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Holocellulase Activity from *Schizophyllum commune* Grown on Bamboo: A Comparison with Different Substrates

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Abstract The natural biodiversity that is found in tropical areas offers countless biotechnological opportunities; especially if we take in account that many biomolecules from several microorganisms have supported for many years, different industrial applications in areas such as pharmacology, agro-industry, bioprocess, environmental technology, and bioconversion. In order to find new lignocellulolytic enzymes and evaluate bamboo fibers as substrate, *Schizophyllum commune* a fungus with broad distribution was isolated and grown during 15 days in liquid culture medium containing 1% lignocellulosic fibers from bamboo, banana stem, and sugarcane bagasse. The enzymatic activity of xylanase, mannanase, polygalacturonase, CMCase, FPase, and avicelase were evaluated. Sugarcane bagasse and banana stem showed to induce higher hollocellulase activity when compared with bamboo as the main carbon source. The physical mechanism that the fungus uses to degrade bamboo was observed not only in fibers naturally infected but also in healthy fibers that were treated and untreated with enzyme solution. SEM analysis showed the structural disruption and invasion of the vascular bundles, parenchyma cells, and parenchymatous tissues as a consequence of the presence of this fungus and the catalytic action of its enzymes into the plant tissue.

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Introduction

During past years, lignocellulosic materials have gained attention as a renewable, abundant, and economical material in order to obtain new valuable chemicals, new materials, and as a source for biofuel production [22, 29]. In this context, the improvement of physical, chemical, and biotechnology tools should be rapidly addressed to process and use the big amount of agro-industrial wastes that are produced worldwide. In addition to the high economic value that can be obtained from some specific products (enzymes, single cell proteins, pigments, antibiotics, etc.), the use of agro-products has a positive impact on the preservation of environmental quality, considering the development of technologies geared toward sustainable transformation of natural resources [9].

Because fungi are known to produce significant amount of cellulolytic enzymes, they have been studied for different industrial applications in areas as pharmacology, agro-industry, bioprocess, environmental technology, and bioconversion. Conversion of lignocellulosic biomass involves a combination of numerous enzyme-catalyzed hydrolytic reactions that allow the natural conversion of cellulose and hemicellulose to fermentable sugars [4]. For this reason, biodiversity studies focused on different aspects related with the hydrolytic process of lignocellulosic biomass such as: microorganism as source of cellulolytic enzymes and the substrates that could be degraded; could allow us to find new potential findings to be used in order to improve some biotechnological process of mass conversion.

The complete hydrolysis of holocellulose requires the catalytic action of different enzymes acting on particular substrates as it has been reported in previous studies involving filamentous fungi [20, 27] and Basidiomycetes [26]. *Schizophyllum commune,* a fungus with broad distribution and mold of Phylum Basidiomycota, Schizophyllaceae family, which colonizes diverse trees and rotting wood including bamboo, *Guadua angustifolia* [7], was used in this study. Although, agricultural crops such as corn, wheat, rice, sugarcane, sugar beet, potatoes, among others, are mainly processed into starch and sugar refineries, they are also useful not only as major substrates for the food industry, but also for most industrial fermentation processes and some other chemical processes [16]. This process can convert agricultural raw materials in a variety of valuable chemicals, including biofuels, and organic solvents such as butanol [10, 28]. *Schizophyllum* sp. is not only widely distributed but also an important fungal agent for its role in the decomposition of lignocellulosic residues [11]. Several studies have found that high concentrations of nitrogen during cell culturing of white rot fungi are essential for optimum enzyme production [6, 30].

The biotechnology, through enzymatic technology, offer the possibility of bioconversion of new agro-industrial wastes such as straw, bran, fruit peels, corn husks, sugarcane bagasse, wood wastes, among others, which are rich lignocellulosic materials that are being undervalued like the bamboo fibers that could be appropriate alternatives as a new energy sources [32, 34].

In this article, we presented the holocellulase activity (enzymes that show activity against holocellulose as xylanase, mannanase, polygalacturonase, endoglucanase, and exoglucanase) from *S. commune* fungus produced on different lignocellulosic fibers (banana stem, sugarcane bagasse, and bamboo). By using scanning electron microscopy (SEM), the physical mechanism that *S. commune* uses to degrade bamboo and the enzymatic effect on tissues were observed.

