

PRODUCTION OF XYLANASE AND CMCASE ON SOLID STATE FERMENTATION IN DIFFERENT RESIDUES BY *THERMOASCUS AURANTIACUS* MIEHE

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ABSTRACT

The use of waste as raw material is important for government economy and natural balance. The purpose of this work was to study the production of CMCase and xylanase by a Brazilian strain of *Thermoascus aurantiacus* in solid state fermentation (SSF) using different agricultural residues (wheat bran, sugarcane bagasse, orange bagasse, corncob, green grass, dried grass, sawdust and corn straw) as substrates without enrichment of the medium and characterize the crude enzymes. The study of the extracellular cellulolytic and hemicellulolytic enzymes showed that *T. aurantiacus* is more xylanolytic than cellulolytic. The highest levels of enzymes were produced in corncob, grasses and corn straw. All the enzymes were stable at room temperature by 24 h over a broad pH range (3.0-9.0) and also were stable at 60°C for 1 h. The optimum pH and temperature for xylanase and CMCase were 5.0-5.5 and 5.0 and 75°C, respectively. The microorganism grew quickly in stationary, simple and low cost medium. The secreted extracellular enzymes presented properties that match with those frequently required in industrial environment.

Key words: *Thermoascus aurantiacus*, xylanase, CMCase, solid state fermentation, agricultural residues

INTRODUCTION

The ability of some microorganisms to metabolize lignin and hemicelluloses make them potentially important to take advantage of vegetable residues. Agricultural and agro-industrial waste, like sugarcane bagasse (a fibrous residue of cane stalks left over after the crushing and extraction of the juice from the sugar cane) (23), wheat bran, rice peel, corn straw, corncob, fruit peels and seeds, effluents from paper industry and orange bagasse, have increased as result of industrialization, becoming a problem regarding space for disposal and environmental pollution. However, those residues represent an alternative source for the microbial growth aiming the production of biomass or enzymes.

Hemicelluloses and cellulose represent more than 50% of the dry weight of agricultural residues (7); they can be converted into soluble sugars either by acid or enzymatic hydrolysis, so they can be used as a plentiful and cheap source of renewable

energy in the world. For many residues xylan is the main component of the hemicellulose fraction. It is degraded by xylanases produced by fungus, bacteria, seaweed, protozoa, gastropod and arthropods. These enzymes also have application in maceration of vegetables, in clarification of juices and wines, in extraction of juices, scent and pigments, in biobleaching of pulp (15).

The complete degrading of cellulose by fungi is made by a cellulolytic enzyme system. The role and the action mechanism of the components of the system have been the center of many studies for the last 3 decades. It has been established that there are three main types of enzymes found in the cellulases system that can degrade the cellulose: exo- β -1,4-glucanase, endo- β -1,4-glucanase and β -glucosidase. Studies have shown that the endoglucanase act internally on the chain of cellulose cleaving β -linked bonds liberating non-reducing ends, and exoglucanases act removing cellobiose from this non-reducing end of cellulose

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chain. Finally, β -glucosidase completes the saccharification by splitting cellobiose and small cello-oligosaccharides into glucose molecule (2,7,16).

In some cases, the use of enzymes of mesophilic organisms can be a disadvantage, once they generally undergo denaturation in temperatures higher than 55°C, resulting in low efficiency of hydrolysis, demanding large quantities of enzyme and increased cost to conduct the hydrolysis under aseptic conditions. The employment of thermostable enzymes to carry out hydrolysis at higher temperatures is advantageous because it increases the speed of reaction and avoids microbial contamination contributing to increase technical and economical viability of the process (8).

Different strains of the *Thermoascus aurantiacus* fungus have been reported to be good producers of thermostable endoglucanase and/or xylanase (8-10,13-16,22,24-26), however there are few accounts of simultaneous production of CMCase and xylanase by this fungus on solid state fermentation.

The purpose of this work was to study the production of the endoglucanase CMCase (carboxymethyl cellulase) and xylanase of an isolated Brazilian strain of *Thermoascus aurantiacus* in solid state fermentation (SSF) using different agricultural residues as substrates without enrichment of the medium and characterize the crude enzymes.

