African Journal of Biotechnology Vol. 11(53), pp. 11595-11603, 3 July, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897//AJB12.422
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Screening and identification of cellulase producing yeast-like microorganisms from Brazilian biomes

R. Goldbeck[1]*, C. C. P. Andrade[1], G. A. G. Pereira[2] and F. Maugeri Filho[1]

[1]Laboratory of Bioprocess Engineering, Faculty of Food Engineering, University of Campinas – UNICAMP, 13083-862, Campinas-SP, Brazil.
[2]Laboratory of Genomic and Expression, Institute of Biology, University of Campinas – UNICAMP, 13083-870, Campinas-SP, Brazil.

Accepted 25 May, 2012

The main goals of the present study included the screening and identification of cellulase producing wild yeasts, isolated from samples collected from different Brazilian biomes. They were selected according to their capabilities of degrading carboxymethyl cellulose (CMC) and micro-crystalline cellulose (SERVACEL®), as single carbon sources in solid medium. After the step of solid medium selection, yeast cells were grown in liquid medium containing cellulose (SERVACEL®); in shake flasks at temperature of 30°C and 150 rpm agitation for 288 h. Three specific activities were evaluated: endoglucanase (CMCase), total activity (filter paper activity), and cellobiase. From a total of 390 strains of wild yeasts previously isolated, 16 strains performed cellulose hydrolysis, verified by the colorless halo in the solid medium. Among these 16 strains, 5 stood out as presenting higher levels of enzyme activity. The following step, screening in liquid medium, indicated only one strain as a potential producer of cellulases, named as AAJ6, for which the highest hydrolytic activity on carboxymethyl cellulose (0.33 U/ml) and filter paper (0.039 U/ml) was recorded. Afterwards, this wild yeast strain (AAJ6) was molecularly identified by sequencing the ITS1-5.8S-ITS2 and D1/D2 domains of the subunit (26 S) ribosomal DNA. Sequencing resulted in the identification of this strain as yeast-like fungus *Acremonium strictum.*

Key words: *Acremonium strictum*, screening, identification, yeast-like, cellulases.

INTRODUCTION

In recent years the interest in production of cellulases has increased due to several potential applications, such as the production of bioenergy and biofuels as well as application in the textile and paper industries (Zhou et al., 2008). Growing concerns regarding the shortage of fossil fuels, greenhouse gas emissions and air pollution by incomplete combustion of fossil fuels have also resulted in an increasing focus of producing bioethanol from lignocellulose, and especially the possibility of using cellulases to perform enzymatic hydrolysis of the lignocellulosic materials (Kitagawa et al., 2011). Cellulases are highly specific biocatalysts that act in synergy to release sugars, especially glucose which is of great industrial interest due to the potential for its conversion to, for example, bioethanol (Castro and Pereira, 2010). There is a wide variety of micro-organisms in nature which produce cellulases; some are known as truly cellulolytic, which are capable of degrading natural cellulose. In laboratory conditions, filter paper is used as inductor substrate for the production of exo-glycosidases and to measure total activity of the cellulolytic complex (Ruegger and Tauk-Tornisielo, 2004).

Although many bacteria and fungi degrade cellulose, the final products are generally carbon dioxide and methane when grown in media contains such carbo-hydrates. Only a few strains of fungi have been seen as major producers of enzymes, which can extensively

*Corresponding author. E-mail: rosanag@fea.unicamp. Tel: +55 19 3521 4052. Fax: +55 19 3521 4027.

degrade from insoluble cellulose to soluble sugars (Lee et al., 2005). In this context, particular attention should be given to the wild flora, which can be of great interest for enzyme production (Peixoto, 2006). Explorations of biodiversity in the search for new biocatalysts by selecting microorganisms from plants or animal cells represents a method for discovering new enzymes which may permit the development of bio-catalysis on an industrial scale. Currently, there is great interest in finding microorganism species that are not yet known as interesting producers of inputs to industry in general, and also in biotechnological processes can replace many chemical processes, in terms of production of key products. As an example is the replacement of acid hydrolysis for enzymatic hydrolysis, as is well known in the industry (Peixoto, 2006).

Brazil possesses the greatest biodiversity on the planet and home to seven biomes, forty-nine already classified as ecoregions, and an incalculable number of ecosystems. Due to the large Brazilian biodiversity, the species of fauna and flora may never be completely known, and the number of species not yet identified may reach the order of tens of millions. It is estimated that less than 5% of microorganisms existing on earth have been identified. In this context it is essential to implement programs capable of better utilizing Brazilian biodiversity. Currently, there is great interest in finding microorganism species that are not yet cataloged as interesting producers of inputs to industry in general, as well as optimizing production processes of these inputs from known microorganisms (Hernalsteens, 2006). Based on this information, the objective of the present study included screening and identification of cellulose producing wild yeasts isolated from different regions of Brazil.

