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Mixed Submerged Fermentation with Two Filamentous Fungi for Cellulolytic and Xylanolytic Enzyme Production

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Abstract

The efficient saccharification of lignocellulosic materials requires the cooperative actions of different cellulase enzyme activities: exoglucanase, endoglucanase, β-glucosidase, and xylanase. Previous studies with the fungi strains *Aureobasidium* sp. CHTE-18, *Penicillium* sp. CH-TE-001, and *Aspergillus terreus* CH-TE-013, selected mainly because of their different cellulolytic and xylanolytic activities, have demonstrated the capacity of culture filtrates of cross-synergistic action in the saccharification of native sugarcane bagasse pith. In an attempt to improve the enzymatic hydrolysis of different cellulosic materials, we investigated a coculture fermentation with two of these strains to enhance the production of cellulases and xylanases. The 48-h batch experimental results showed that the mixed culture of *Penicillium* sp. CH-TE-001 and *A. terreus* CH-TE-013 produced culture filtrates with high protein content, cellulase (mainly β-glucosidase), and xylanase activities compared with the individual culture of each strain. The same culture conditions were used in a simple medium with mineral salts, corn syrup liquor, and sugarcane bagasse pith as the sole carbon source with moderate shaking at 29°C. Finally, we compared the effect of the cell-free culture filtrates obtained from the mixed and single fermentations on the saccharification of different kinds of cellulosic materials.

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Index Entries: Cellulolytic activity; mixture fermentation; bagasse pith; saccharification.

Introduction

Cellulose is the most abundant organic compound and is annually replenished. Vast quantities of waste residues are generated by the forestry and agricultural industries, making cellulose a cheap raw material that can be used to obtain potentially different and attractive biotechnologic products. There is currently great interest in the degradation of lignocellulosic materials to provide suitable raw materials for food, fuel, and chemical products. Much of this interest is centered on the hydrolysis of cellulose by cellulase and xylanase enzymes. There are a number of studies on the use of agricultural wastes to produce enzymes of importance in the bioprocessing industry *(1–3)*. Several different attempts have been made to take biotechnologic advantage of the utilization of cellulose, but, at present, the results are neither satisfactory nor economically viable *(4)*. Some recent assessments of the process for the enzymatic saccharification of cellulose have revealed that a major cost (40–60%) is in the production of the cellulases. Much research effort has focused on improving the activity and productivity of cellulose in order to reduce the cost component of the enzyme in the overall process. For the successful industrial application of an enzyme, increasing productivity is important, especially for bulk enzymes, such as cellulases *(5)*.

Although many microorganisms can grow on cellulose or produce enzymes that can degrade amorphous cellulose, relatively few produce the entire complement of extracellular cellulases able to degrade crystalline cellulose in vitro *(6)*. In recent years, interest in mixed culture fermentation systems has been a revitalized, and it is becoming clear that the deliberate construction and establishment of stable microbial communities is a viable approach to many biotechnologic problems *(7)*.

Even though differences exist in properties of the enzymes from different fungi, the main features of cellulose degradation are the same. We have already reported the effect on sugarcane bagasse pith saccharification of using different mixtures of culture filtrates obtained by individual fermentation by these cellulolytic fungi strains *(5)*.

Although significant gains have been made in enzyme production of enzymes to hydrolyze cellulose, a considerable amount of work is still required to enhance the production efficiency of these enzymes. Because the mixed culture offers an alternative for the attainment of well-balanced multienzyme cellulase systems to enable complete saccharification of lignocellulosic materials, we studied the mixed culture action between two different cellulolytic fungi strains with the aim of increasing the efficiency of the cellulase and xylanase system for soluble sugar production with sugarcane bagasse pith as raw material.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma (St. Louis, MO). Solid culture medium for strain propagation was obtained from Bioxon (México).

Microorganisms

Two filamentous fungi strains, *Penicillium* sp. CH-TE-001 and *Aspergillus terreus* CH-TE-013, were supplied from the Unidad Profesional Interdisciplinaria de Biotecnologia culture collection. These strains were grown at 29 ± 1°C for 5 d on potato dextrose slants and stored on the same medium at 4°C. Subcultures were prepared at monthly intervals. A spore preparation was used as inoculum for growth assays.

