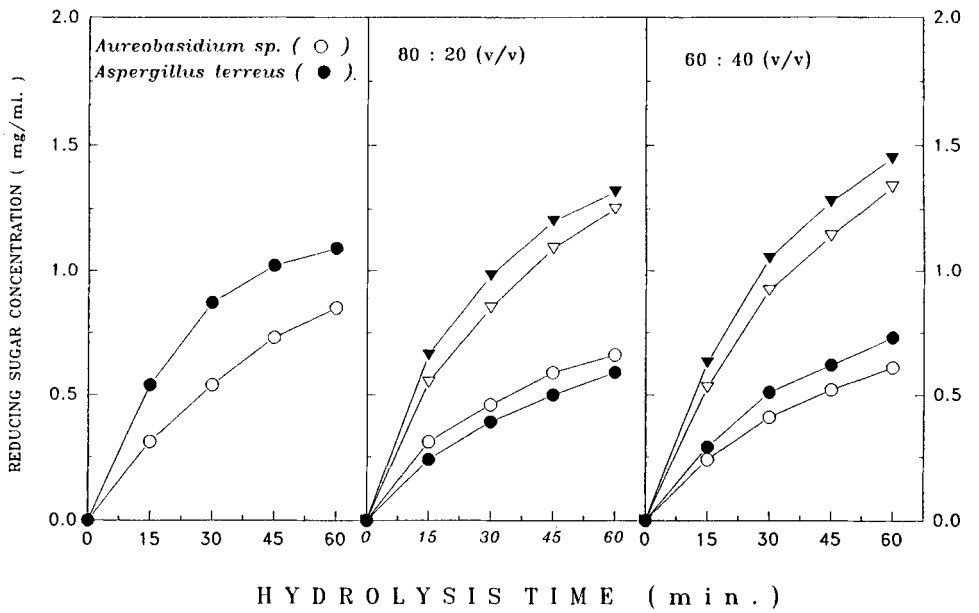

Fig. 3. Saccharification of native sugar cane bagasse pith (50 mg) by culture filtrates of *Aureobasidium* sp. (○) and *A. terreus* (●), alone and with the indicated volumetric combination (▼), pH 4.8, 50°C, and shaking of 120 rpm. Theoretical sum of the action of each filtrate, acting separately (▽).

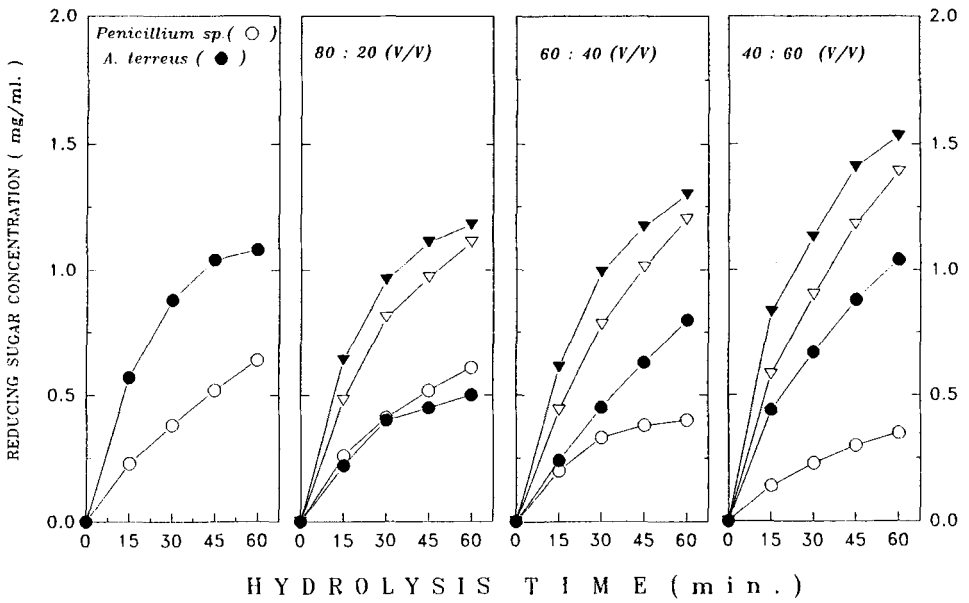

Fig. 4. Saccharification of native sugar cane bagasse pith (50 mg) by culture filtrates of *Penicillium* sp. (○) and *A. terreus* (●), alone and with the indicated volumetric combination (▼), pH 4.8, 50°C, and shaking of 120 rpm. Theoretical sum of the action of each filtrate, acting separately (▽).