Materials and Methods

Substrate

Bamboo (*G. angustifolia*) fibers, sugarcane bagasse and banana stem were collected from a local farm around Brasilia—DF (Brazil). These plant materials were ground and autoclaved at 121°C for 20 min. After autoclaving, they were mixed with the liquid cultures to form a homogeneous blend ready for enzymatic hydrolysis experiments. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Culture Conditions

A *Schizophyllum commune* wild strain was collected in the locality of Santágueda, Caldas-Colombia (5° 05′N–75° 40′W, Altitude: 1010 masl, Temperature: 23.5°C, Relative humidity: 71%) and was replicated on Sabouraud dextrose agar (SDA) medium. The inocula were prepared by adding two pieces (1 cm^2) of an active growing culture into 250 ml Erlenmeyer flask containing 50 ml of the liquid culture medium (peptone 10 g/l, D-glucose 20 g/l, and chloramphenicol 0.05 g/l) at pH 7.0 [17]. The culture medium was also supplemented with sterilized 1% (w/v) healthy-bamboo fibers, 1% (w/v) banana stem and 1–15% (w/v) sugarcane bagasse. All treatments were incubated by using rotational agitation 150 rpm at 37°C, excepted the experiment with different concentrations of sugarcane bagasse that was maintained without agitation. After 15-day of growth, all cultures were clarified by centrifugation at 10,000×*g* during 30 min. The supernatant was then concentrated by ultrafiltration using an Amicon System fitted with 300 kDa cut-off-point membranes [26]. The resulting concentrate was kept at −80°C and then used for enzymatic assays.

Enzymatic Assays

Carboxymethyl cellulase (CMCase), xylanase, polygalacturonase, and mannanase activities were determined by using the dinitrosalicylic acid (DNS) method by measuring the amount of reducing sugars that were released during the enzymatic reaction at 540 nm using a Unicam UV2 spectrophotometer [21]. In brief, 50 µl of enzyme solution was mixed with 100 µl of 1% (w/v) CMC, oat spelt xylan or pectin and 0.5% (w/v) galactomannan at 50°C for 30 min, respectively. Enzymatic activity on filter paper (FPase) was determined by DNS method, incubating 150 µl of enzyme extract with filter paper (Whatman N°1) as the substrate at 50°C for 1 h [18]. Avicelase activity was determined by DNS method, mixing 50 µl of microcrystalline cellulose suspension (1% w/v) with 100 µl of enzyme

sample at 50°C for 2 h. The enzymatic activity was expressed as μmol of reducing sugar released per min per ml of enzyme solution, i.e., as IU ml^{-1}. Glucose, xylose, mannose and galacturonic acid were used as standards. The protein content of the enzyme solutions was determined by the Bradford's method, using the BioRad protein assay with bovine serum albumin (BSA) as standard [5]. For all assays, triplicate reactions were performed separately.

Scanning Electronic Microscopy

The physical mechanism that *S. commune* used to decompose bamboo was observed by SEM, using naturally infected fragments of bamboo showing different stages of fungal growth. In addition, healthy-bamboo fibers were treated by immersion into an enzyme cocktail from a liquid medium obtained after 15-day of fungal growth, and maintained during 15 days at 50°C and relative humidity of 80% to allow the hydrolysis process. Appropriate control with fibers immersed in liquid extract without fungal inoculation was used. Bamboo fragments and fibers were washed, fixed with acetaldehyde, dehydrated using grade ethanol series, and metalized with gold particles by using vacuum. Thereafter, a topographic visualization by using a JEOL JSM-S 5000 equipment (JEOL, Tokyo—Japan) with a 10 kV potential was performed.