Cellulase and Xylanase Production

Four grams of cellulosic material (native sugarcane bagasse pith collected locally in the Emiliano Zapata sugar mill, Morelos, México) was placed in a 500-mL flask and then 180 mL of fermentation medium was added. Inoculum was prepared by harvesting spores from 1-wk-old PDA slants of the respective strain in the proportion 3:1 in sterile distilled water containing 0.1–0.3 mL of Tween-80. Fermentation flasks were inoculated with the following medium composition in grams per liter of tap water: sugarcane bagasse pith (20), corn syrup liquor (10), KH_2PO_4 (7.5), K_2HPO_4 (7.5), $NH_4{\cdot}_2SO_4$ (1.4), Tween-80 (5 mL) (pH was adjusted to 5.5 before sterilization). Flasks of 500 mL with 180 mL of the fermentation culture medium were incubated in a rotary shaker at 180 rpm and 29°C for 6 d.

A 1-mL sample of the inoculum was added to each flask for single culture and a 1-mL final sample inoculum for mixture culture obtained by mixing of 1 mL of each strain, respectively. Samples were periodically removed (after 2, 4, 5, and 6 d) and analyzed for pH, FPase, carboxymethylcellulase (CMCase), β-glucosidase, xylanase activities, and soluble protein.

Analytical Methods

Culture broth samples were centrifuged at 10,000 rpm for 5 min, and the supernatant was then filtered through a 0.45-µm polysulfone filter (MFS) and used to determine cellulase and xylanase activities.

Cellulolytic Activity

Filter paper activity was determined by the method of Mandels et al *(6)* as follows: Culture supernatant (0.5 mL) was added to 1 mL of 0.075 *M* citrate buffer, pH 4.8, at 50°C for 60 min. The enzymatic reaction was terminated by adding 3 mL of dinitrosalicylic acid (DNS) reagent.

Table 1
Values Corresponding to Maximum Enzymate Activities After 4–6 d of Fermentation[a]

Filamentous fungi strain	Enzyme activity (U/mL) FP	CMC	*p*-NPG	Birchwood Xylanes	Soluble protein (mg/mL)
Penicillium sp. CH-TE-001	0.40	0.82	0.38	1.45	0.54
Aspergillus terreus CH-TE-013	0.38	1.28	0.90	3.96	0.60
Mixed culture of *Penicillum* sp. CH-TE-001 and *A. terreus* CH-TE-013	0.55	1.41	1.05	5.02	1.14

[a]Enzyme activity units were estimated according to IUPAC *(10)* recommendations.

The carboxymethylcellulose (CMC) saccharifying activity (CMCase) was measured by our reported method *(7)*. Briefly, the assay determination of reducing groups on CMC is done by incubating 0.5 mL of culture filtrate and 1 mL of CMC at 7.5% suspension in 0.075 *M* citrate buffer (pH 4.8) for 60 min at 50°C.

β-Glucosidase activity was determined as in ref. *8* as an aryl-β-glucosidase by means of the hydrolysis of *p*-nitrophenyl-β-D-glucoside (*p*-NPG) measured as the amount of 1 µmol of *p*-nitrophenol liberated by the enzyme/min, and xylanase activity was determined using birchwood xylanes as substrate by measuring the reducing sugars (as xylose) as in ref. *9*.

The enzymate activities were calculated as indicated by IUPAC, using the recommendations of Ghose *(10)* 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 were defined as 0.185/(enzyme releasing 1 mg of glucose)/U/mL of enzyme solution.

Determination of Soluble Protein and Reducing Sugar Concentration

Assays were performed with the cell-free supernatant. Soluble protein content was determined without precipitation according to Lowry et al. *(11)* in an aliquot of cell-free culture filtrate after overnight dialysis at ±4°C, using bovine serum albumin (Sigma) as standard. All reducing sugar determinations were performed by DNS method *(12)*.

Saccharification Experiment

Enzymatic saccharification of filter paper and native sugarcane bagasse pith was carried out in assay tubes at 50°C agitated at 150 strokes/min.