MATERIALS AND METHODS

Organism and culture conditions

The microorganism *Thermoascus aurantiacus* Miede was isolated from decayed wood in Manaus, AM-Brazil (22). The fungus was sewed in tubes containing slanting Sabouraud agar and it was incubated for 5 days at 50°C. 5 mL of sterilized distilled water was added to the five day old culture and slightly scratched with a loop to obtain mycelia suspension. One mL of this suspension was used as inoculum and transferred to a 500 mL Erlenmeyer flask containing 20 g of solid medium (SM). The SM were prepared by mixing the 10 mL of sterilized water with 10g of one of the following residues: Wheat bran, sugarcane bagasse, orange bagasse (a fibrous residue of orange peel left over after the extraction of the juice from the orange fruit), corncob, green grass, dried grass, eucalyptus sawdust or corn straw following a manual homogenization. Residues were from agro industries and farms from region of São José do Rio Preto, SP. All the agricultural residues were washed, dried and powdered to a diameter of 2.0 mm. After 4 days (or as indicated) of incubation at 50°C, 100 mL of distilled water was added to the flasks and they were maintained under occasional shaking for 1 h at room temperature. The flask contents were filtered using Whatman no.1 filter paper. The clear filtrate was designed crude enzyme and used for assays of enzyme activities. Fermentation was carried out in duplicate and average values from duplicates are presented in this work.

Enzyme assays

The activities of xylanase, carboxymethyl cellulase (CMCase), filter paper activity and avicelase were assayed by incubating 0.1 mL of appropriately diluted enzyme (to make sure the activity is initial rate) with 0.9 mL of a solution containing 0.5% of the respective substrate, xylan (Birchwood - Sigma), carboxymethyl cellulose (Hércules, 7H35F), filter paper Whatman no. 1 and avicel (Merck), in 0.1 M acetate buffer, pH 5.5. After incubation at 60°C for 10 min the reducing substances released were assayed by 3,5-dinitrosalicylic acid (DNS) (18). Controls were prepared with 10 min boiled enzyme. One IU of activity toward the substrate mentioned above was defined as 1 μ mole of xylose (xylanase) or glucose (CMCase, filter paper activity and avicelase) equivalent released per minute under the above assay conditions, by using a xylose or glucose standard curve.

Optimum temperature and pH of the enzymes

To determine the optimum temperature and pH of enzymes the crude CMCase and xylanase activities were measured under standard assay conditions, except that temperature was ranging from 40 to 90°C, and the pH ranging from 2.0 to 8.0, respectively. The following 0.1 M buffer systems were used: McIlvaine (pH 2.0-8.0), sodium acetate (pH 4.0-5.5); Tris-/HCl (pH 8.0-/9.0). To test the heat stability of the enzymes, the crude CMCase and xylanase were incubated at various temperatures ranging from 40 to 80°C for 60 min. The reaction was stopped in ice-cold water and the remaining activity was measured under standard assay conditions. To check the pH stability, 1 mL of the sample solution was added to 9 mL of the buffer solutions at various pH values, ranging from 2.0 to 8.0 for 24 h at room temperature. The remaining activity was measured under standard assay conditions.

Enzymatic hydrolysis of CMC and xylan

10 mL of crude enzyme (0.5 U/mL) were incubated in 90 mL of a suspension of CMC or xylan in acetate buffer 0.1M, pH 5.5 in order to obtain a final concentration of 1% in substrate (including the volume of enzyme). At indicated intervals of time, samples were withdrawn and heated in a boiling water bath for 10 min to inactivate the enzyme, centrifuged at 10000 rpm and the extension of saccharification on supernatant was measured by quantifying the reducing substances using DNS (18). The rate of hydrolyze (%) was calculated regarding to an acid hydrolysis of the suspension with 1N H₂SO₄ under ebullition for 30 min. Under these conditions the reducing substances released were considered to be 100%.

RESULTS AND DISCUSSION

Effect of the growth temperature on enzyme production

Aiming at finding the best temperature for enzyme production, the fungus was grown in various temperatures ranging from 30 to 60°C. The *Thermoascus aurantiacus*, grew

and produced enzymes more efficiently at 50°C (Fig. 1). Similar results were reported by other authors regarding some thermophilic strains of fungus, such as *Humicola isolens* (12), *Humicola grisea* var. *thermoidea* (27). Gomes (8), reported the use of 47°C for the strain of *T. aurantiacus*, in liquid fermentation.