MATERIALS AND METHODS

Sampling for microorganisms

Hernalsteens and Maugeri (2007) sampled flowers, fruit and soil from tropical Brazilian biomes, including: the Atlantic Rainforest (stretches along the Brazilian coast); the Cerrado (tropical savanna eco-region); the Pantanal (the world's largest wetland) and the Amazon Forest (complex biome due to the great diversity of vegetation present, considered the “lungs of the world”), aiming to isolate yeasts. The isolated yeasts were maintained in agar slant (GYMP Medium: 2.0% glucose, 0.5% yeast extract, 1.0% malt extract, 0.2% monobasic sodium phosphate, 2.0% agar and pH 5.5) under refrigeration at 5°C (stock culture). All cultures were stored in the bank of cultures in the Laboratory of Bioprocess Engineering (LEB) - FEA/UNICAMP and corresponded to a total of 390 strains.

Solid medium selection

Selection of yeasts was based on the break-down of carboxymethyl cellulose (CMC) and cellulose (SERVACEL®), in separate experiments. The selective solid culture medium consisted of carboxymethylcellulose (CMC), 10 g/L or SERVACEL®, 20 g/L; yeast extract, 0.6 g/L; KH_2PO_4, 7.0 g/L; K_2HPO_4, 2.0 g/L; $MgSO_4.7H_2O$, 0.1 g/L; $(NH_4)_2SO_4$,1.0 g/L; agar, 15.0 g/L and pH 5.0 (Peixoto, 2006). The Petri dishes with the selective medium described above were inoculated with the yeasts, and incubated at 30°C for 96 h. Then the plates were revealed using a Congo red solution (1.4 g/L) in Tris-HCl 0.1 M pH 8.0. The cellulase producing yeasts were selected according to a colorless halo around the colonies.

Index of enzymatic activity

The enzymatic activities were estimated according to the method reported by Anagnostakis and Hankin (1975) who proposed an Enzymatic Activity Index (EAI), which is the ratio between the halo diameter and the colony diameter. The experiments were performed in triplicate and data was statistically analyzed with STATISTICA® 6.0, according to the Tukey's tests at 5% of probability ($p \leq 0.05$).

Liquid medium selection

For the selection in liquid culture, the medium consisted of: SERVACEL®, 20.0 g/L; yeast extract, 0.60 g/L; KH_2PO_4, 7.0 g/L, K_2HPO_4, 2.0 g/L; $MgSO_4.7H_2O$, 0.15 g/L; $(NH_4)_2SO_4$, 1.0 g/L; $FeSO_4.7H_2O$, 0.01 g/L; and KCl, 0.50 g/L and pH 5.5 (Peixoto, 2006). The inoculums were cultivated on GYMP agar slants at 30°C for 96 h. Fermentations were then performed in shake flasks at 30°C and 150 rpm, and monitored for 288 h. Samples were removed every 48 h. The samples collected periodically were centrifuged at 4°C and 18.200 x g for 10 min, and the supernatant (crude enzymatic extract) submitted to assays for CMCase (endoglucanase activity), FPase (filter paper activity) and cellobiase activity.

Endoglucanase activity

A carboxymethyl cellulose (CMC, 1%) solution was prepared in 0.2 M sodium acetate buffer (pH 4.2). One milliliter (1 ml) of the CMC solution was incubated with 1 ml of crude enzymatic extract at 50°C for 10 min (Ogawa, 1982) and the amount of sugar was measured using the 3,5-dinitrosalicylic (DNS) reagent method, according to Miller (1959).

Filter paper activity

Total cellulase activity was determined by the filter paper assay procedure (Mandels and Sternberg, 1976). The assay system had a total volume of 2 ml, consisting of 1 ml of crude enzymatic extract, 1 ml of 0.2 M sodium acetate buffer (pH 4.2) and 50 mg of Whatman filter paper N° 1, incubated for 60 min at 50°C. The amount of reducing sugar was measured by the 3,5-dinitrosalicylic (DNS) reagent method according to Miller (1959).

Cellobiase activity

Cellobiase activity was measured according to a reaction mixture containing a solution of cellobiose 20 mM in acetate buffer 0.2 M, pH 5.2. One milliliter (1 ml) of the cellobiose solution was mixed with 1 ml of the crude enzymatic extract and incubated at 50°C for 30 min. Reducing sugar was measured using a commercial enzymatic kit containing glucose oxidase (Henry, 1974). One unit of endoglucanase, filter paper or cellobiase activity is defined as the amount of the enzyme that released 1 μmol of glucose per minute from the substrate, at the three experimental conditions described above.