Saccharification was started by adding 0.5 mL of cell-free culture filtrates into 1 mL of 0.075 *M* citrate buffer, pH 4.8, containing 50 mg of substrate. Sodium azide (0.02% [w/v]) was added to the reaction mixture to avoid bacterial contamination. Samples were withdrawn at 1 h for analysis of reducing sugars.

Results and Discussion

Microbial conversion of cellulose is still an important area of biotechnologic research. The use of microbial strains producing enzymes with new properties is an urgent aspect of this research. All cellulolytic enzymes studied so far are characterized by a multiplicity of enzyme components whose exact number varies from one organism to another. The nature, origin, and requirements for these multiple forms, which are still not clear, are largely discussed by Bisaria and Mishra *(13)*. For instance, each component of the cellulase system in *Trichoderma reesei* has been found to be present in a multiplicity of forms with apparent duplication of function. The nature and origin of these isoenzymic forms continue to be the subject of much speculation.

Previous studies have demostrated the ability of cross-synergistic action of cellulases from three distinct cellulolytic fungi strain in the saccharification of native sugarcane bagasse pith *(5)*. Accordingly, synthetic enzyme mixtures can be made that bring about maximum hydrolysis of cellulose. In this context, it may be added that mixed culture fermentations have been used to produce high-activity enzyme mixtures that display increased saccharification efficiencies *(14–16)*. Mixed cultures are advantageous when the feedstock is complex and variable since they yield higher productivity owing to increased culture stability and greater resistance to contamination. The use of mixed cultures of lignocellulosic microorganisms looks promising for increasing the protein content compared with pure cultures, and many of them have been reported to be more efficient in degrading lignocellulosic substrates and in producing high-activity enzymes *(17–19)*.

Furthermore, the general nutrient requirements are likely to be less since individual organisms will feed each other. Figures 1 and 2 show respectively the enzyme activities and soluble protein content after 6 d of inoculation with each strain, *Penicillium* sp. CH-TE-001 and *A. terreus* CH-TE-013, separately. The strains were grown under liquid fermentation bagasse sugarcane pith in a medium formulated with industrial materials. The cellulase and xylanases activities obtained were similar to those in our previous report *(5)*.

Figure 3 shows the enzyme activities and soluble protein content after 6 d of inoculation with the aforementioned mixture culture with the same culture medium and fermentation conditions. The mixed fermentation produced larger activities of cellulase and xylanase activities after 2 d of culture with shaking at 29°C. Note that after 2 d of inoculation with the