Results and Discussion

Hollocellulase Activity

The maximum enzymatic activities of xylanase, pectinase, CMCase, FPase, and avicelase were obtained using sugarcane bagasse as carbon source (Fig. 1). The higher mannanase activity was found using banana stem as carbon source. With exception of pectinase all enzymes were produced in lower amounts when bamboo was used as substrate. Previous experimental reports have shown that some substrates or heavy metals can generate physiological and reproduction changes on basidiomycetes as *S. commune*, seriously affecting not only its ability to colonize the plant tissue but also affecting the activity of endogenous cellulolytic enzymes involved in the infection process [3]. It is not clear yet, how much of these specific metals are present in bamboo tissue or how those compounds could affect the development of this fungus and its natural ability to degrade cellulolytic material. Additional work must be addressed in order to get a better understanding of this process. Haltrich et al. [12] did optimize the enzymatic activity of *S. commune* fungus grown on cultures containing microcrystalline cellulose as a carbon source. The enzymatic package of this fungus has the complete capacity to decompose lignocellulosic residues, exhibiting interesting properties to be used in enzymatic treatments [24, 25, 33]. The growth of *S. commune* on culture containing cellulose as carbon source increased the enzymatic activity of cellulase, xylanase, and mannanase. In contrast, pure xylan did not induce xylanase or mannanase activities [13].

It is clear that sugarcane bagasse induces higher enzymatic activities of the main enzymes assayed (Fig. 1). The results suggest a direct correlation between sugarcane bagasse concentration and the production of pectinases and avicelase while no correlation was observed with xylanases, mannanase, FPases, and CMCase activity (Fig. 2). Likewise, other studies with *Agaricus brasiliensis* and *Pleurotus sajor-caju* grown on different concentrations of lignocellulosic carbon sources, such as sugarcane bagasse, banana stem, and cotton waste (textile industry) showed more xylanase and pectinase activity [25]. Bailey et al. [2] demonstrated that xylanases production from *Trichoderma reesei* was increased when the fungus was grown by using a medium containing cellulose and xylan as the main carbon sources. In addition, the mesophilic fungus *Penicillium corylophilum* produced highest holocellulase activities when it was grown in the presence of dirty cotton residue [27]. Under different carbon sources, *Trichoderma harzianum* showed a higher endoglucanase activity when compared to avicelase and β-glucosidase [1].

In spite of the fact that both banana stem and bamboo have higher concentration of cellulose, hemicellulose and lignin (cellulose: 52.9 and 46.8%; hemicellulose: 17.4 and 25.8%; lignin: 9.4 and 23.9%, respectively) [27, 32] than sugarcane bagasse (cellulose: 34.6%; hemicellulose: 16.2%; lignin: 5.1%) [27], the total cellulase production by the fungus on bagasse was higher when compared to the others. It has been shown that the activity of enzymes that participate during the infection process is mediated by induction factors and regulated by both environment conditions [6] and genetic factors [19]. Also, this activity is in part differentiated by the constitution of the host material defining the host-pathogen interaction that could occur [12]. The low enzymatic activity observed when only bamboo was used as supplement could be explained, due to the fact that this is a more complex plant material and that the infection process and final enzymatic hydrolysis depend directly of variables such as: substrate features, enzyme production, enzymatic synergy and inhibition [33]. It is clear that hydrolytic enzymes play an important role during the final degradation process of biological residues [8]. Nevertheless, the presence of higher concentrations of lignin into the structure affects directly the complete decomposition process by reducing the holocellulase activity [4].