Effect of different nutrient sources on production of enzymes

The production of enzyme and other commercially important products by filamentous fungi in submerged fermentation have long been established. In recent years studies on solid state fermentation (SSF) have increased significantly (20). The choice of the kind of fermentation depends on the physiological adaptation of the organism. Generally, in submerged cultivation the growth form of filamentous fungi varies between pelleted and filamentous, each form having its own characteristics, which can affect the rate of enzyme production by influencing the mass transfer rate (20). While in submerged fermentation (SmF), the fungus is exposed to hydrodynamic forces, in SSF, growth is restricted to the surface of the solid matrix. SSF is defined as the culture in which a microorganism grows on a moist insoluble solid material in the absence or near absence of free water (1). Our isolated strain of *T. aurantiacus* showed a very low CMCase and xylanase activities when it was grown on liquid medium (data not shown). However, this fungus was a good producer of these enzymes on SSF. When SSF was used, any substrate produced more activity than in SmF. This may be due SSF provides the fungus with an environment closer to its natural habitat (wood and decayed organic matter), which stimulates this strain to produce more hemicellulolytic enzymes. As is shown in Table 1, the *T. aurantiacus* produced xylanase and CMCase independently of the material used as nutrient

Table 1. Effect of different substrate on enzyme production. After 4 days of incubation the clear filtrate was used for assays of enzyme activities. Fermentation was carried out in duplicate.

Nutrient sources	Avicelase (U/ml)	CMCase (U/mL)	Xylanase (U/mL)
Corn cob	0.86	60	107
Corn straw	0.65	59	97
Dried grass	0.65	60	99
Green grass	0.65	59	102
Orange bagasse	0.30	4	8
Eucalyptus Sawdust	0.30	9	37
Sugarcane bagasse	0.60	3	11
Wheat bran	1.50	30	64

source (as it was used only distilled water to moisten the substrate all the nutrients necessary to the growth of fungus came from the raw material used). Orange bagasse, sugar cane bagasse and sawdust were very poor substrate to the growth of the fungus, consequently the enzyme production on these substrates were very low too. Avicelase activities were significantly lower among all the lignocellulosic materials tested as carbon sources (Table 1). In this work, we did not supply any nutrients or saline solution to the growth medium, and only distilled water was used to humidify the solid substrates. In a previous work on pectinases production (17), a good growth on orange bagasse or sugar cane bagasse containing-medium was obtained when these media were supplied with nutrient solution, containing NH_4NO_3 , $\text{NH}_4\text{H}_2\text{PO}_4$ and MgSO_4 . It has been reported that thermophilic fungi require buffering agent (i.e., phosphate) for growth in solid medium (16). After the first 24 h of fermentation, the pH of the medium may drop and cease the growth of the fungi. The low pH of the medium reduces the solubility of CO_2 , which is important for the anaplerotic enzyme pyruvate carboxylase (11). The measurement of biomass on SSF on insoluble substrate is done by indirect approach (16), so in this work only visual growth of the fungus was done. Good growth and high production of xylanase were obtained from the medium containing corncob followed by green grass, dried grass, corn straw and wheat bran, respectively. Independent researchers have recognized corncob as a useful and cost-effective medium ingredient, because it is largely produced as a by-product during the corn processing (3,15,22). The cost of substrate should be considered for enzyme production (3). Grasses also have shown to be a good substrate for enzyme production. In spite of the duplicate used in this experiment, the level of fluctuation of detectable enzyme involved in such kind of experiment may be accepted as normal. Thus, the corncob, grasses, and corn straw are in the same level as good substrate for xylanase and CMCase production. The

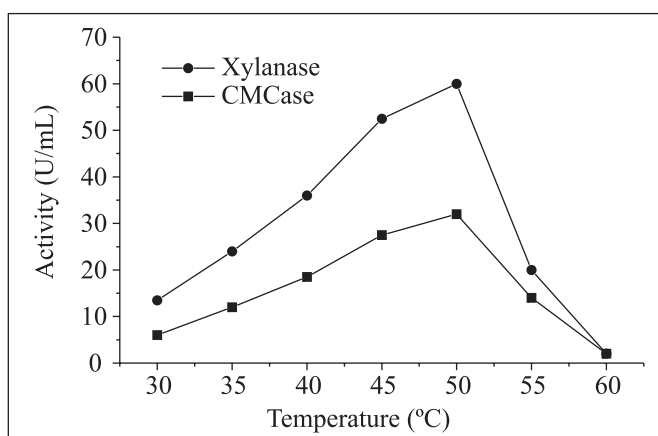


Figure 1. Effect of temperature of growth on enzyme production. After 4 days of incubation at the indicated temperature, the clear filtrate was used for assays of enzyme activities. Fermentation was carried out in duplicate.

