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Endocellulase and Exocellulase Activities of Two *Streptomyces* Strains Isolated from a Forest Soil

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Abstract

Two *Streptomyces* strains, M7a and M23, from a Brazilian forest soil were evaluated for the cellulase production of their supernatants after growth in a microcrystalline cellulose medium, using carboxymethylcellulose and filter paper as substrates at different temperatures and pH values. Endoglucanase and exoglucanase activities were compared to a commercial *Trichoderma reesei* cellulase using fluorogenic conjugated substrates. Similar specific activities were observed for the enzyme preparations of strain M23 and *T. reesei*. For M7a the activities were about seven times higher than those obtained for *T. reesei*. Extracellular or cell-associated cellobiase activities were not detected in both strains.

Index Entries: Brazilian forest soil; cellulolytic strains; endocellulases; exocellulases; *Streptomyces*.

Introduction

Cellulose is the most abundant carbohydrate polymer in the biosphere, and its estimated synthesis rate is approx 4×10^7/yr. Considering this scenario, this biopolymer represents a potential solution to our needs for energy resources and raw material for chemicals *(1,2)*. However, the biotechnological applications of cellulose have been hindered by the technical and economical difficulties of its enzymatic hydrolysis. This is considered to require the action of at least three hydrolytic enzymes: endoglucanase (endo 1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4), which randomly

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attacks the cellulose chain and splits β-1,4-glucosidic linkages; exo-glucanase (exo-1,4-β-D-glucan-4-cellobiohydrolase, EC 3.2.1.91), which releases either cellobiose or glucose from the nonreducing end of cellulose; and cellobiase (β-glucosidase, EC3.2.1.21), which hydrolyzes cellobiose and other soluble cellodextrins to glucose *(3)*. A synergistic interaction of these enzymes is necessary for the complete hydrolysis of native cellulose to glucose *(4)*.

Among prokaryotes, the actinomycetes constitute an important part of the microbial community responsible for the degradation and recycling of natural biopolymers such as cellulose. Many *Streptomyces* strains secrete large quantities of extracellular proteins, including hydrolytic enzymes such as cellulases. Although streptomycetes are known to degrade lignin, hemicellulose, and cellulose *(5)*, relatively few of their cellulase systems have been studied compared to fungi *(6–8)*.

The objective of the present work is to report some properties of cellulases produced by *Streptomyces* strains isolated from a latosol Mata Atlântica forest soil.

Materials and Methods

Screening

Actinomycetes were obtained by serial dilution of a latosol forest soil (Mata Atlântica, Rio de Janeiro, Brazil). For the isolation of the microorganisms, using the conventional dilution-plate procedure, 5 g of screened soil (1-mm sieve) were added to 30 mL of sterile water and shaken at 140 rpm for 20 min. Tenfold dilutions were prepared from the resulting suspension up to 10^{-4}. Portions of 0.1 mL of each dilution were spread with the aid of a Drigalsky loop on the surface of agar plates containing 20 mL of starch-casein-agar pH 7.0 *(9)* and glycerol-asparagine-tyrosine-agar pH 7.0 *(10)*. Both media were supplemented with amphotericin B at 100 μg/mL. In the dispersion and differential centrifugation technique *(11)*, dispersion of soil microorganisms was achieved in three successive stages, allowing the realization of three distinct supernatants: S_1, supernatant of extraction, after shaking a mixture of soil and water with sodium cholate and chelating resin (Sigma), submitted to centrifugation; S_2, the previous residue ultrasonicated in a low-energy ultrasonic bath in sodium cholate, then shaken and submitted to centrifugation; S_3, the residue from S_2 shaken in sterile distilled water and submitted to centrifugation. All three supernatants were subjected to the same dilution and inoculation scheme used in the conventional procedure. The plates (five replicates for each dilution) were incubated at 28°C for 7, 14, 21, and 28 d, when the growing actinomycetes colonies were streaked on yeast extract-malt extract-agar medium *(12)* for isolation. The pure cultures were lyophilized or maintained as a spore suspension in 20% glycerol at –20°C. For selection of cellulolytic strains, each culture was inoculated in duplicate in test tubes containing a liquid mineral medium and a filter paper strip as the sole carbon source *(13)*. The more

active strains were identified using morphological and chemotaxonomic procedures *(14)*.