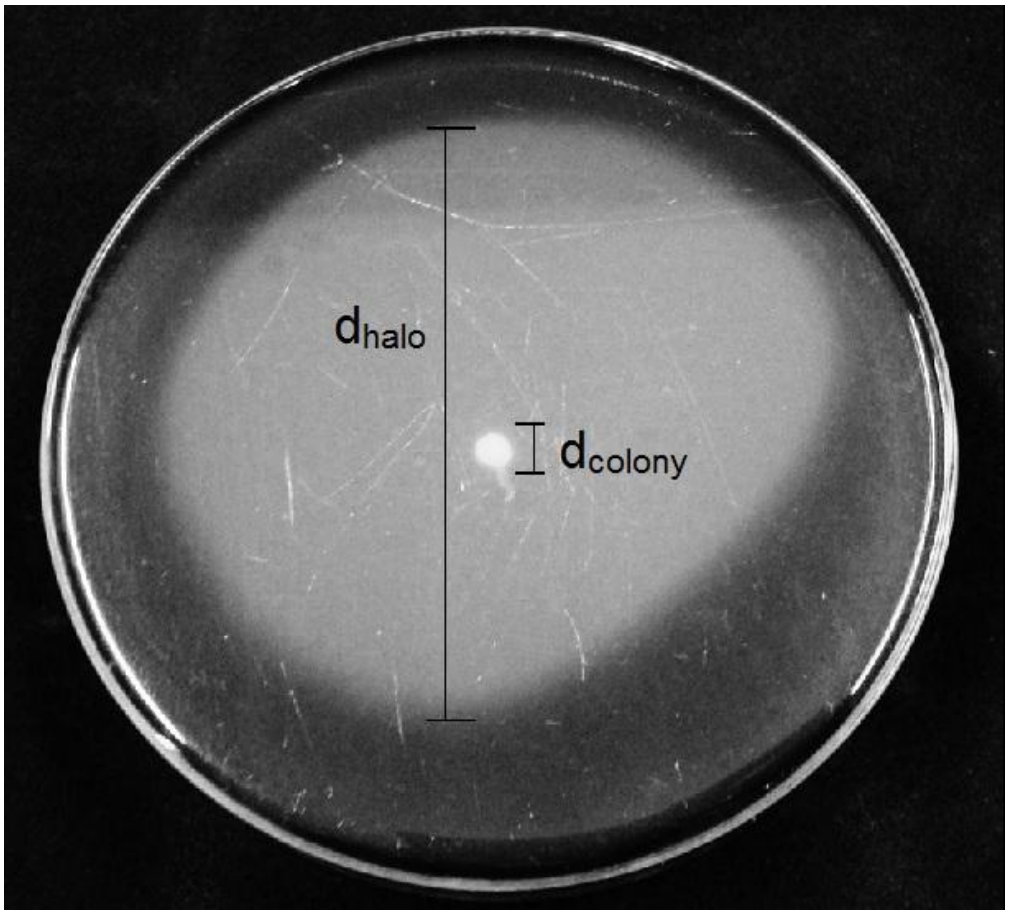

Figure 1. Halo of hydrolysis formed around the colony when grown for 96 h at 30°C.

Molecular identification

The yeast strain was identified by the molecular method as follows: a portion of a colony was cultured in 4 mL of 2% YEPD (Yeast Extract Peptone Dextrose) medium, overnight, under agitation, at 30°C. Afterwards, the cells were centrifuged and ressuspended in 200 ml of breaking buffer and DNA extraction as described by Sambrook et al. (1989). Identification was carried out by sequencing the gene that encodes the 5.8 S regions of ribosomal ribonucleic acid (RNA) and the spacer regions *ITS-1* and *ITS-2*. To do so, the universal primers *ITS1* (5′ TCCGTAGGTGAACCTGCGG 3′) and *ITS4* (5′ TCCTCCGCTTA-TTGATATGC 3′) were used. The D1/D2 domains of the 26 S subunit were also sequenced by using the primers *NL1* (5′ GCATATCAA-TAAGCGGAGGAAAAG 3′) and *NL4* (5′ GGTCCGTGTTT-CAAGACGG 3'), according to the methodology described by Kurtzman and Robnett (1997); Esteve-Zarzoso et al. (1999) and Leaw et al. (2006). DNA fragments from polymerase chain reaction (PCR) were purified by the enzymatic method ExoSAP with Exo 1 (Exonuclease 1) and SAP (Shrimp Alkaline Phosphatase); and DNA quantification was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) at the wavelength of 260 nm. The reaction followed the sequencing method of Sanger and was performed using the MegaBACE device (GE Healthcare), at the Brazilian National Synchrotron Light Laboratory (LNLS), according to local protocol. Nucleotide sequences were assessed and compared using the BLAST algorithm (Basic Local Alignment Search Tool) with the sequences deposited in the NCBI data bank (http://www.ncbi.nlm.nih.gov) for the genus and species already identified.