difference in level of enzyme production by different raw material is determined by many factors involved in the process, such as the presence of activator or inhibitor, surface area, diffusion of catabolite, pretreatment, content and sugar composition of this substrate (3,8). In this study, despite of using different substrates the fixed time (4 days) was used, and is possible that the fungus might have a different lag phase, regarding to some of these substrates, in this case, if that substrate was sampled later a higher enzyme concentration might have been achieved. However, it was our interest to compare the different substrates with the wheat bran, which had produced enzymes in four days of fermentation (Fig. 2). Comparing those substrates named above with the largely used wheat bran, our results have shown that, for xylanase and CMCase production corncob, green or dried grass and corn straw showed higher production than wheat straw. The filtrated culture from this substrate is cleaner than that obtained from wheat bran. These properties are important for purification of enzymes since production of enzyme in wheat straw results in induction of various enzymes including protease, and pigment that make the purification process difficult. The study of the CMCase and xylanase enzymes of the *T. aurantiacus*, showed that the fungus is much more xylanolytic than cellulolytic. As the CMCase act only at amorphous region of cellulose (16) and there was a very low filter paper activity (less than 0.2 U for all tested substrates, data not shown) and a low avicelase activity in the enzymatic filtrate, its possible use in prebleaching of paper pulp would be an interesting subject to be assessed (4).

Time course of the xylanase and CMCase production in SSF

As shown in the Fig. 2, the fungus produced a high xylanase activity. The maximum production of xylanase and CMCase

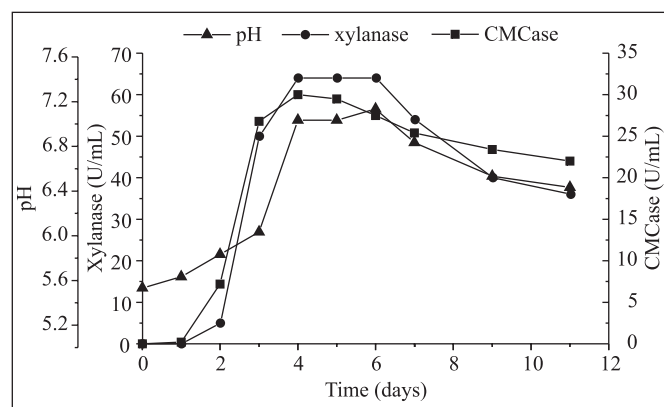


Figure 2. Time course of the xylanase and CMCase production by *T. aurantiacus* on SSF. After the indicated time of incubation the clear filtrate was used for assays of enzyme activities and pH. Fermentation was carried out in duplicate.

occurred on the 4th day. For xylanase, the maximum production extended until the 6th day, when then, started to decline; while the maximum production of CMCase was reached on the 5th day. The pH variation was also followed and its profile showed an increase, from 5.0 to 7.0 from the first to the 4th day, when it stabilized until 6th day and then, started to decline again.

Temperature and pH characteristics of the enzymes

Effect of pH in the activity and stability of crude enzymes: The results are shown in Fig. 3. Xylanase shown its optimum pH at 5.0-5.5 and its activity fell to 50% when pH ranged from 5.5 to 6.5 (Fig. 3-a). This optimum pH was a little higher than that reported by Gomes (8) to his strain of *T. aurantiacus*, which was pH 4.5. Fig. 3 (a) still shows that optimum pH for the crude CMCase activity is 5.5. However in pH 4.0 the activity was almost completely lost and in pH 7.0 the enzyme still retained 58% of its activity. The optimum pH found for the CMCase is comparable to those for the other *T. aurantiacus* strains (9,27). Results presented in Fig. 3 (b), show that xylanase was 100% stable within a wide range of pH, that is from pH 3.5 pH to 8.0.