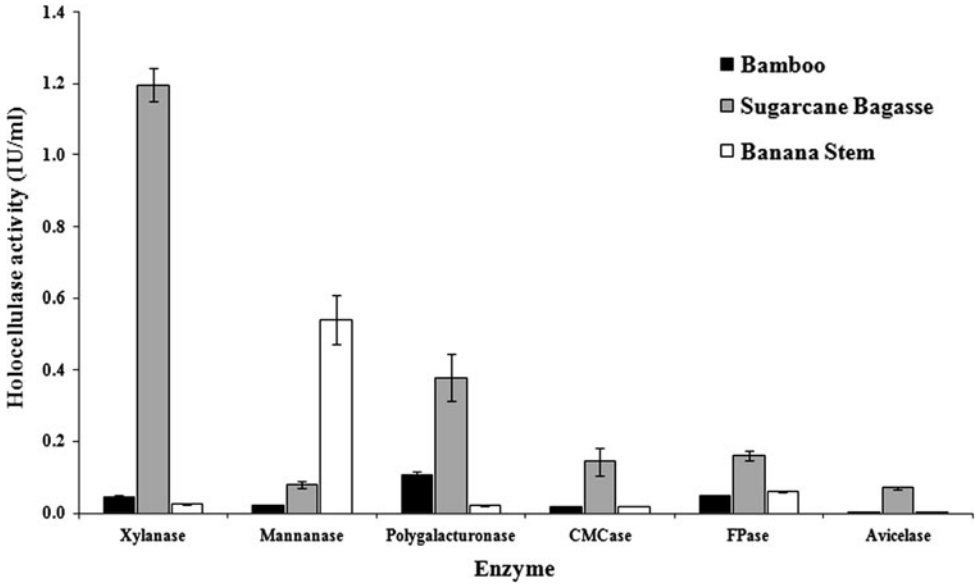

Fig. 1 Holocellulase activity of the enzyme solution from *S. commune* on different carbon source in submerged cultivation for 15 days

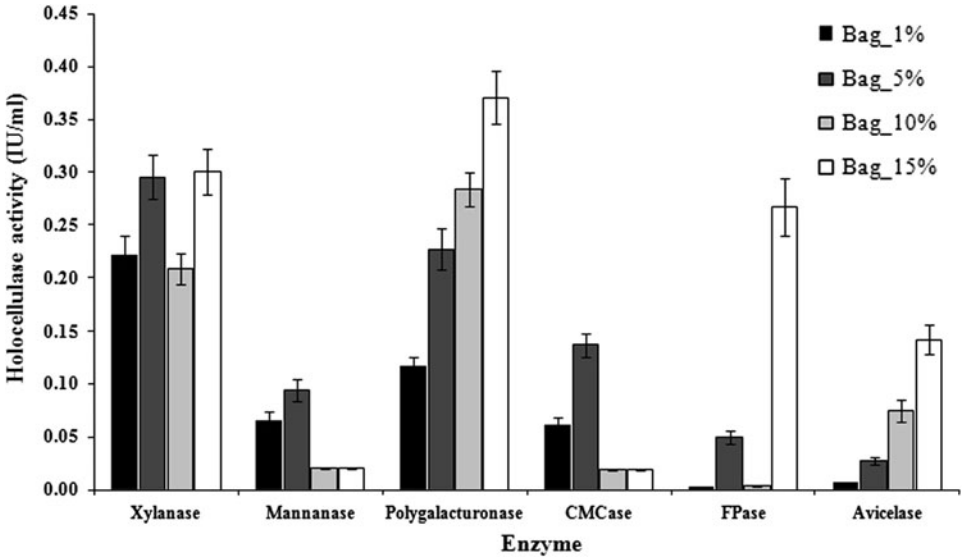

Fig. 2 Holocellulase activity of the enzyme solution by *S. commune* grown on different concentration of the sugarcane bagasse carbon source in submerged cultivation for 15 days

Scanning Electronic Microscopy

Bamboo is a cheap and fast-grown resource that has been widely used in homes building and for numerous industrial applications (household products, paper industries, chemical industries, energy) [32]. Few studies have been focused for evaluating the infection process and cellulose degradation in this lignocellulosic material. For this reason, bamboo was selected as starting material to visualize the physical mechanisms that take place during the degradation of healthy-bamboo fibers by cellulolytic enzymes from *S. commune*. Structural disruption of the bamboo fibers was clearly observed after hydrolysis treatment (Fig. 3). It is important to point out that the complementary action of both, physical mechanisms, and enzymatic activity of cellulases and xylanases play a key role in the degradation of several lignocellulosic materials as observed in previous studies [13]. It is also important to mention that the composition of *G. angustifolia* (bamboo) is 51% parenchyma, 40% fiber, and 9% of conductive tissues [32], useful to improve any bioconversion processes that includes this plant material. Previous studies of chemical characterization and morphology were initiated in the 1970s, in order to get a better understanding of the physical mechanisms that *S. commune* uses during the infection process [31].