Microscopy

The morphology of *Acremonium strictum (*AAJ6 strain), grown on SERVACEL® during exponential growth phase, was observed in a Carl Zeiss Jena optical microscope, Variant Jenamed model, with 120X magnification.

RESULTS AND DISCUSSION

Solid medium selection

For selection in solid medium, the wild yeasts were grown in Petri dishes in order to identify those able to degrade carboxymethyl cellulose (CMC) and micro-crystalline cellulose (SERVACEL®). After incubation, the plates were revealed with a Congo red solution and halo formation was analyzed. The formation of a clear halo surrounding the colony indicates hydrolytic activity. Diameters of the halos were measured in three different directions to determine the average value (Figure 1). Analyzing Figure1, it can be visualized that a large halo

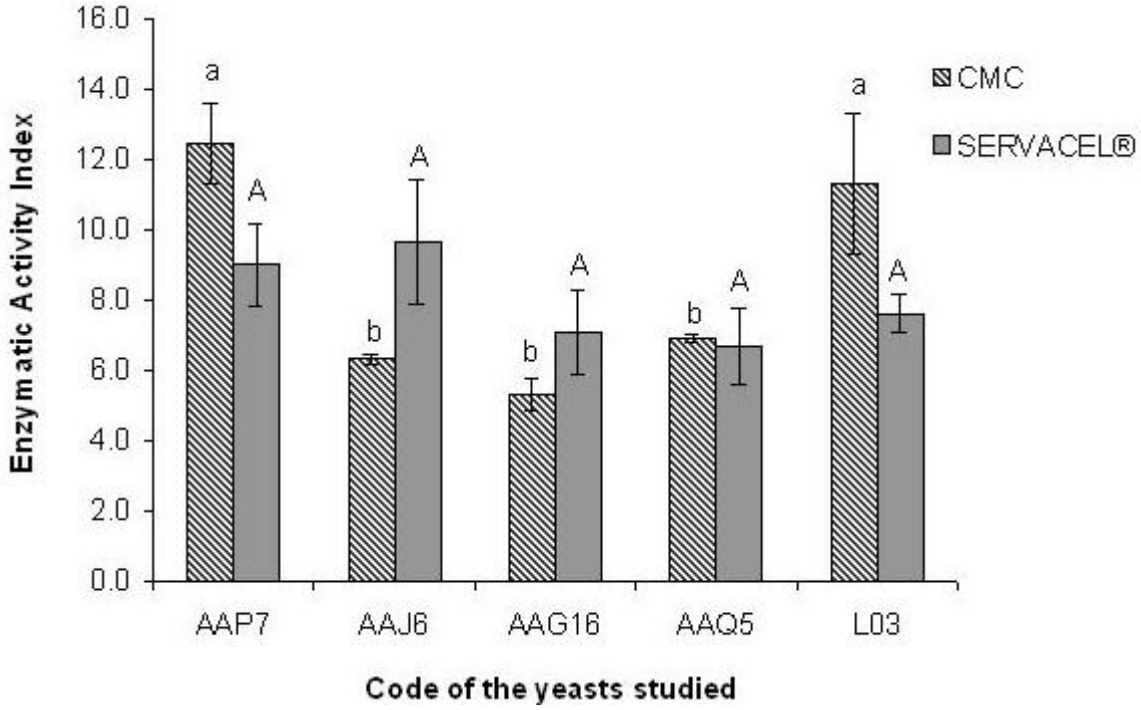

Figure 2. Enzymatic Activity Indices (EAI) for the five yeast strains grown on solid medium. *Different letters indicate significant difference at 5% level of significance ($p \leq 0.05$) among the strains, for both substrates (Carboxymethylcellulose and SERVACEL®). Capital letters were used for tests with SERVACEL® and lower-case letters used for tests with Carboxymethyl cellulose (CMC), evaluated by the Tukey's test.

of hydrolysis formed after 96 h of incubation on agar with carboxymethyl cellulose (CMC), however, the colony showed little increase in the same period. A similar result was observed for Ruegger and Tauk-Tornisielo (2004), who studied different strains of fungi isolated from soil, and many microorganisms showed colonies with smaller diameter and large halo hydrolysis resulting in higher values of enzymatic activity index (EIA).