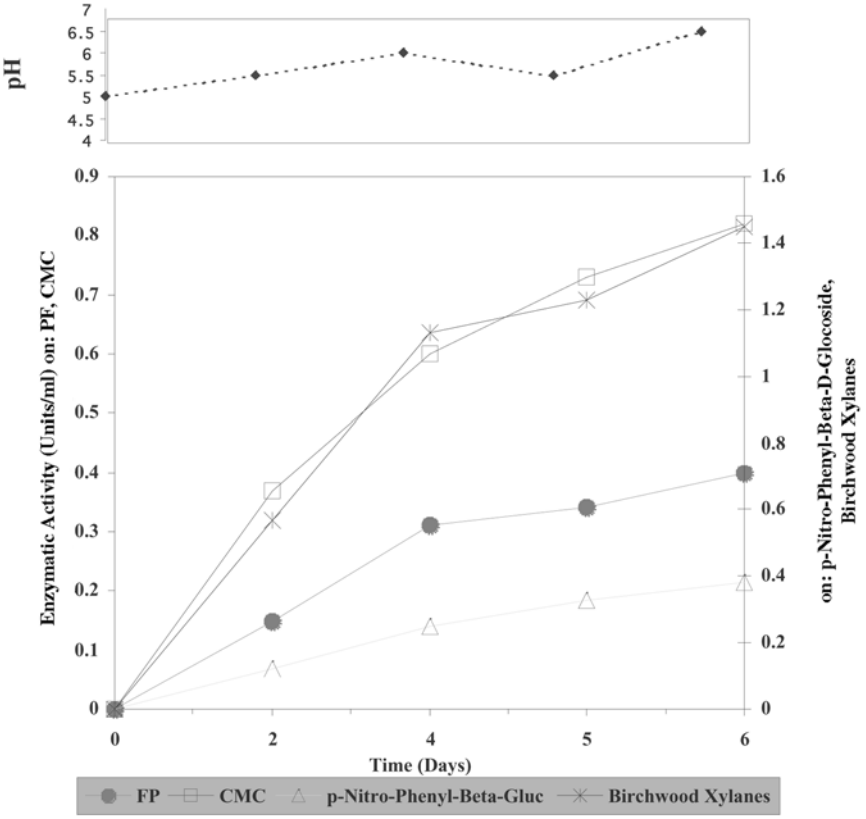

Fig. 1. Cellulase and xylanase production (U/mL equivalents) and Ph profile by submerged fermentation using *Penicillium* sp. CH-TE-001 at 29°C in shaking flask (180 rpm) in culture medium containing sugarcane bagasse pith, corn syrup liquor, and mineral salts.

mixed fungi strains, all of the enzymate activities as well as a high soluble protein content in the culture filtrates were obtained (results not shown). Probably this effect could be a complementary action among the cellulolytic and xylanolytic activities acting together for the complete hydrolysis of cellulose fiber during the early stage of fermentation. Another possibility is a competition for the sole carbon source in the culture medium in the induction enzymatic stage or a specific synergistic effect of the enzymate activities on reducing sugars obtained by cellulose hydrolysis. Another point is that in a mixed culture the growth of the participating organisms is not the total sum of their growths in single cultures, since the metabolites produced by one organism could affect the growth of the other.

Since another one of our goals was the utilization of agroindustrial byproducts, such as sugarcane bagasse pith, in a saccharification reaction to produce soluble sugars useful for other purposes, we initially compared the capacity of hydrolysis of cellulases obtained by individual culture of