Fermentations

The selected strains were grown in 50-mL Erlenmeyer flasks with 20 mL of microcrystalline cellulose medium *(15)* containing 1.5 g/L of KH_2PO_4; 2.0 g/L of K_2HPO_4; 1.4 g/L of $(NH_4)_2SO_4$; 2.0 g/L of yeast extract; 1.0 g/L of proteose peptone; 10 g/L of microcrystalline cellulose (type no. 38, Sigma); 2 mL of Tween-80; and 1 mL of trace elements *(16)*, pH 7.0. After sterilization, $MgSO_4 \cdot 7H_2O$ (30 mg) and $CaCl_2 \cdot 2H_2O$ (30 mg) were added aseptically. Experiments were also performed replacing yeast extract and Tween-80 with vitamin solutions *(17)*. Growth media were inoculated with 1 mL of a spore suspension (1.55×10^7 cells/mL) from a 7-d culture in malt extract-agar. Cultures were incubated in a shaker at 28°C and 150 rpm. One Erlenmeyer flask was withdrawn every 24 h, up to 7 d, for determination of enzyme activity on the supernatant (crude enzyme). Cells were separated by centrifugation at 3000 rpm for 10 min and filtered through Millipore filters (GSWP 0.22 μm). The cell pellets were washed twice with 0.05 *M* potassium phosphate buffer, pH 7.0, and resuspended in 10 mL of the same buffer for disruption using a low-energy ultrasonic apparatus. During disruption, the samples were kept cold in an ice bath. The extracts were centrifuged at 3000 rpm for 10 min and filtered as just described. The homogenates were used for determining β-glucosidase activity.

Enzyme Assays

The substrates used were carboxymethylcellulose (CMC) (sodium salt, Sigma); a substituted, soluble, not crystalline cellulose; and filter paper (FP) (Whatman no. 1), a crystalline more resistent cellulose. Endocellulase activity was assayed in a reaction mixture containing 500 μL of 1% CMC, 100 μL of the crude enzyme, and 700 μL of 0.05 *M* citrate-phosphate buffer, pH 7.0 *(18)*. Reducing sugar concentration was determined after incubation for 60 min at 30, 42, 50, 70, and 100°C. The cellulase complex (endo- and exocellulase) was assayed by measuring the release of reducing sugars *(19)* using a reaction mixture containing 25 mg of FP as substrate, 100 μL of the crude enzyme, and 700 μL of 0.05 *M* citrate-phosphate buffer, pH 5.6. Enzyme activity was determined after 48 h of incubation at 30, 42, 50, 70, and 100°C using 1.33 mg/mL of thimerosal to prevent microbial contamination. Enzyme activity was also evaluated in various pH values (3.0, 4.0, 5.6, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0), at 42°C, using CMC and FP as substrates, in the same conditions as described previously. Results were presented as units per liter in which 1 U of activity is the amount of enzyme required to liberate, under the conditions of the assay, 1 μmol of reducing sugar expressed as glucose equivalent liberated per minute. For each pH value, the corresponding buffer was used: pH 3.0, 4.0, and 6.0, citric acid-sodium citrate; pH 5.6 and 7.0, citric acid-Na_2HPO_4 (Mcllvaine); pH 8.0, Na_2HPO_4–NaH_2PO_4; pH 9.0 and 10.0, glycine-NaOH; pH 11.0 and 12.0,

phosphate buffer solutions *(20)*. Supernatants and cellular homogenates were used to evaluate extra- and intracellular β-glucosidase activities. With this purpose, glucose concentration was measured after incubation of supernatant or homogenates (100 μL) at 42°C for 30 min with 900 μL of 0.02 *M* cellobiose in 0.05 *M* citrate-phosphate buffer, pH 5.6.

Enzyme Kinetics

The degradation kinetics of conjugated fluorogenic substrates were used to follow exo- (4-methylumbelliferylcellobioside, Sigma, M6018) and endocellulase (4-methylumbelliferylcellotrioside, Sigma, M1532) activities *(21)*. Reaction mixtures containing 10 μL of the crude enzyme plus 190 μL (100 m*M* in 0.05 *M* citrate-phosphate buffer, pH 5.6) of the relevant substrates were incubated at 37°C in a fluorescence apparatus (Fluoroskan II, excitation: 355 nm and emission: 460 nm). Measurements were automatically performed every 5 min for 50 min. A commercial cellulase (Sigma, C8546) from *Trichoderma reesei* (optimum temperature at 37°C and optimum pH 5.5) was also used for comparison. Specific activities were expressed as units/microgram of protein. One unit of enzymatic activity was defined by the release of 1 μmol of 4-methylumbelliferone per minute.