From a total of 390 previously isolated wild yeast strains, only 16 performed cellulose hydrolysis, verified by a colorless halo in the solid medium. Among these 16 strains, 5 stood out as presenting high levels of enzyme activity. These five strains presented distinguishable colorless halos around their colonies in both substrates (carboxymethyl cellulose and cellulose). Ruegger and Tauk-Tornisielo (2004) studied the cellulase activity of fungi isolated from soil of the ecological station of São Paulo, Brazil, and among the fungi studied, 36 strains (45%) showed zones clearer around the colonies, this halo corresponds to the degradation of CMC (carboxymethyl cellulose). Enzymatic activity index (EAI) varied from 1.4 to 6.0; the best results obtained were with *Trichoderma hamatum* and *Penicillium herquei*.

The enzymatic activity index (EAI) was established based on the ratio between the halo diameter and the colony diameter. Colonies with the highest EAI are those with higher extracellular enzyme activity (Ceska, 1971; Ruegger and Tauk-Tornisielo, 2004; Oliveira et al., 2006). The EAI is a semi-quantitative parameter commonly used to assess enzyme production by microorganisms in solid medium (Anagnostakis and Hankin, 1975; Lopes et al., 2011). Microorganism selection considered enzyme producers based on the direct correlation between diameters of the degradation halo and degradative ability of microorganisms (Ceska, 1971; Chen et al., 2004; Oliveira et al., 2006). Figure 2 shows values of enzyme activity recorded for the five selected strains when grown on solid medium containing carboxymethylcellulose or cellulose (SERVACEL®) as the single carbon sources for the different experiments.

The experiments were performed in triplicate and data assessed according to the Tukey's test. Figure 2 shows the average enzymatic activity indices (EAI) for the five yeasts, in which the letters indicate if there is significant difference among strains at 5% significance for both substrates (carboxymethyl cellulose and cellulose). Different letters indicate significant differences ($p \leq 0.05$). In experiments performed with carboxymethyl cellulose as the sole carbon source, the yeast strains named AAP7 (isolated from the Cerrado) and L03 (isolated from the Pantanal) showed the highest enzymatic activity indices, 12.46 and 11.33, respectively. Both showed significant differences ($p \leq 0.05$) when compared with the other strains isolated from the Cerrado (AAJ6, AAG16 and