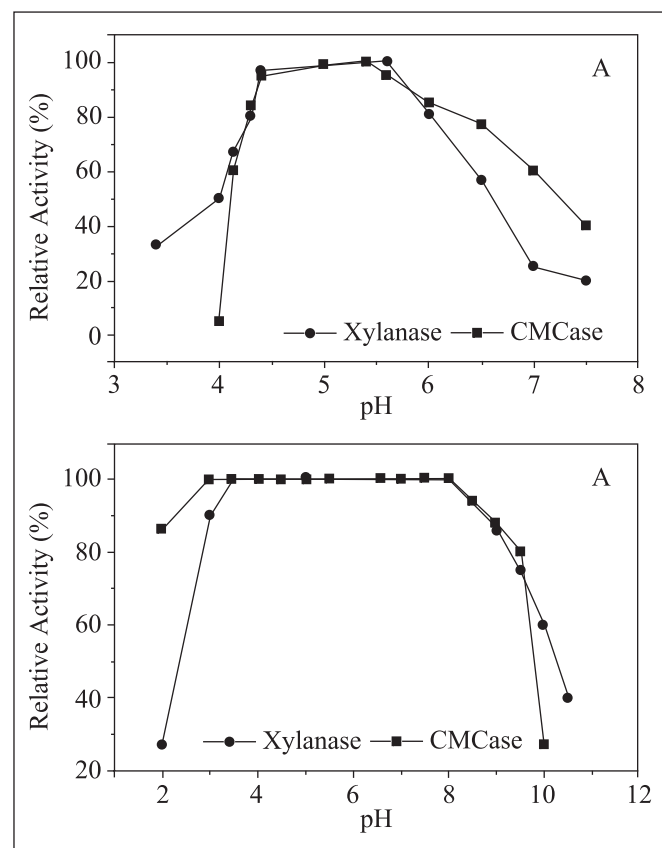


Figure 3. Optimum pH (A) and pH stability (B) of the crude enzyme.

Even at pH 2.5 and pH 10.0 the enzyme retained 62% and 60%, respectively, of the maximum activity after the treatment. This result for stability is close to the result obtained by Gomes (8), which reported stability over a broad pH range, exhibiting more than 80% of its total activity between pH 3.0 and 9.0. The results obtained with the crude CMCase show that the enzyme was 100% stable on a wide range of pH values, from 3.0 to 8.0. The crude enzyme still retained 85% of the maximum activity in pH 2.0 and 90% of the activity after the treatment at pH 9.0, but at pH 10.0 only 25% of the activity was recovered. Our result on pH stability has shown CMCase less stable than others related by Gomes (9) (i.e. 5 to 8) and Kawamori (14). The data have shown that the xylanases and CMCases were very stable to pH under the tested conditions, mainly in the pH range between 3.0 and 7.0, which account for the most frequent conditions in the industry.

Effect of the temperature in the activity and stability of crude enzymes: Fig. 4 (a), shows that the optimum temperature of crude xylanase was 75°C. By comparing this value with the available reports on activity of crude xylanase in literature, it was verified

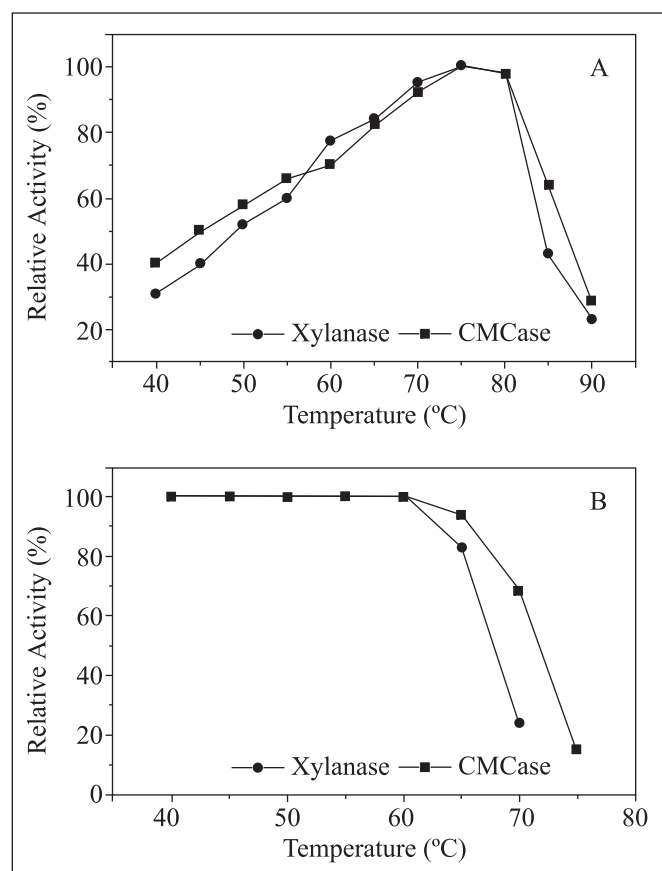


Figure 4. Optimum temperature (A) and thermostability (B) of the enzymes.