Analysis

Reducing sugars were measured using the Nelson Somogyi method *(19)*. Glucose release was detected using high-performance thin-layer chromatography (HPTLC). Runs were performed in butanol:ethanol: H_2O (40:11:19), and 0.2% orcinol was used as the glucose reagent *(15)*. Glucose determinations were performed using a Glucose Analyzer (Beckman II). Protein determination was performed according to Bradford *(22)*, using bovine serum albumin (Sigma) as standard. Enzyme assays and analytics were conducted in triplicate and the results expressed as average values. Variations in the multiple assays were <5%.

Results

Among the 154 actinomycetes studied, strains M7a and M23 were selected for their higher cellulolytic activity and were identified as *Streptomyces* spp. *Streptomyces* M7a was isolated using the dispersion and differential centrifugation technique and starch-casein-agar medium, whereas *Streptomyces* M23 was isolated by the conventional dilution-plate procedure and glycerol-tyrosine-asparagine-agar medium. Preliminary experiments showed the importance of the yeast extract and Tween-80 in the growth medium because no CMCase activity from both M7a and M23 strains was detected in medium in which these were replaced by vitamin solutions, and the same results were obtained in a medium without cellulose.

Fermentations were carried out for 5 d since the highest CMCase and FPase levels were observed for both strains within this time interval. Figure 1 shows the effect of the temperatures on enzyme activity. For *Strep-*

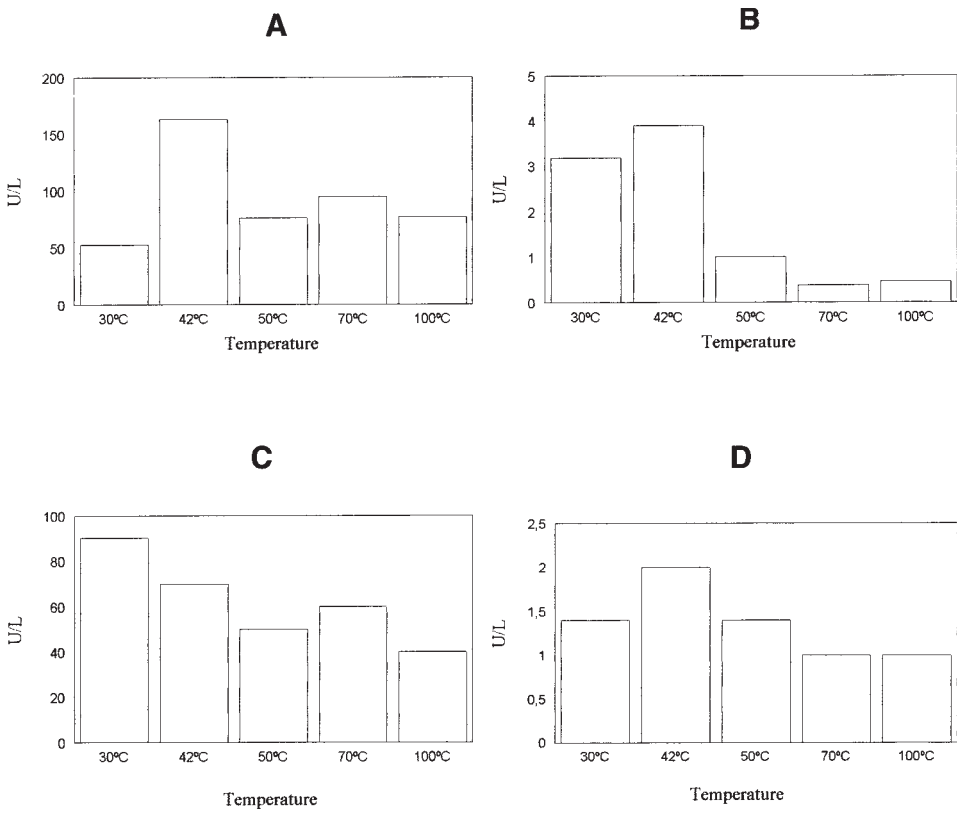

Fig. 1. Cellulolytic activity of *Streptomyces* M7a **(A)** CMCase and **(B)** FPase and *Streptomyces* M23 **(C)** CMCase and **(D)** FPase incubated at pH 5.6 and different temperatures.

tomyces M7a, CMCase and FPase activities were higher at 42°C at which values of 163 and 3.9 U/L were observed, respectively. The endocellulolytic activity decreased to an average value of 86 U/L within the temperature range of 70–100°C. The maintenance of 50% activity in comparison to the levels observed at 42°C may indicate the existence of a thermotolerant component in the *Streptomyces* M7a cellulolytic system. Lower levels of both cellulolytic activities were observed for strain M23 since the highest CMCase and FPase activities of 90 and 2 U/L were observed at 30 and 42°C, respectively. Significant CMCase activity levels were also observed within the temperature range of 70–100°C.

