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Screening of Potential Antibiotic Action of Cellulolytic Fungi

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Abstract

Twenty different strains of filamentous fungi were initially selected for evaluation of cellulolytic activity using a single test in a simple mineral salts culture medium with filter paper as the only carbon source. Those fungi strains that were capable of completely breaking the filter paper strip within 4–8 d were assayed also for antimicrobial action, using *Staphyloccocus aureus* ATCC 6538P according to the so-called agar piece method. We screened three different strains with both capacities: the production of cellulolytic activity and antibiotic action. The experimental results suggest that the fungi *Penicillium* sp. F0PC01, *Aspergillus* sp. F0Q001, and *Cephalosporium* sp. F03800 have both capabilities because they grew rapidly on cellulose as the only carbon source and were able to produce an area of growth inhibition in *S. aureus* of approx 2.04, 1.57, and 2.39 cm, respectively, on agar plates using the agar piece method. Subsequently, the antibiotic production obtained with those cellulolytic strains was evaluated by submerged fermentation at the flask level, in a simple culture medium containing lactose without biosynthesis precursor, obtaining 3670, 2830, and 4060 antibiotic units/mL, referred to as penicillin G, whereas for cellulolytic activity, the results were 1.34, 1.81 and 0.57 FPU/mL, respectively.

Index Entries: Cellulolytic activity; antimicrobial action; filamentous fungi; bioassays.

Introduction

The biosynthesis of bioactive and chemotherapeutic compounds with the aid of microorganisms is an ever-increasing and most important branch

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of microbial biotechnology and the fermentation industry. A major aim of industrial antibiotic production is directed to screening programs to generate new producer organisms either from natural sources or from established cultures *(1)*.

The carbon source represents a comparatively large part of the overall manufacturing cost of the production of antibiotics (penicillin). Cellulose is one of the most plentiful organic materials on the earth. It is considered a renewable material and is a cheap raw material that can be used to obtain potentially different and attractive biotechnological products. Thus, there is currently great interest in finding degradation methods of lignocellulosic materials to provide suitable raw materials for food, fuel, or chemical needs. Several different attempts have been made to take biotechnological advantage of the utilization of cellulose, but until now, the results have been neither satisfactory nor economically viable *(2)*.

The usefulness of cellulosic materials is dependent on their chemical or biological hydrolysis to glucose. We focused on the second alternative of utilization of cellulosic materials, which is related to direct fermentation by cellulolytic microorganisms, taking into account that little work has been done related to obtaining metabolite by direct fermentation without a previous hydrolysis step. In addition, there are no reports related to the objectives defined herein. The utilization of biological cellulose represents a promising alternative if we consider that in Mexico, there are important quantities of lignocellulosic materials such as sugarcane bagasse and corn straw, both considered secondary and waste products of agricultural industry. To take advantage of these raw materials, we have proposed the direct fermentation of cellulose by cellulolytic microorganisms simultaneously with antibiotic production capabilities in order to save energy in the antibiotic production process as well as to reduce the amount of agricultural wastes.

In this article, we report on the methodology used initially to examine some of the 20 strains of cellulolytic fungi registered at the Unidad Profesional Interdisciplinaria de Biotecnología (UPIBI) microbial collection with respect to the potential antimicrobial action and the preliminary results obtained on the production of antibiotic and cellulase activity by submerged fermentation with the strains *Penicillium* sp. F0PC01, *Aspergillus* sp. F0Q001, and *Cephalosporium* sp. F03800 in a simple lactose medium without biosynthesis antibiotic precursor after 12 d of fermentation time.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma (St. Louis, MO). USP reference standard penicillin G-pure potassium salt (mol wt 327.47) with an activity of 1595 antibiotic units/mL (AnU/mL) was also obtained from Sigma. Solid culture media for bioassays and strain propagation were obtained from Bioxon (México).