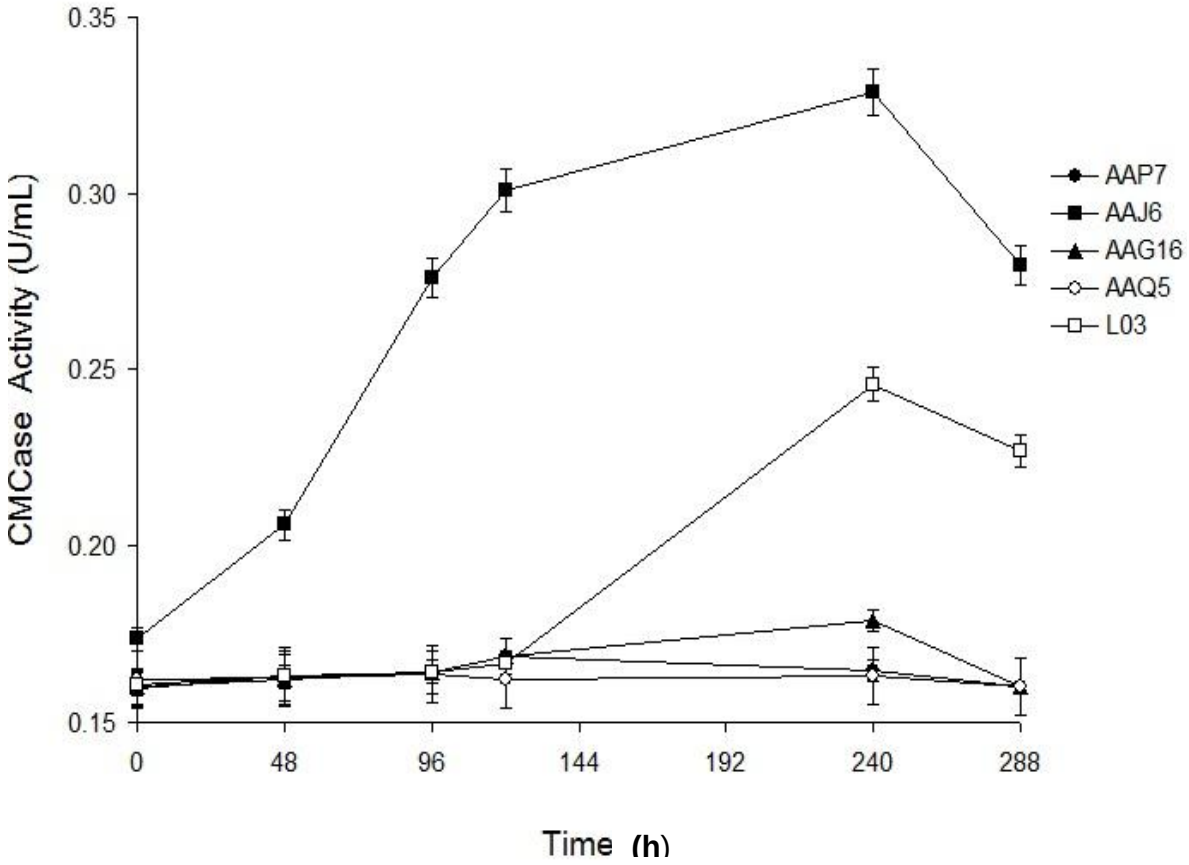

Figure 3. CMCase activity in relation to the fermentation time for the five strains studied.

AAQ5). Regarding the experiments with SERVACEL®, the EAI for the five yeast strains showed no significant difference at 5% significance.

Some authors recommend an EAI≥2.5 for considering a microorganism as a producer of enzymes in solid medium (Peixoto, 2006; Oliveira et al., 2006; Lopes et al., 2011). The five yeast strains selected showed EAI much higher than 2.5 (EAI 5 times greater than the minimum suggested). Therefore, these five were all considered cellulase producing strains and were assayed in liquid medium fermentations.

Values of the enzymatic activity index (EAI) found in this study were higher than those described in the literature because in this work the plates were incubated for 96 hours instead of 48 hours, as reported in the literature. Lopes et al. (2011) studied the screening of xylanase producing yeast non-conventional isolated from different Brazilian regions and reported enzymatic activity indices (EAI) varied from 2.5 to 6.7 for the yeast incubated for 48 h at 30°C and revealed using a Congo red solution.

However, in fact the intent this work was only use this method as a tool to screening cellulase producing microorganisms among the samples, and not to compare with data from the literature, so that the incubation time was in accordance with our needs.

Liquid medium selection

The fermentations were performed in medium containing SERVACEL® cellulose as the sole carbon source and monitored for 288 h. Samples were collected every 48 h for further analysis of CMCase, FPase, Cellobiase activities, as shown in Figures 3, 4 and 5. When viewing Figures 3 and 4, it can be observed that there is a similar behavior for CMCase and FPase activities for all strains. The highest activities were observed at 240 h of fermentation for both enzymes, and after 240 h, the enzymatic activity begins to drop. The strain AAJ6 stood out by producing the highest CMCase and FPase activities, 0.33 and 0.039 U/ml, respectively. These results are similar to those found for Ruegger and Tauk-Tornisielo (2004) who recorded an activity of CMCase 0.034 U/ml and FPase 0.016 U/ml for the microorganism *Penicillium purpurogenum* and CMCase of 0.036 U/ml and FPase of 0.018 U/ml for *Chloridium virescens* after 14 days of cultivation at 28°C.

Regarding cellobiase activity (Figure 5), the yeast strain L03 showed greatest activity of 0.039 U/ml after