As soon as the fungus penetrates the plant tissue, it will produce a full spread of hyphae on the tissue, which is always facilitated by the synergistic action of digestive enzymes [31] that allows the fungus to colonize the parenchyma through natural holes located in the parenchyma of the bamboo internode (Fig. 4b), generating disruption of parenchyma cells and parenchymatous ground

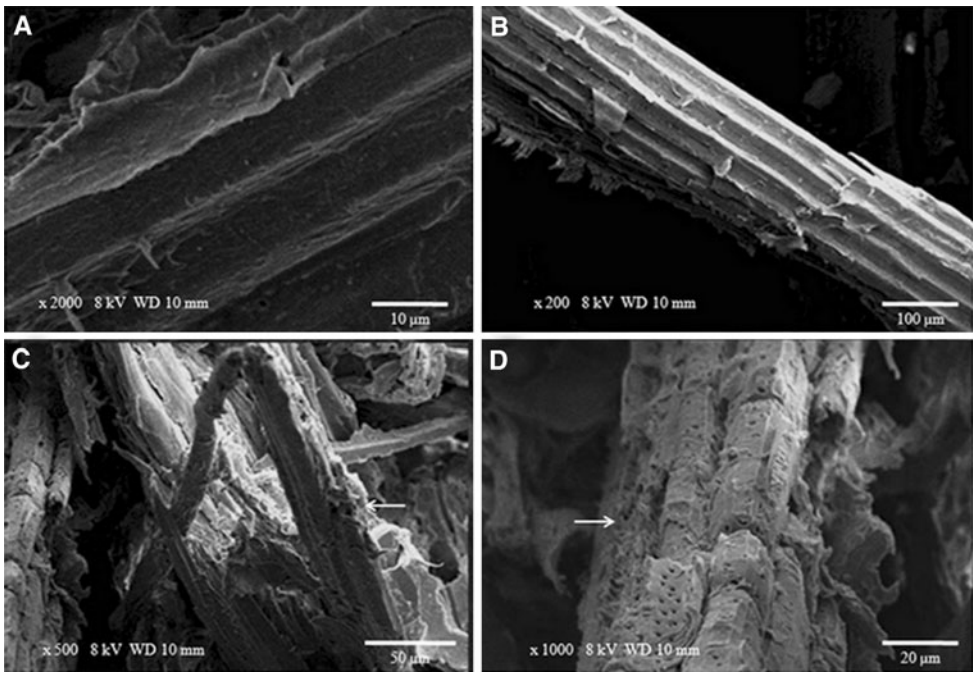

Fig. 3 SEM topographical image. **a**, **b** Control, fibers immersed in liquid extract without fungal inoculation. **c**, **d** healthy-bamboo fibers treated by immersion into an enzyme cocktail from a liquid medium obtained after 15-day of fungal growth, and maintained during 15 days at 50°C. Degradation localized in the tissues after hydrolysis treatment with an enzyme cocktail (*white arrows*)