The temperature of 42°C was chosen to be used in the subsequent experiments aiming to evaluate CMCase and FPase activities at different pH values. According to the data presented in Fig. 2, the highest equivalent CMCase activities for strain M7a (235 and 230 U/L) were observed at two distinct pH values, 7.0 and 11.0, respectively, whereas the best conditions for FPase were at pH 5.6 and 9.0 (2 and 1.14 U/L, respectively). For strain M23, higher CMCase activities were observed within the pH range

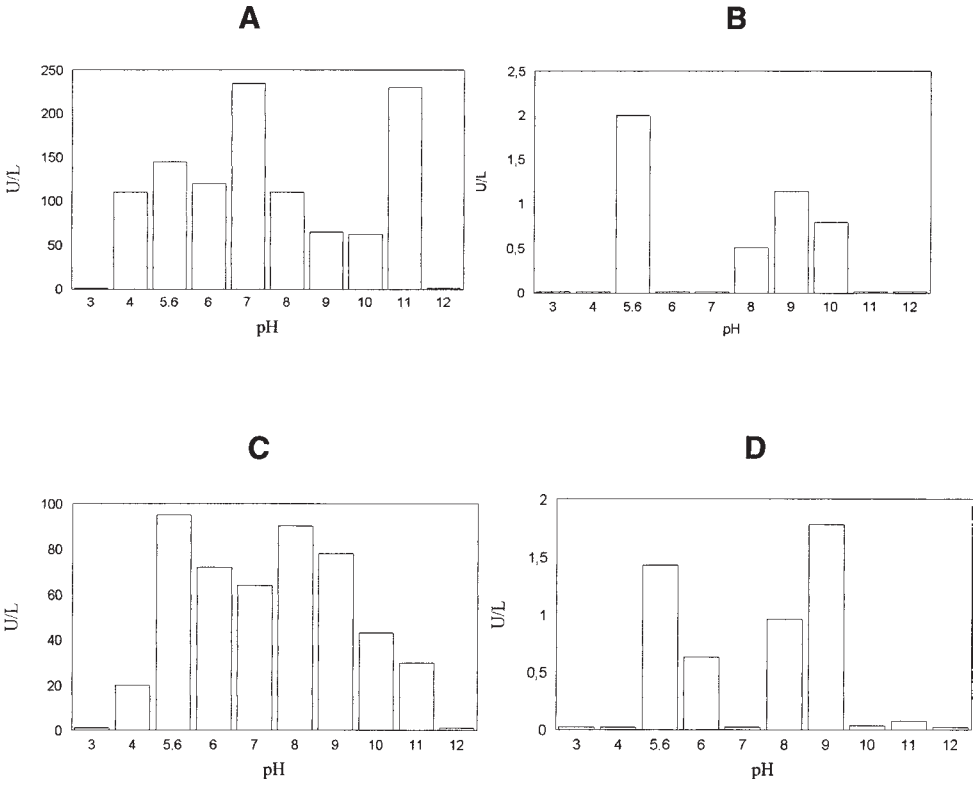

Fig. 2. Cellulolytic activity of *Streptomyces* M7a **(A)** CMCase and **(B)** FPase and *Streptomyces* M23 **(C)** CMCase and **(D)** FPase at 42°C and different pH values.

of 5.6–9.0 (64–95 U/L) and the highest FPase activities were observed at pH 5.6 and 9.0 (1.43 and 1.78 U/L, respectively). The overall analysis of the cellulolytic activities from both strains indicated that its cellulases are active on a wide pH range, from 5.6 to 11.0 with prevalence within the range of 5.6–9.0. Although the *Streptomyces* cellulases display activity below pH 7.0, they show a clear tendency to act under alkaline conditions.