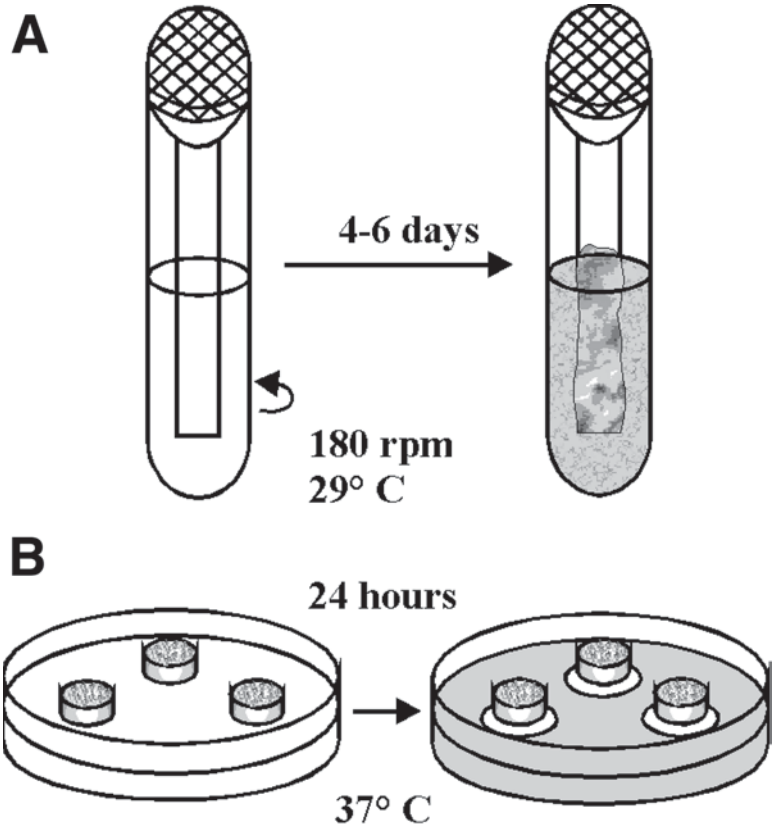

Fig. 1. Diagram showing preliminary tests used for examination of preselected filamentous strains. Conditions of the procedure are described in Materials and Methods. **(A)** Filter paper disruption; **(B)** agar piece bioassay.

Microorganisms

All the filamentous fungi and the *Staphyloccocus aureus* ATCC 6538P strains were supplied from the UPIBI culture collection. The filamentous fungi 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 (filter paper disruption and antibiotic action). *S. aureus* ATCC 6538P was maintained by weekly transfers to fresh sterile slants of Grove and Randall agar medium no. 1 according to Kavanagh *(3)*.

Bioassays

Filter Paper

The procedure for the examination of the cellulolytic filamentous fungi strains was carried out in 18 × 150 mm glass tubes, according to García-Kirchner *(4)*, using filter paper (Whatman no. 1) cut into 12 × 1 cm strips, as indicated in Fig. 1A, using 10 mL of the following saline culture medium:

0.2 g of KH_2PO_4, 0.14 g of $(NH_4)_2SO_4 \cdot 7\,H_2O$, 0.03 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $CaCl_2 \cdot 6H_2O$, and distilled water to 100 mL. Then, 1 mL of sterile trace elements solution (0.20 mg of $CoCl_2 \cdot 6H_2O$, 0.50 mg of $FeSO_4$, 0.17 mg of $MnSO_4 \cdot 7H_2O$, 0.2 mg of $ZnCl_2$, and distilled water to 100 mL) was added after sterilization. Medium pH was adjusted to 5.0 before sterilization. The spore-inoculated culture tubes were incubated in a rotary shaker at 150 rpm and 29°C until growth and/or filter paper strip dispersal.

Antimicrobial Action

The cellulolytic filamentous fungi were preselected by measuring their antibiotic production on agar plates using the agar piece method according to Trilli et al. *(5)*, as indicated in Fig. 1B, placing three potato dextrose agar (PDA) disks over antibiotic no. 1 agar medium containing *S. aureus* ATCC 6538P recent inoculum in order to measure the growth inhibition zone after 24 h of incubation at 37°C. The procedure was carried out under aseptic conditions.