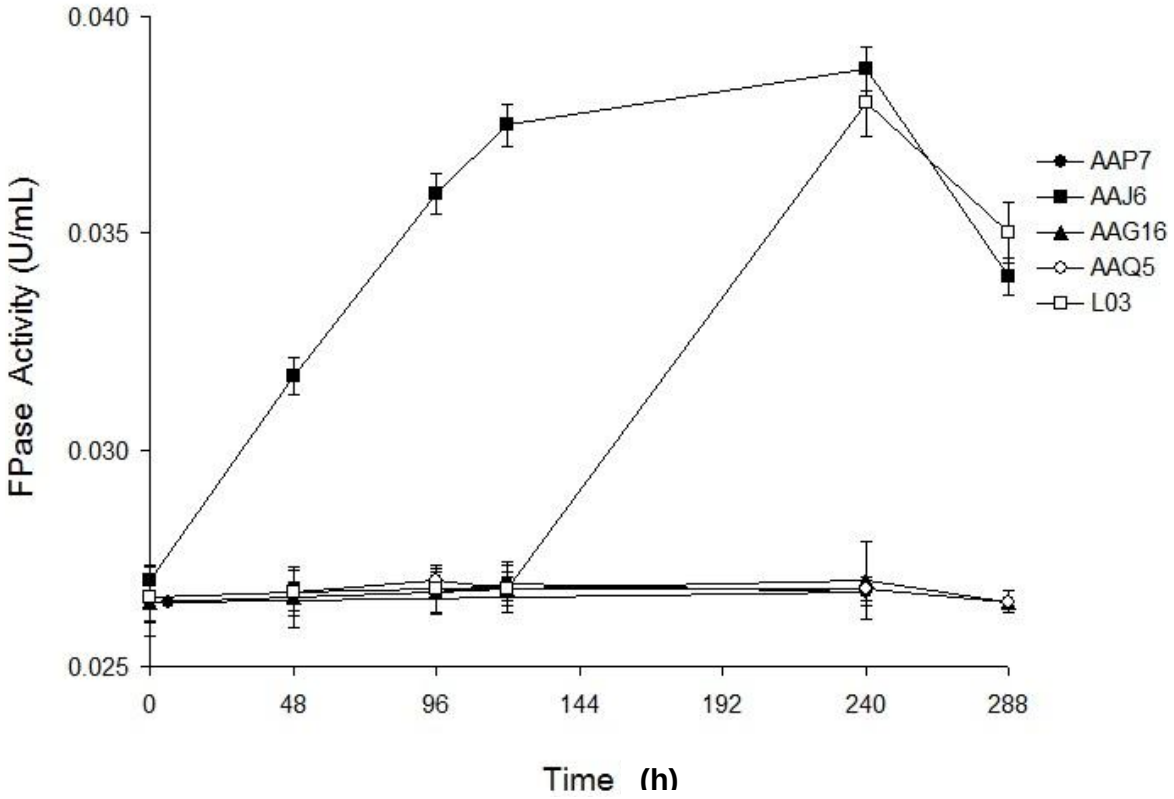

Figure 4. FPase activity in relation to the fermentation time for the five strains studied.

240 h of cultivation, followed by the AAG16 strain (0.021 U/ml) at 120 h of cultivation. After 240 h of fermentation, the cellobiase activity from strain L03 begins to decrease and for the other strains the activity begins to fall at roughly 120 h. Silva et al. (2009) evaluated the production of cellulases by *Trichoderma harzianum* in submerged fermentation using crystalline cellulose as an inducer, and the following results were obtained: CMCase activity of 0.57 U/ml, FPase activity of 0.055 U/ml and cellobiase activity of 0.022 U/ml. Such results are similar to those found for the wild strains studied, although filamentous fungi have greater potential for cellulase production than yeasts.

Regarding the assays in solid and liquid medium, it is observed that the strain AAP7 showed the highest enzymatic activity index in solid medium, but did not perform well in liquid medium; whereas the strains AAG16 and LO3, which performed relatively well in solid medium, reproduced cellobiase activity in the liquid medium. The strain AAJ6 showed good enzymatic activity index on solid medium and high enzymatic activities for CMCase (endoglucanase) and FPase. However, it can be concluded that the enzymatic activity index (EAI) in this study was not an appropriate parameter for comparative assessment of enzymatic activities between different strains, but can be used as an efficient and useful tool for selecting strains (Ruegger and Tauk-Tornisielo, 2004). Additional liquid fermentations were performed with the AAJ6 and L03 strains in order to confirm the previously obtained results and it was again observed that the activities of strain AAJ6 were superior. The AAJ6 strain (isolated from Cerrado) was selected as a potential producer of cellulases and was subjected to molecular identification.

Molecular identification

Molecular identification was based on ribosomal targets: the ITS1-5.8S-ITS2 and D1-D2 domains of the RNA operon. In recent years, the number of ITS sequences available in public databases has increased significantly, and the expanding database may improve the quality and accuracy of fungal identification (Hinrikson et al., 2005; Leaw et al., 2006). The D1/D2 recombinant deoxyribonucleic acid (rDNA) region has been sequenced for almost all known yeasts, including non-pathogenic species. As an identification tool, the sequencing of this region is relatively simple to apply and yields clear results (Kurtzman and Robnett, 1997; Leaw et al., 2006). The BLAST searches, even as phylogenetic analysis, are used for identification by searching public databases for the closest known sequences to the unidentified yeast (Suh et al., 2006). A BLAST search in the NCBI database