Figure 3 presents enzyme kinetics for cellobiohydrolase (exocellulase) and endocellulase, measured through the release of 4-methylumbelliferone from fluorogenic conjugates, for M7a, M23, and *T. reesei*. Table 1 presents data on endocellulase- and exocellulase-specific activities for M7a, M23, and *T. reesei*, obtained by this method. Equivalent activity levels were observed for the enzyme preparations from strain M23 and a *T. reesei* commercial cellulase preparation. Cellulases from strain M7a are sevenfold more active than the ones of strain M23. No extracellular or cell-associated cellobiase activities were observed. In addition, no glucose was detected by HPTLC or using a glucose analyzer, in reaction mixtures containing cellobiose as substrate.

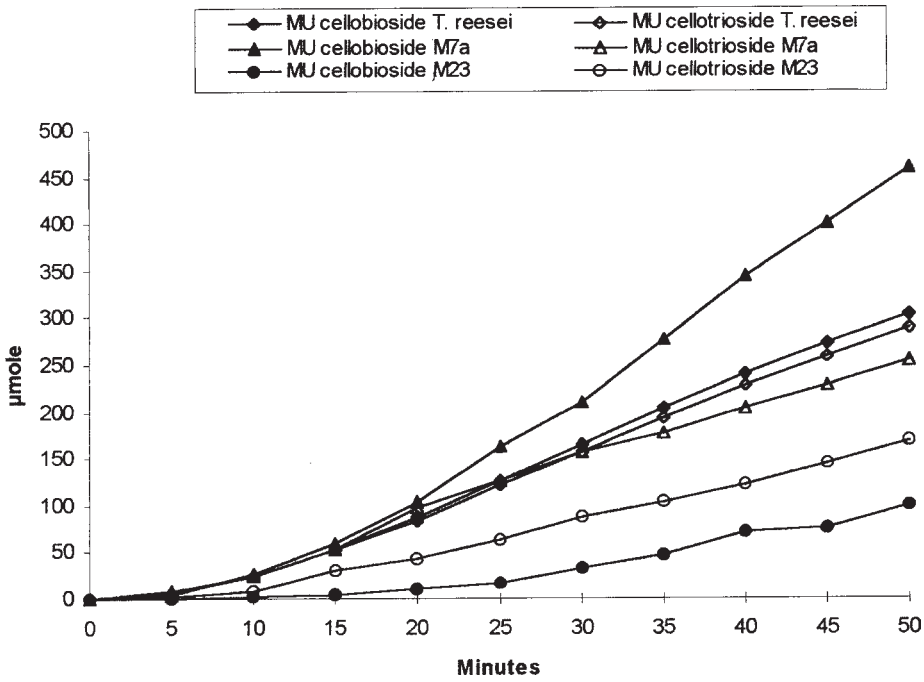

Fig. 3. Kinetics of MU cellobioside and MU cellotrioside hydrolysis on the action of cellobiohydrolase and endocellulase of *Streptomyces* M7a, *Streptomyces* M23, and *T. reesei*. For *T. reesei*, commercial cellulose was used. MU, methylumbelliferyl.

Table 1
Specific Activities of *Streptomyces* M7a, *Streptomyces* M23, and *T. reesei*

	Endocellulase activity (U/µg protein)	Exocellulase activity (U/µg protein)
Streptomyces M7a	1.70	1.80
Streptomyces M23	0.34	0.07
T. reesei	0.27	0.26

Discussion

The use of yeast extract (0.5%) in the culture medium *(15)*, for growth of cellulolytic actinomycetes in the present study, was of extreme importance for the detection of their enzymatic activities. Stutzenberger *(23)* has verified that the addition of yeast extract (0.1%) to the medium allowed growth and production of cellulases by the actinomycete *Thermomonospora curvata*. Several other investigators have also utilized yeast extract in the composition of culture media for the production of cellulases by actinomycetes *(15,24–26)*.

Similarly, the use of Tween-80 may have favored the production of extracellular cellulases. Walter and Schrempf *(8)* verified that the use of this

surfactant increases the production of extracellulases of the avicelase type, CMCase and *p*-nitrophenyl-β-D-cellobiosidase, up to 10 times in *Streptomyces reticuli*. This same effect has been previously observed in fungi such as *Caprinus cinereus (27)*. Tween-80 may interact with the cellular membrane and surface of insoluble substracts, altering the membrane permeability and allowing the liberation or solubilization of extracellular enzymes *(28)*.