that *T. aurantiacus* under study has an optimum temperature for xylanase activity higher than other mesophilic fungus, i.e. 70°C for *T. terrestris* and *S. cellulophilum*, 55°C for *T. Reesei*, and 55°C for *Penicillium* sp (5). Our result for optimum temperature is smaller than that reported by Gomes, (8) for his strain of *T. aurantiacus*, which was at 80°C. The optimum temperature of the CMCase was 75°C and at 85°C the crude enzyme still had 60% of the original activity. The optimum temperature found for the CMCase is in good agreement with the reports for the other *T. aurantiacus* strains (9,14). Effect of the temperature in the stability of crude enzymes is shown in the Fig. 4 (b). Xylanase remained 100% stable until the treatment at 60°C, starting to undergo denaturation from this temperature, and at 70°C only 22% of the initial activity was recovered after the treatment. The results of the crude CMCase show that the enzyme was 100% stable to the treatment at 60°C and started to undergo denaturation from this temperature. At 70°C, 68% of the initial activity was still recovered after the treatment. Comparing with work of Mishra and Rao (19) all the enzymes presented good thermostability and xylanase of *Termonascus aurantiacus* was more thermostable than xylanase of *T. Reesei* QM 9414 (50°C for one hour) and that of *P. funiculosum* (50°C for 30 minutes) (19).

The enzymes of this strain of *T. aurantiacus* appeared to be as stable as other *T. aurantiacus* already reported, however the methodologies used in other works were different, making the comparisons difficult. It is accepted that conditions of growth and the nature of medium may stabilize the enzyme against thermal denaturation. The presence of protease induced by different composition of the medium may also affect the enzyme stability (8,9).

Determination of the hydrolysis of various glucan by the crude enzyme

The results are presented in Fig. 5. The temperature of saccharification was 60°C. Degradation of the different β -glucans by the crude enzyme confirm that many hydrolyzing enzymes were present in the filtrate. Arabinoxylan, xylan and CMC were hydrolyzed in an extension of 60, 60 and 30%, respectively, at the end of 55 hours. The hydrolysis of CMC, 30%, was not very different from published results of other authors (27) and only glucose appeared as a product in this hydrolyzed. This result was confirmed by paper chromatography, indicating the presence of β -glucosidase in the filtrate, which was confirmed by later analysis with PNPG substrate (data not presented in this paper).

Various hemicellulases that occur extensively in bacteria and mesophilic and thermophilic fungus, have been investigated regarding the bioconversion of agricultural biomass in fuels and in useful chemical products (2,4,7,15). An extensive revision on the production of cellulases and xylanases for microorganisms is found in the work of Elisashvili (6). Wojtczak *et al.* (26) carried out a comparative study of thermostability of

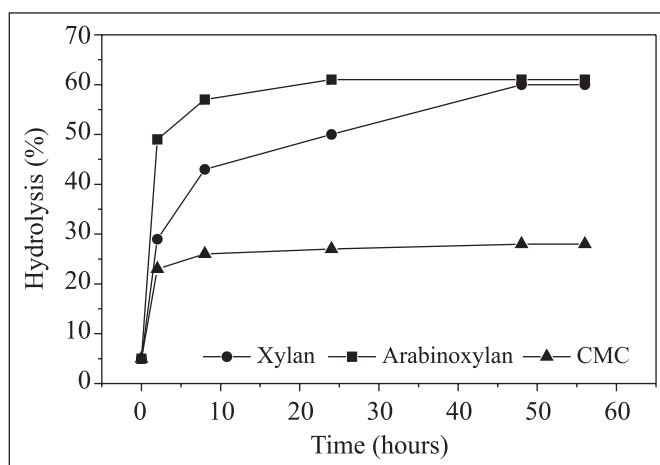


Figure 5. Hydrolysis curves of different glucans by crude enzymes. Aliquots were removed at times 0, 2, 8, 24, 48 and 56 hours. Hydrolysis were carried out in duplicate.

cellulases of various thermophilic and mesophilic fungi. The results show that despite the high temperature growth of the thermophilic, not all presented optimum temperatures of activities higher than of the mesophilic fungus.