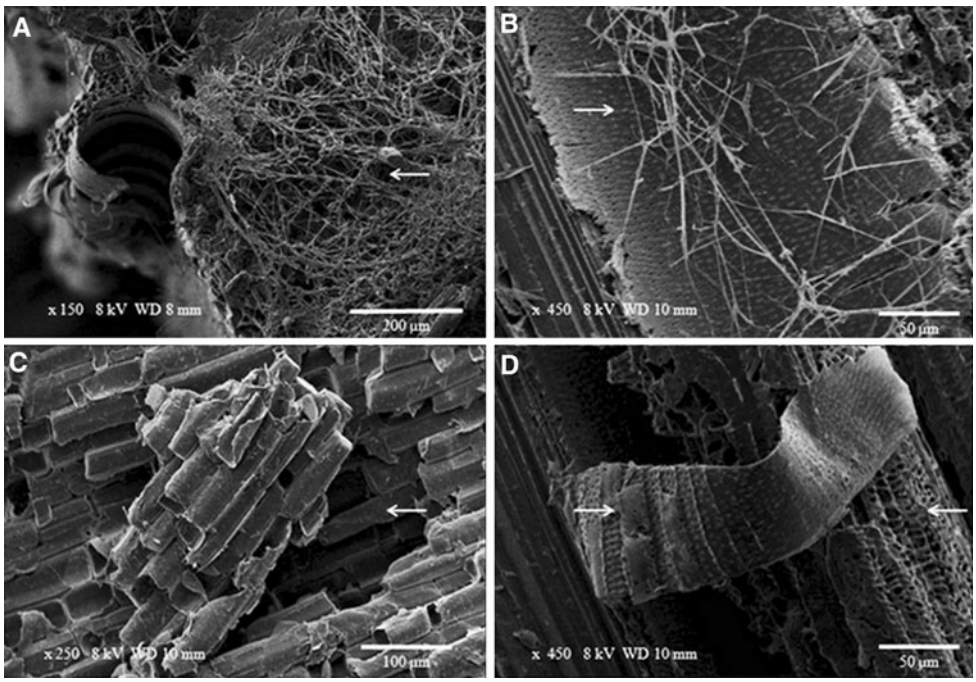

Fig. 4 SEM topographical image from naturally infected fragments of bamboo showing different stages of fungal growth. **a** Carpophore initial phase. Primary fruiting body formation (*white arrow*). **b** Infection of the bamboo vascular bundles. Spread of mycelia (*white arrow*). **c**, **d** Disruption of bamboo parenchyma cells and parenchymatous ground tissues (*white arrows*)

tissues (Fig. 4c, d). The infection process continues with the formation of fruiting bodies, typical carpophores, at the level of vascular bundle structure as described for *Schizophyllum* sp. [15] (Fig. 4a). Other authors have also established the complete infection process that occurs during infection by basidiomycetes fungi [14].

Enzymes such as laccases allow depolymerizing aromatics compounds exposing hemicelluloses and cellulose chains to the action of enzymes such as xylanases, pectinases, avicelases, which can release short polymers which are suitable for the enzymatic action of endoglucanases and glucanases [26]; by this way the laccases facilitate the access to polysaccharides by the holocellulases. However, it has been shown that some isolates of endophytic fungi have the ability to develop non-selective processes of decomposition patterns similar to those described above, on host plants [23].

The exact mechanism underlying the decay patterns observed in infected bamboo samples is still unclear. It is possible that the degradation mechanisms in bamboo,

resulting from attack by *S. commune*, are related to the action of both, physical and enzymatic process in order to achieve the complete degradation of bamboo fibers.

Conclusions

Schizophyllum commune has potential to be an important source of catalytic enzymes for the degradation of lignocellulosic materials, in order to produce a second generation of biofuels. It is necessary to explore our biodiversity and also to characterize the most important biomolecules that are produced by wild microorganisms and its role in biomass degradation, in order to get access to a new available energy sources. Because sugarcane bagasse and banana stem have showed to induce a higher hollocellulase activity, those plant-materials could be considered in future research activities focused on production of cellulolytic enzymes. However, the composition, availability, and worldwide offers of bamboo, requires further and additional studies to be considered as a potential lignocellulosic material for bioconversion which will require previous pretreatments before enzymatic treatment. The structural disruption of the bamboo tissues is clearly visible by using SEM on bamboo fragments naturally infected by *S. commune* and bamboo fibers partially hydrolyzed with enzyme solutions from cell-culturing.

In addition, it is necessary to get a better understanding of the biodegradation process of this kind of lignocellulosic materials in order to transform this abundant and cheap material in several added-value products.