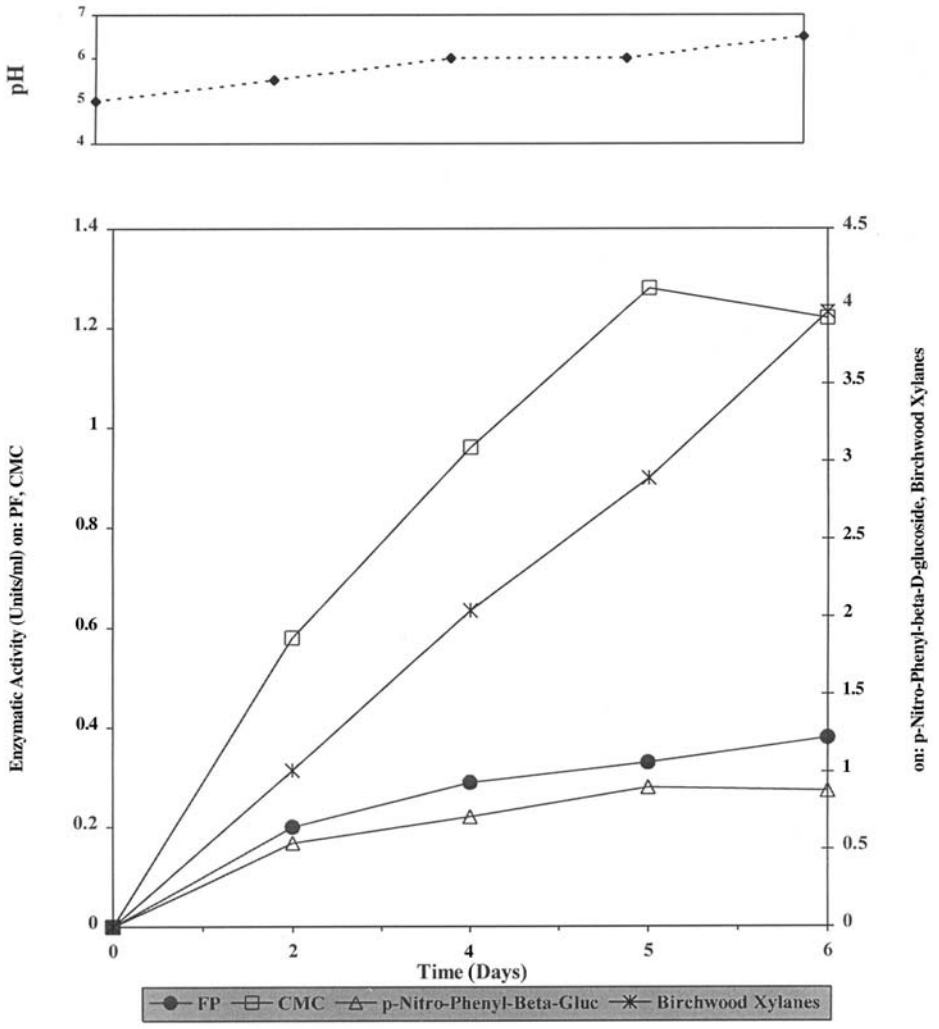

Fig. 2. Cellulase and xylanase production (U/mL equivalents) and pH profile by submerged fermentation using *A. terreus* CH-TE-013 at 29°C in shaking flask (180 rpm) in culture medium containing sugarcane bagasse pith, corn syrup liquor, and mineral salts.

each strain with respect to the cellulases and xylanases obtained by mixed fermentation. The experimental results showed that at short times of hydrolysis reaction cellulose saccharification is acomplished by the concerted action of culture filtrates obtained from mixed fermentation. Figure 4 indicates the efficiency of the conversion of native sugarcane bagasse pith to reducing sugars by the cellulases obtained by mixed fermentation. As can be seen, a major capacity of reducing sugar production with the culture filtrate by mixture culture of *Penicillium* sp. and *A. terreus* strains was obtained. Similarly, it was possible to increase the filter paper and the

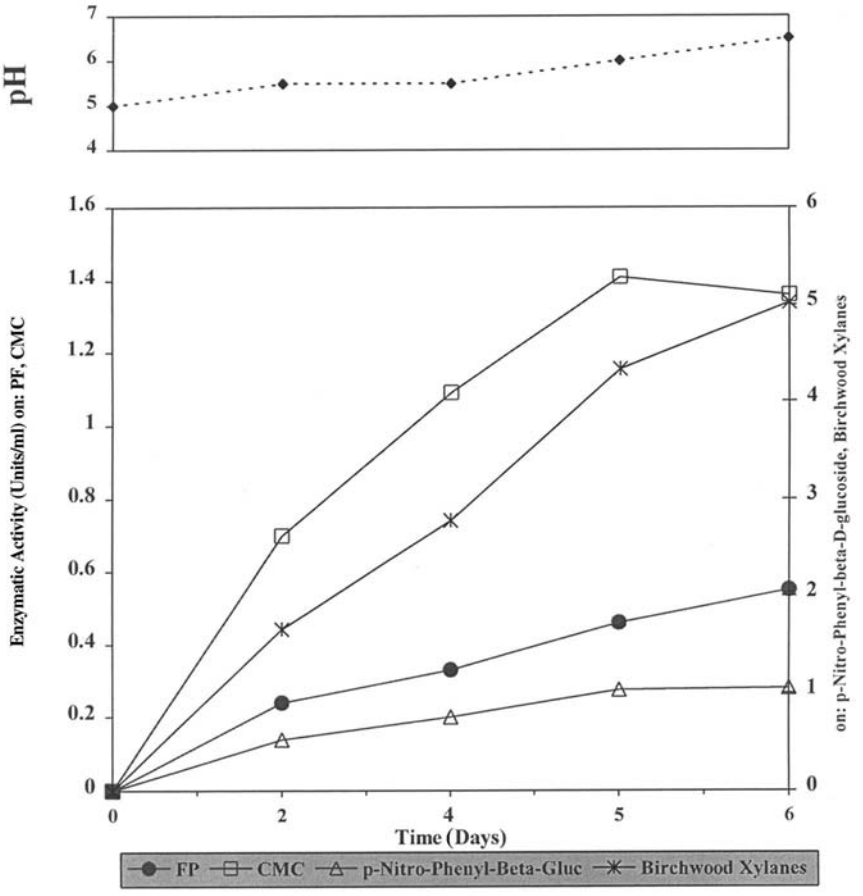

Fig. 3. Cellulase and xylanase production (U/mL equivalents) and pH profile by submerged fermentation using coculture of *Penicillium* sp. CH-TE-001 and *A. terreus* CH-TE-013 at 29°C in shaking flask (180 rpm) in culture medium containing sugarcane bagasse pith, CSL,a nd mineral salts.