Several actinomycetes cited in the literature have been able to produce cellulolytic enzymes, most of them belonging to the genus *Streptomyces*, such as *S. antibioticus (29)*, *S. flavogriseus (15)*, *S. lividans (30)*, *S. reticuli (31)*, and *S. higroscopicus (32)*. Most of the *Streptomyces* cellulases are active against amorphous or soluble cellulose (such as CMC), therefore being able to produce endoglucanases but unable to degrade microcrystalline cellulose, which demands the presence of exoglucanase. The main final product of both enzymes is cellobiose, which is the substrate for β-glucosidase. On the other hand, *S. reticuli (8,31)* and *S. higroscopicus (15)* produce endoglucanases, exoglucanases, and β-glucosidases, which allows the efficient and synergistic degradation of cellulose to glucose.

The strains M7a and M23 were able to degrade CMC (endo) and FP (endo and exo); however, they were not able to degrade cellobiose. Although 42°C has shown to be the optimum temperature for the enzymatic system of both strains, using either CMC or FP, the wide temperature range for enzyme activity (30–100°C) must be stressed. The tolerance of the cellulase system to high temperatures observed in both M7a and M23 is not usually found in the majority of actinomycete cellulases. Wachinger et al. *(33)* found for *S. reticuli* optima activities of the cellulase system in the range of 45–50°C, being extremely sensitive outside of this range. Ishaque and Kluepfel *(15)* found for *S. flavogriseus* optimum activity at 40°C for FPase and 50°C for CMCase, considerable decreases being shown below 35°C or above 50°C. *T. curvata* has presented a high cellulase optimum temperature (65°C), but at 55°C and 75°C its activity was reduced by one-third *(23)*. Strains that produce enzymes with tolerance to high temperatures are a fundamental importance in the treatment of municipal solid wastes *(34)* because activity at 65°C takes advantage of the maximum microbial thermophilic respiratory activity. Temperatures above 70°C are very restrictive with respect to microbial sources, since the spectrum of the organisms decreases rapidly with an increase in temperature *(35)*. Temperatures below 60°C are of no interest owing to the low hygienical efficiency. Thus, the utilization of thermotolerant cellulase systems would be a valuable option.

The cellulases produced by the *Streptomyces* strains that were isolated in this work were quite stable in the alkaline pH range. Alkaline enzymes, such as proteinases, lipases, and cellulases, have been used in the formulation of household detergents for the improvement of their efficiency. This allowed the reduction of the phosphate level in the conventional formulation of detergents, without decreasing their efficiency *(26)* and with environmental importance. The presence of alkaline cellulases in actino-

mycetes has been poorly studied. Horikoshi *(36)*, Park et al. *(37)*, and Damude et al. *(38)* have studied a semialkaline cellulase produced by a *Streptomyces* strain. Nakai et al. *(24)* have also isolated a *Streptomyces* strain with three types of cellulases, all having CMCase activity but only one with an optimum activity in an alkaline pH (8.5). Silva et al. *(26)* have isolated a *Streptomyces* sp. producing an extracellular constitutive alkaline CMCase, with optima activities at pH values of 6.0 and 7.0 at 35°C. At 55°C it was stable in the pH range of 5.0–9.0.

The low efficiency of the cellular systems, lacking β-glucosidase to degrade cellulosic materials, is probably owing to the strong inhibition produced by their final product, cellobiose. In nature, this problem is overcome by the presence of mixed populations producing the enzyme, and thus removing cellobiose from the environment. Several studies have shown that β-glucosidase activities produced by a variety of *Streptomyces* strains seem to be cell associated *(16,32)*. In this study, the presence of β-glucosidase activity extra- or intracellularly was not detected in both strains M7a and M23.

The use of fluorogenic conjugated substrates has confirmed the presence in both M7a and M23 of endo- and exoglucanase activities. $MU(Glc)_3$ and $MU(Glc)_2$ have been used, respectively, for these detections *(2,18)*. Although Van Tilbeurgh et al. *(18)*, among others, have claimed the lack of specificity of these kinds of substrates, they observed that endocellulase from *T. reesei* is not active against $MU(Glc)_2$, exocellulase activity being the only possibility, since cellobiase is not present. In our experiments, we have demonstrated that our strains have a cellulolytic activity against $MU(Glc)_3$ and $MU(Glc)_2$, possibly of the endo and exo types, respectively, which conclusively were nearly one order of magnitude greater than those obtained for commercial *T. reesei* cellulases, using the same conditions. In addition, their cellulolytic systems have the advantages of being thermotolerant and active in a wide pH range. Despite the absence of β-glucosidase, the cellulolytic systems we studied could be evaluated for applications that require only partial hydrolysis of cellulose.

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