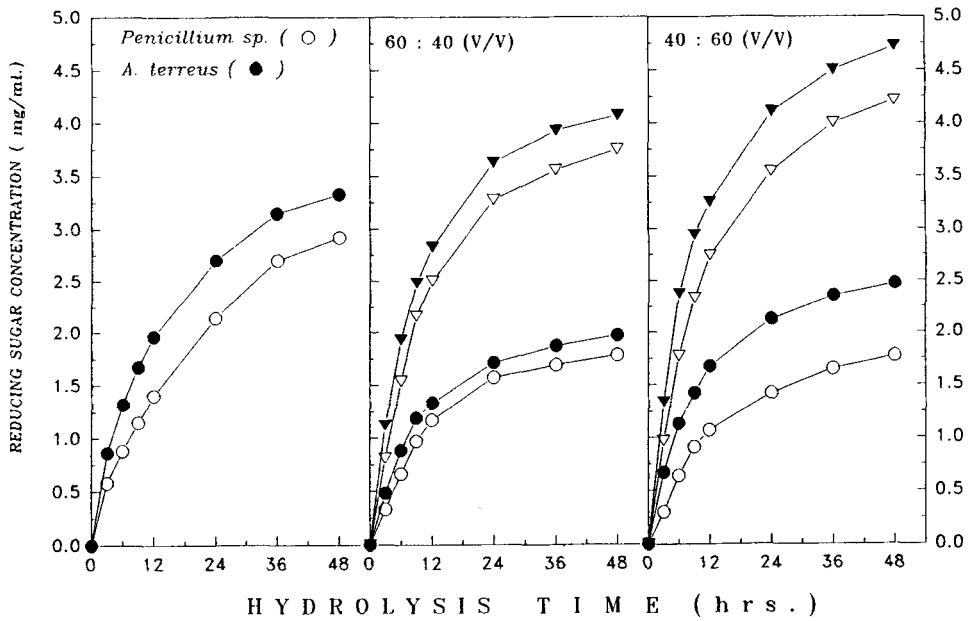

Fig. 5. Saccharification of native sugar cane bagasse pith (20 g) by culture filtrates from *Penicillium* sp. (○) and *A. terreus* (●), alone and with the indicated volumetric combination (▼), pH 4.8, 50°C, in a stirred reactor of 120–150 strokes/min. Theoretical sum of the action of each filtrate, acting separately (▽).

Table 3
Reducing Sugar Concentration at Short Time of Hydrolysis
on Sugar Cane Bagasse Pith, and DSE for Cellulase Mixtures

Mixture of culture filtrates	Volumetric Relation, V/V	Time, min									
		Reducing Sugars, mg/mL					DSE[a]				
		0	15	30	45	60	0	15	30	45	60
Aureobasidium sp.	80:20	0	0.46	0.72	0.90	1.02	0	1.15	1.14	1.10	1.07
+ *Penicillium* sp.	60:40	0	0.48	0.75	0.95	1.09	0	1.17	1.14	1.13	1.09
Aureobasidium sp.	40:60	0	0.65	0.98	1.20	1.32	0	1.18	1.15	1.10	1.06
+ *A. terreus*	60:40	0	0.63	1.05	1.28	1.45	0	1.19	1.14	1.12	1.08
Penicillium sp.	80:20	0	0.64	0.96	1.13	1.15	0	1.33	1.19	1.14	1.06
+ *A. terreus*	40:60	0	0.61	0.99	1.17	1.30	0	1.39	1.27	1.16	1.08
	60:40	0	0.83	1.13	1.41	1.53	0	1.43	1.33	1.19	1.10
Aureobasidium sp.	100	0	0.31	0.54	0.73	0.85	NA	NA	NA	NA	NA
Penicillium sp.	100	0	0.23	0.38	0.52	0.64	NA	NA	NA	NA	NA
A. terreus	100	0	0.57	0.88	1.04	1.08	NA	NA	NA	NA	NA

[a]DSE = (Reducing sugar concentration released by the mixture of cellulases/sum of reducing sugar concentration released by each individual cellulase).

Table 4
Percentage of Saccharification of Nonpretreated Sugar Cane Bagasse Pith by Culture Filtrates from *Penicillium* sp., *A. terreus*, and Their Mixtures

Source of culture filtrate[a]	Concentration of protein, mg/mL	Cellulolytic Enzyme Activities,[c] U/mL				% Saccharification of native sugar cane bagasse pith, h				
		FPase	CMCase	β-glucosidase	Xylanase	3	6	12	14	48
Pennicillium sp.	0.50	0.185	0.703	0.380	1.240	3.17	4.80	7.64	11.68	15.94
A. terreus	0.54	0.194	1.073	0.870	2.708	4.69	7.21	10.70	14.74	18.18
Mixtures[b] of *Pennicillium* and *A. terreus*:										
60:40	0.52	—	—	—	—	6.11	10.53	15.45	19.81	22.22
40:60	0.52	—	—	—	—	7.26	12.94	17.74	22.38	25.66

[a]After 6 d of cultivation for both filamentous fungi.
[b]At the volumetric ratios indicated.
[c]The enzymatic activities were determined by methods indicated, and the U/mL of each activity was estimated as recommended by IUPAC (*26*).