Culture Conditions: Antibiotic Production

Inoculum was prepared by harvesting spores from 1-wk-old PDA slants of the respective strain at a ratio of 3:1 in sterile distilled water containing 0.1–0.3 mL of Tween-80. Fermentation Erlenmeyer flasks were inoculated to give 10^6–10^7 spores/mL. A liquid medium contained the following ingredients (in grams per liter of distilled water): 30.0 g/L of lactose, 1.0 g/L of $(NH_4)_2SO_4$, 7.5 g/L of KH_2PO_4, 0.7 g/L of $MgSO_4 \cdot 7H_2O$, 0.05 g/L of $MnSO_4 \cdot 4H_2O$, 0.05 g/L of $ZnSO_4 \cdot 7H_2O$, 0.18 g/L of $FeSO_4 \cdot 2H_2O$, 0.008 g/L of $CuSO_4$, 0.05 g/L of $CaCl_2$, 2.0 g/L of $CaSO_4$. pH was adjusted to 5.5 before sterilization. Erlenmeyer flasks (500 mL) with 180 mL of the fermentation culture medium were incubated in a rotary shaker at 180 rpm and 29°C for 12 d. Ten-milliliter samples were removed each 48 h for 12 d under the described incubation conditions under aseptic conditions for determination of pH, antibiotic assay, cellulolytic activity, reducing sugars, and soluble protein.

Antibiotic Assay

Mycelium and other solids present were removed by filtration (Whatman no. 1 filter paper), and the antimicrobial action of culture broths was measured by the cylinder method with the same strain of *S. aureus* as organism on antibiotic medium (Bioxon) at 37°C as indicated in USP XXIII *(6)* using a penicillin G secondary standard. Total antibiotic equivalent potency was calculated from the clear zone of the standard curve in the range of 0.5–10 AnU/mL. The diameter of the clearing zone is proportional to the logarithm of the amount of penicillin plus a constant factor *(7)*.

Cellulolytic Activity

Filter paper activity was measured by the method of Mandels et al. *(8)* as follows: culture supernatant (0.5 mL) was added to 1 mL of 0.075 *M* citrate

Table 1
Results of First Prescreening Stage for Filamentous Fungi Strains

Filamentous fungi strain	Filter paper degradation (d)	Growth inhibition of *S. aureus* (diameter [cm])[a]
Fusarium sp. F00009	7	1.80
Cephalosporium sp. F03800	4	2.39
Penicillium sp. F05900	8	2.54
Aeremonium sp. F06900	6	1.53
Nocardia sp. F07800	5	2.17
Aspergillus sp. F0Q001	4	1.57
Penicillium sp. F0PC01	4	2.04

[a]The diameters of the inhibition halos indicated are averages of five replicas.

buffer, pH 4.8, at 50°C for 60 min. The enzymatic reaction was terminated by the addition of 3 mL of dinitrosalicylic acid (DNS) reagent. One unit of cellulolytic activity (FPase) was defined as the amount of enzyme releasing about 2 mg of glucose/mL of culture filtrate in 60 min.

Determination of Soluble Protein and Concentration of Reducing Sugars

Assays were performed with the cell-free supernatant. Soluble protein content was determined without precipitation according to Lowry et al. *(9)* in an aliquot of cell-free culture filtrate after ±4°C overnight dialysis, using bovine serum albumin (Sigma) as standard. Total reducing sugars was estimated colorimetrically using DNS reagent *(10)*.

Results and Discussion

During the first stage of the study, 20 different filamentous fungi strains isolated from different places (soil, lignocellulosic materials, cheese) were chosen by their cellulolytic activity over different kinds of cellulose. Initially using two simple microbiological techniques (*see* Fig. 1), seven different filamentous fungi were prescreened with regard to the following capacities: to grow rapidly on cellulose as the only carbon source and to present antimicrobial action. Table 1 gives the results obtained with these two preliminary evaluation techniques. It was proved that all the fungi strains were able to grow on filter paper cellulose at different rates, but only three entirely disrupted the filter paper strip. In relation to the antimicrobial effect, the seven fungi strains similarly inhibited the growth of *S. aureus*. The preselection technique of agar disk plates was useful in discriminating low producers of antibiotic action and nonproducing strains, rather than selecting improved strains. Thus, this method was used to reduce the number of strains to be tested in shake flask liquid fermentation.

Finally, the growth capacity on cellulose was the selection judgment considering only the fungi strains, that in less time, were able to grow on

filter paper (4 d). Subsequently, only three strains were selected to evaluate their capacities to produce cellulolytic activity and antimicrobial effects by submerged fermentation using a culture medium containing lactose and mineral salts. In Fig. 2, the data show the microbial growth related to the consumption of reducing sugar and the amount of soluble protein during the fermentation time. The consumption of lactose could be related to the production phase of the secondary metabolism, in which each strain showed a different behavior within 8–10 d of growth. Figure 3 shows that the higher levels of antibiotic production for the screened strains—*Penicillium* sp., *Aspergillus* sp., and *Cephalosporium* sp.—were obtained after 12 d and 8 d of fermentation, respectively. In this fermentation using the culture medium containing lactose as the only carbon source, it was possible to relate the induction of different levels of cellulolytic activity (FPase activity) during the primary metabolic phase (after 4 d of culturing). Moreover, we can observe a direct relationship between the cellulolytic activity and the soluble protein in cell-free culture filtrates, which can be helpful in the monitoring of cellulolytic activity production.