Different strains of *Thermoascus aurantiacus* had been said to be good producers of endoglucanase and/or xylanases (8,9,13-16,22,24-26). Many of these reports were in SmF and/or using optimized nutrient and fermentation condition. However, there isn't a complete report on simultaneous production of endoglucanase and xylanase in SSF in wastes by this fungus. Many of the investigations on SSF named in literature are using mesophilic microorganism. The problem in the use of enzymes from mesophilic organisms is that they generally undergo denaturation in temperatures higher than 55°C, resulting in low efficiency of hydrolysis, in the requirement of large amount of enzyme and increased cost to lead hydrolysis under aseptic conditions (5). The use of thermostable enzymes to carry out hydrolysis at higher temperatures is advantageous because it increases the reaction speed and it prevents microbial contamination thus contributing to increase the economical and technical viability of the process (5,8). In this context, despite of a direct comparison under identical conditions, was not performed, the thermostability of CMCase and xylanase produced by this strain of *T. aurantiacus*, appeared to be superior to some others thermophilic fungi already described (3,5,16,21). High thermostability of crude enzyme might be attributed to the kind of fermentation process, presence of substrate, cofactors, salts and extra proteins in the medium or different geographical origins (9,16).

The fungus *T. aurantiacus* can be cultivated in various types of culture mediums of low cost for the metabolic

attainment desired (8,9,25). Although various groups of known lignocellulolytic microorganisms have presented its enzymatic systems well characterized and some of them are already used in industrial scale, worldwide researches have demonstrated that the activities of new isolate have been comparable or superior to the traditional strains. Nature represents an interminable source of xylanolytics and cellulolytics microorganisms and especially tropical countries as Brazil, which presents a very diversified microbial flora, certainly shelters species of unknown microorganisms of optimum industrial interest. In this context, the exploration of new isolated strains is very important to provide microorganism with properties that match with the conditions existing in the industrial environment.

CONCLUSION

The thermophilic fungus *T. aurantiacus* was submitted to SSF using different residues as raw material. Maximum yield of xylanase was obtained from the medium containing corncob followed by green grass, dried grass, corn straw and wheat bran, respectively. Independent researchers have recognized corncob as a useful and cost-effective medium ingredient, because it is largely produced as a by-product during the corn processing. The study of the extracellular hemicellulolytic enzymes of the *T. aurantiacus*, showed that the fungus is much more xylanolytic than cellulolytic one. All the enzymes were stable over a broad pH range and temperature. Finally, the microorganism is promising for industrial application since it grows quickly in stationary condition in simple and of low cost substrates and secretes the enzymes extracellularly and its set of enzymes displayed properties that match those frequently required for industrial application.

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RESUMO

Produção de xilanase e CMCase por fermentação em estado sólido em diferentes resíduos pelo fungo termofílico *Thermoascus aurantiacus* miehe

O emprego de resíduos como matéria prima é importante como estratégia governamental e para o balanço ambiental. O propósito deste trabalho foi estudar a produção de CMCase e xilanase de uma linhagem de *Thermoascus aurantiacus* isolado de solo brasileiro em fermentação em estado sólido (SSF) usando diferentes resíduos agrícolas (farelo de trigo, bagaço de cana, bagaço de laranja, sabugo de milho, grama verde, grama

seca, serragem de eucalipto e palha de milho) como substratos sem enriquecimento dos meios e caracterizar as enzimas. O estudo das enzimas hemicelulolíticas extracelulares mostrou que o fungo *T. aurantiacus* é mais xilanolítico do que celulolítico. Ele produziu maiores níveis das enzimas em meios contendo sabugo de milho, grama e palha de milho. Todas as enzimas foram estáveis por 24 h à temperatura ambiente numa ampla faixa de pH (3,0 – 9,0) e também foram estáveis a 60°C por 1 h. O pH ótimo e temperatura ótima para xilanase e CMCCase foram 5,0 – 5,5 e 5,0 e 75°C, respectivamente. O microrganismo cresceu muito bem estacionariamente no meio simples, de baixo custo. As enzimas estáveis secretadas extracelularmente apresentam as características necessárias para sua aplicação industrial.

Palavras-chave: *Thermoascus aurantiacus*, xilanase, CMCCase, fermentação em estado sólido, resíduos agrícolas

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