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REFERENCES

1. Giovanozzi-Sermanni, G., Bertoni, G., and Poosi, A. (1989), in *Enzyme Systems for Lignocellulose Degradation*, Coughlan M. P., ed., Elsevier, London, pp. 373–382.
2. Brenmen, A. H., Hoagland, W., and Schell, D. J. (1987), *Biotechnol. Bioeng. Symp.* **17,** 53–70.
3. Wright, J. D. and Power, A. J. (1985), *Biotechnol. Bioeng. Symp.* **15,** 511.
4. Cuskey, S. M., Frein, E. M., Montenecourt, B. S., and Eveleigh, D. E. (1984), in *Overproduction of Microbial Metabolites*, Krumphanzyl, V., Sihyta, B., and Vaneck, A. eds., Academic, New York, pp. 405–416.
5. Fan, L. T., Lee, Y. H., and Gharpuray, M. M. (1987), *Adv. Biochem. Eng.* **23,** 157–187.
6. Grohman, K., Wyman, C. E., and Himmel, M. E. (1992), in *Emerging Technologies for Materials and Chemical Biomass*, Rowel, R. M., Schults, T. P., and Narayan, R., eds., ACS Symp. Ser. No. 476, pp. 354–392.
7. Alber, J. P., Beguin, P., and Millet, J. (1988), *Biochemistry and Genetics of Cellulose Degradation*, Academic, New York.
8. Kubick, C. P., Eveleigh, D. E., Esterbauer, U., Steiner, W., and Kubiceck-Pranz, E. M. (1990), *Trichoderma Cellulases; Biochemistry, Genetics, Physiology and Application*, The Royal Chemicals Society, Cambridge, UK.
9. Beldman, A. G. V., Voragen, T. M., Rombouts, F. M., and Pilnik, W. (1988), *Biotechnol. Bioeng.* **31,** 173–178.
10. Wood, T. M., Mc Crae, S. I., and Bhat, K. M. (1989), *Biochem. J.* **260,** 37–43.
11. Woodward, V., Luna, M., and Lee, N. E. (1988), *Biochemistry* **255,** 895–899.
12. Henrissat, B., Driguez, H., Viet, C., and Schulein, M. (1985), *Bio/Technol.* **3,** 722–726.
13. Wood, T. M. and Mc Crae, S. I. (1979), *Adv. Chem. Ser.* **181,** 181–209.
14. Irwin, D. C., Spezio, M., Walker, L. P., and Wilson, D. B. (1993), *Biotechnol. Bioeng.* **42,** 1002–1013.
15. Wood, T. M., McCrae, S. I., and Macfarlane, C. C. (1980), *Biochem. J.* **189,** 51.
16. Wood, T. M. and McCrae, S. I. (1978), *Biochem. J.* **171,** 61.
17. Gilbón, A., Larios, G., and Huitrón, C. (1981), *Tecnol. Aliment.* **16(5),** 24–30.
18. García-Kirchner, O. (1983), Producción de Celulasas y su Utilización en la Sacarificación de Materiales Celulósicos. Tesis de Licenciatura. Fac. de Química, UNAM, México.
19. Huitrón, C., Saval, S., and Acuña, M. E. (1984), *Ann. NY Acad. Sci.* **434,** 110.
20. García-Kirchner, O. (1985), Memorias del Congreso Nacional de Microbiología. Universidad Autónoma de Puebla-Sociedad Mexicana de Microbiología. Pue., Pue. México.
21. Lowry, O. H., Rosenbrough, M. V., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 256.
22. Mandels, M., Andreotti, R., and Roche, C. (1976), *Biotechnol. Bioeng. Symp.* **6,** 21–33.
23. Larios, G. and Huitrón, C. (1981), *Rev. Tecnol. Aliment (México)* **16(5),** 24–29.
24. Larios, G., Gilbón, A., Lara, Y., and Huitrón, C. (1987), *Enzyme Eng.* **6,** 353,354.
25. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
26. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
27. Zetelaki, K. (1976), *Proc. Biochem.* July/August: 11–18.
28. García, M. D. V., Ogawa, T., Shinmyo, L., and Enatsu, T. (1974), *J. Ferment. Technol.* **52,** 378–386.
29. Mandels, M. and Weber, M. (1969), *Adv. Chem. Ser.* **95,** 391–414.
30. Woodward, J., Lima, M., and Lee, N. E. (1988), *Biochem. J.* **255,** 895–899.