References

1. Aslam N, Munir A, Shiekh MA et al (2010) Expression pattern of Trichoderma cellulases under different carbon sources. Pak J Bot 42:2895–2902
2. Bailey MJ, Buchert J, Viikari L (1993) Effect of pH on production of xylanase by *Trichoderma reesei* on xylan- and cellulose-based media. Appl Microbiol Biot 40:224–229
3. Baldrian P (2003) Interactions of heavy metals with white-rot fungi. Enz Microb Tech 32:78–91
4. Berlin A, Balakshin M, Gilkes N ((2006)) Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198–209
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
6. Cavallazzi JR, Kasuya CM, Soares MA (2005) Screening for inducers for laccase production by *Lentinula edodes* in liquid medium. Braz J Microbiol 36:1517–8382
7. Chiu SC, Tzean SS (1995) Glucanolytic enzyme production by *Schizophyllum commune* Fr during mycoparasitism. Physiol Mol Plant P 46:83–94
8. Coughlan M (1991) Mechanisms of cellulose degradation by fungi and bacteria. Anim Feed Sci Tech 32:77–100
9. Dahod SK (1999) Raw materials selection and medium development for industrial fermentation processes. In: Demain AL, Davies JE (Eds) Manual of industrial microbiology and biotechnology, ASM Press, Washington, p 213–220
10. Demain AL (2000) Small bugs, big business: the economic power of the microbe. Biotechnol Adv 18:499–514
11. Fang J, Liu W, Gao PJ (1998) Cellobiose dehydrogenase from *Schizophyllum commune*: purification and study of some catalytic, inactivation, and cellulose-binding properties. Arch Biochem Biophys 353:37–46
12. Haltrich D, Preiss M, Steiner W (1993) Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. Enz Microb Tech 15:854–860
13. Haltrich D, Steiner W (1994) Formation of xylanase by *Schizophyllum commune*: effect of medium components. Enz Microb Tech 16:229–235
14. Hernández E, Garcia C, Novaes-Ledieu M (1990) Chemical characterization of the hyphal walls of the basidiomycete *Armillaria mellea*. Exp Mycol 14:178–183
15. Horton JS, Bakkeren G, Klosterman SJ (2005) Genetics of morphogenesis in basidiomycetes. Appl Mycol Biotech 5:353–422
16. Kamm B, Kamm M (2004) Principles of biorefineries. Appl Microbiol Biot 64:137–145
17. Kolenová K, Vršanská M, Biely P (2005) Purification and characterization of two minor endo-β-1,4-xylanases of *Schizophyllum commune*. Enzyme Microb Tech 36:903–910
18. Mandels M, Andreotii R, Roche C (1976) Measurement of saccharifying cellulase. Biotechnol Bioeng 16:21–33
19. Martínez D, Larrondo LF, Putman N et al (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22:695–700
20. Martins LF, Kolling D, Camassola M et al (2008) Comparison of *Penicillium echinulatum* and *Trichoderma reesei* cellulases in relation to their activity against various cellulosic substrates. Bioresource Technol 99:1417–1424
21. Miller G (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
22. Moldes AB, Cruz JM, Domínguez JM et al (2002) Production of a cellulosic substrate susceptible to enzymatic hydrolysis from prehydrolyzed barley husks. Agr Food Sci 11:51–58
23. Oses R, Valenzuela S, Freer V (2006) Evaluation of fungal endophytes for lignocellulolytic enzyme production and wood biodegradation. Int Biodeter Biodegr 57:129–135
24. Robinson T, Singh D, Nigam P (2001) Solid-state fermentation: a promising microbial technology for secondary metabolite production. Appl Microbiol Biot 55:284–289
25. Siqueira F (2010) Resíduos Agroindustriais com Potencial para a Produção de Holocelulases de Origem Fúngica e Aplicações Biotecnológicas de Hidrolases. Universidade de Brasília, Tese de Doutorado
26. Siqueira FG, Siqueira AG, Siqueira EG et al (2010) Evaluation of holocellulase production by plant-degrading fungi grown on agro-industrial residues. Biodegration 21(5): 815–824
27. Siqueira FG, Siqueira EG, Jaramillo PM et al (2010) The potential of agro-industrial residues for production of holocellulase from filamentous fungi. Int Biodeter Biodegr 64:20–26
28. Soetaert W, Vandamme EJ (2009) Biofuels. In: Stevens C (Ed) Renewable resources series. John Wiley & Sons Ltd., New York, p 242
29. Sreenath HK, Koegel RG, Moldes AB et al (2001) Lactic acid production by simultaneous saccharification and fermentation of alfalfa fiber. J Biosci Bioeng 92:518–523
30. Srinivasan C, D′souza TM, Boominathan K et al (2005) Demonstration of laccase in the white rot Bacidiomycete *Phanerochaete chrysosporium* BKM-F1765. Appl Environ Microb 61: 4274–4277

31. Wessels JG, Kreger DR, Marchant BA et al (1972) Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. Biochim Biophys Acta General Subjects 273:346–358
32. Xiaobo L (2004) Physical, chemical, and mechanical properties of bamboo and its utilization potential for fiberboard manufacturing. Theses, Louisiana State University
33. Yi-heng PZ, Lee RL (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng 88:797–824
34. Zhang Y, Percival H (2008) Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. J Ind Microbiol Biot 35:367–375