One of the tested strains (*Aspergillus* sp.) showed the highest cellulolytic activity, whereas its antimicrobiological action was the lowest. The opposite effect was observed in the strain *Cephalosporium* sp. (*see* Fig. 3). Table 2 summarizes the experimental data obtained. Antibiotic formation was not considered to be regulated by carbon sources nor by any other type of effector. Although the mechanism by which the carbon source controls secondary metabolism is unknown, lactose was found to be a carbon source that improved penicillin production *(11,12)*. However, lactose induced cellulolytic activity production *(13)* under the defined conditions (Fig. 3). This aspect represents a fundamental characteristic of the screened strains because it can be hypothesized that it would produce important levels of antibiotic in a culture medium containing cellulose as the only carbon source. It is also possible that the filamentous fungi grow and synthesize the secondary metabolites, such as antibiotics, only after having passed through part or all of their growth phase.

Conclusion

The experimental results showed that it was possible to prescreen seven different filamentous fungi strains showing separately two capacities: to grow on cellulose as the only carbon source and to present antimicrobial action. Only three strains—*Penicillium* sp. F0PC01, *Aspergillus* sp. F0Q001, and *Cephalosporium* sp. F03800—showed the capacity to produce an acceptable level of antibiotic production, referred to as penicillin G, using a lactose medium after 8–12 d without a biosynthesis precursor by submerged culture at 29°C. Moreover, it was possible to induce cellulolytic activity, referred as FPase activity, after 4 d in the lactose medium for the three screened strains.