native sugarcane bagasse pith saccharification in a quantity of 52 and 52.2%, respectively, with culture filtrates obtained by mixture fermentation. The percentage of increase is major compared with the highest filtrate culture saccharifing activity acting individually and under the same conditions. This is probably the result of a synergism between a β-glucosidase and the other cellulase components, as was observed by Wood and McCrae *(20)*, who reported that the degree of synergism depends on the source of enzyme and the type of component present.

Conclusions

The experimental results obtained demonstrated that the mixed fermentation of *Penicillium* sp. CH-TE-001 and *A. terreus* CH-TE-013 produced

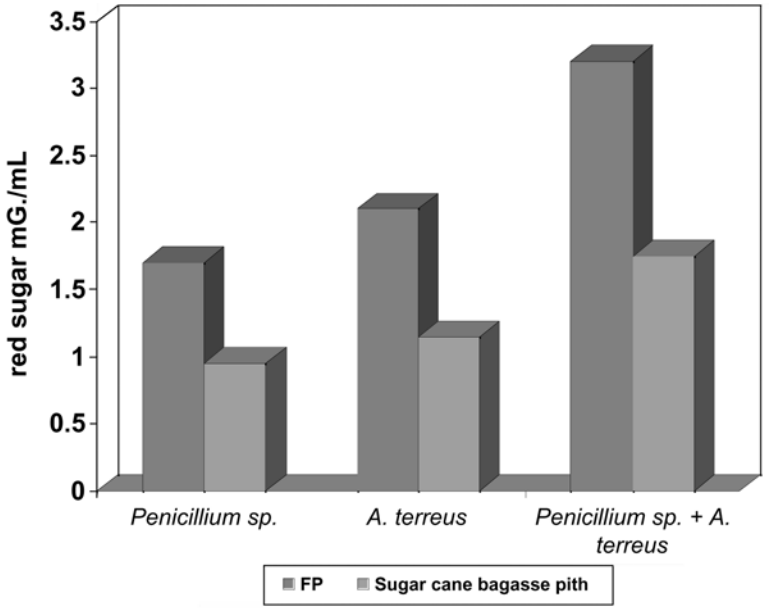

Fig. 4. Saccharification of lignocellulosic materials by *Penicillium* sp. CH-TE-001 and *A. terreus* CH-TE-013 cellulases.

culture filtrates with higher protein content, cellulase (mainly β-glucosidase), and xylanase activities after 2 d in comparison with the individual culture of each strain under the same conditions using a simple medium such as sugarcane bagasse pith as the sole carbon source. Assays will need be done to optimize the culture medium and submerged fermentation conditions, in order to improve the cellulolytic and xylanolytic productivity.

The cell-free culture filtrates obtained by mixture fermentation of the selected strains were able to produce a major saccharification of filter paper (pure lignocellulosic material) and of native sugarcane bagasse pith (heterogeneous and waste lignocellulosic material) with respect to the effect of the culture filtrates obtained on each strain evaluated individually. This is probably the result of a major quantity of soluble protein content in the culture filtrates obtained by mixture fermentation. It was also possible to obtain a heterogeneous cellulolytic and xylanolytic system with complementation in hydrolytic activity on lignocellulosic materials. For this reason, it would be necessary to compare the effect between the filtrate cultures obtained by mixed fermentation and those obtained by mixing the strains in different ratios. Synergism between different cellulasic components exists when they are acting simultaneously on insoluble cellulose *(21)*.

This work demonstrated that it is possible to obtain a major efficient cellulolytic and xylanolytic system for enzymatic hydrolysis of lignocellulosic materials such as sugarcane bagasse pith by mixture fermentation of two different cellulolytic fungi strains, *Penicillium* sp. CH-TE-001 and

A. terreus CH-TE-013, selected mainly because of their different enzymatic capabilities on lignocellulosic materials.

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