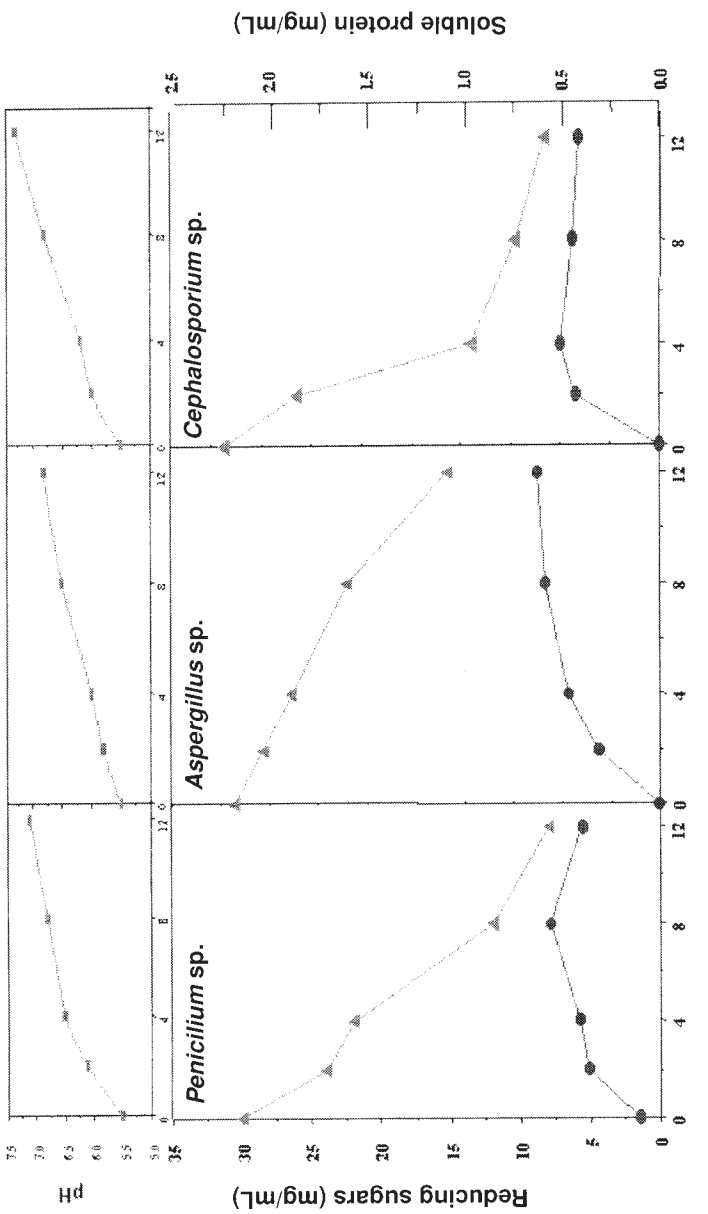

Fig. 2. Reducing sugar (▲), soluble protein (●), and pH (■) profiles of *Penicillium* sp., *Aspergillus* sp., and *Cephalosporium* sp. during antibiotic fermentation at 29°C in the lactose and mineral salts medium.

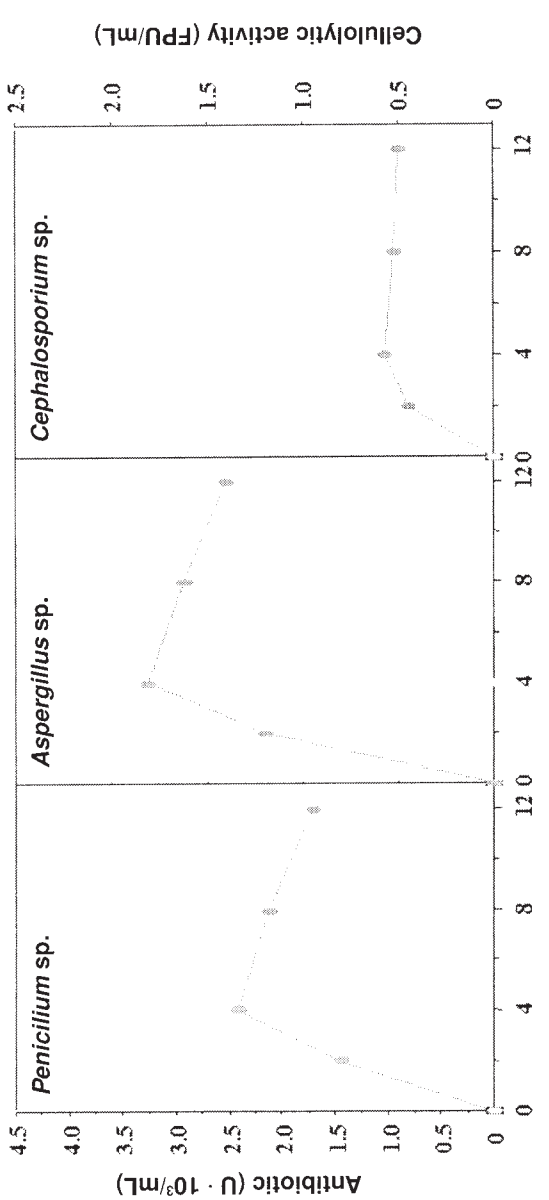

Fig. 3. (—■—) Antibiotic production (penicillin U × 10^3/mL equivalents) and (—●—) cellulolytic activity (FPU/mL) by submerged fermentation using *Penicillium* sp., *Aspergillus* sp., and *Cephalosporium* sp. at 29°C in a shaking flask (150 rpm) in a culture medium containing lactose and mineral salts.

Table 2
Indicated Values Corresponding
to Maximum Cellulolytic Activity After 4 d of Fermentation

Filamentous fungi strain	FPU/mL	Penicillin equivalents (AnU/mL)
Penicillium sp. F0PC01	1.34	3670
Aspergillus sp. F0Q001	1.81	2830
Cephalosporium sp. F03800	0.57	4060

[a]The maximum antibiotic production obtained was at 8 d for *Penicillium* sp. and 12 d for the other two strains.

Further studies will be done using lignocellulosic materials such as agroindustrial crops, e.g., sugarcane bagasse pith, as a fermentation substrate in a simple culture medium for the direct antibiotic fermentation with the screened cellulolytic fungi strains. The significance of this work resides in the selection of three filamentous fungi strains capable of biotransforming the lignocellulosic materials on high-value molecules as the antibiotics. One important objective still pending is the identification of the antibiotic obtained in order to add the correspondent precursor, which could influence the biosynthetic process and thus probably increase the antibiotic productivity. However, if we take into account the microorganism used as proof (*S. aureus*), we can assume a chemical structure like that of the β-lactam ring.

Acknowledgments

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