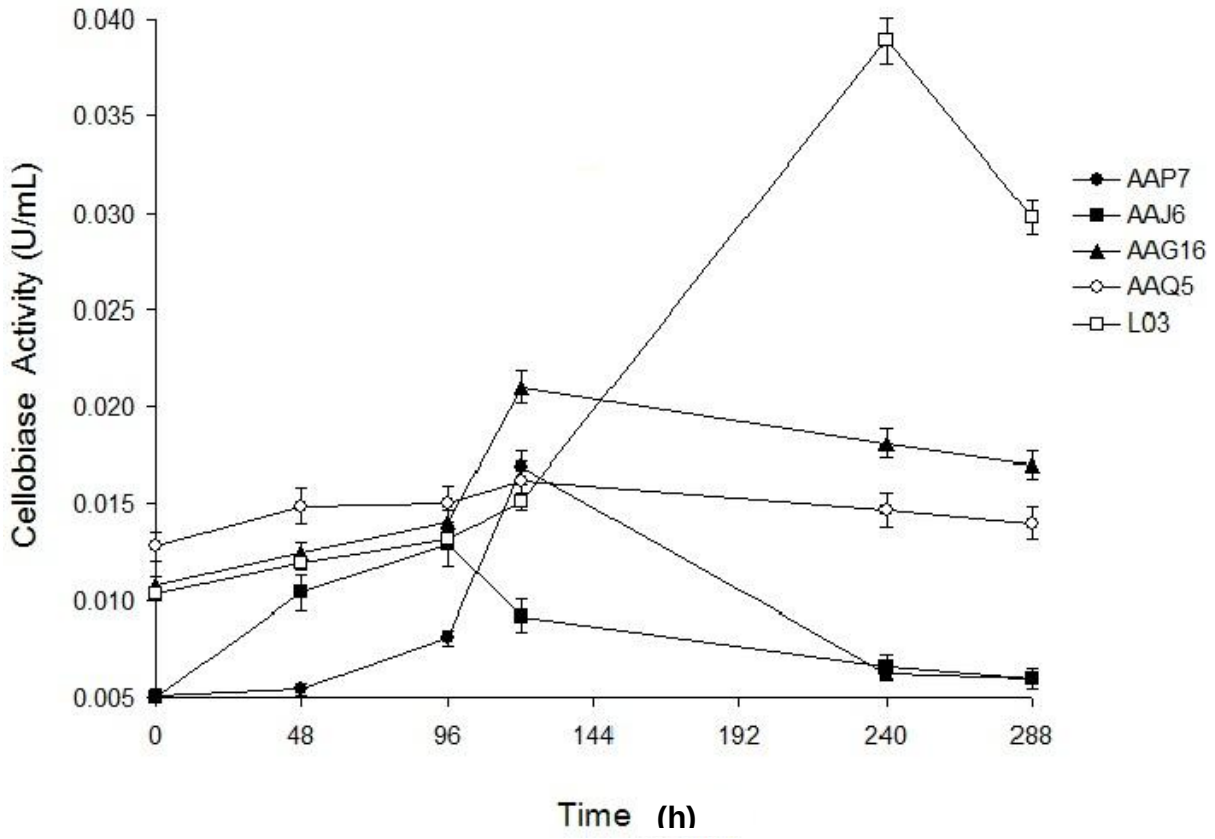

Figure 5. Cellobiase activity in relation to the fermentation time for the five strains studied.

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the ITS1-5.5S-ITS2 sequenced fragment (545 bp) showed 100% sequence identity with *Acremonium strictum.* Similarly, the D1/D2 sequenced fragment (584 bp) also exhibit 100% sequence identity with this same fungal species.

Acremonium strictum also referred to as *Acremonium zeae, cephalosporium sp.* and *Cephalosporium acremonium,* together with *Fusarium verticilliuides*, are colonizers of freshly harvested corn (*Zea maydis*). Infection by this fungus is asymptomatic and usually occurs in the embryo and endosperm of corn grains (Almeida, 2009). Initially, the intention of this study was to identify wild cellulose producing yeast and not a filamentous fungus, due to the fact that the isolated strains were previously classified according to biochemical tests as yeasts. Although *Acremonium strictum* is commonly found as a filamentous fungus, this microorganism under the studied growth conditions showed a yeast-like form. This type of phenomenon, which is called dimorphism, is quite common among fungi and is based on the fact that the same microorganism can appear in different morphological forms, depending on the growth conditions imposed. The fungi may have different morphology according to nutritional conditions and the temperature of development. Dimorphism is expressed by a mycelial growth between 22 and 28°C and yeast from 35 and 37°C (Hurtado and Rachubinski, 2002).

In general, these forms are reversible and important in medical sciences, for example, *Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Sporothrix schenckii* (Pelczar et al., 1997). Recently, it was demonstrated by DNA sequence analysis that the species *Acremonium strictum* displays a broad genetic polymorphism (Das et al., 2010). Like other strains isolated by Hernalsteens (2006) and that used in the present study, the strain AAJ6 is stable in the yeast form under the experimental conditions employed. This feature is extremely desirable for fermentation processes, since they grow predominantly in a unicellular form (Figure 6) and reproduce faster than mould. Moreover, they are normally easy to manipulate, the inoculum can be easily prepared, and yeast cells can be efficiently recycled in fermentation processes (Atala et al., 2001). The microorganism AAJ6 identified as *Acremonium strictum* showed significant potential for cellulase production. Currently, little is reported in literature regarding the production of cellulases from this genus, which permits numerous studies on novel microorganisms in relation to production of enzymes of industrial interest. Thus, the results of this study stimulate new investigations, seeking applications in

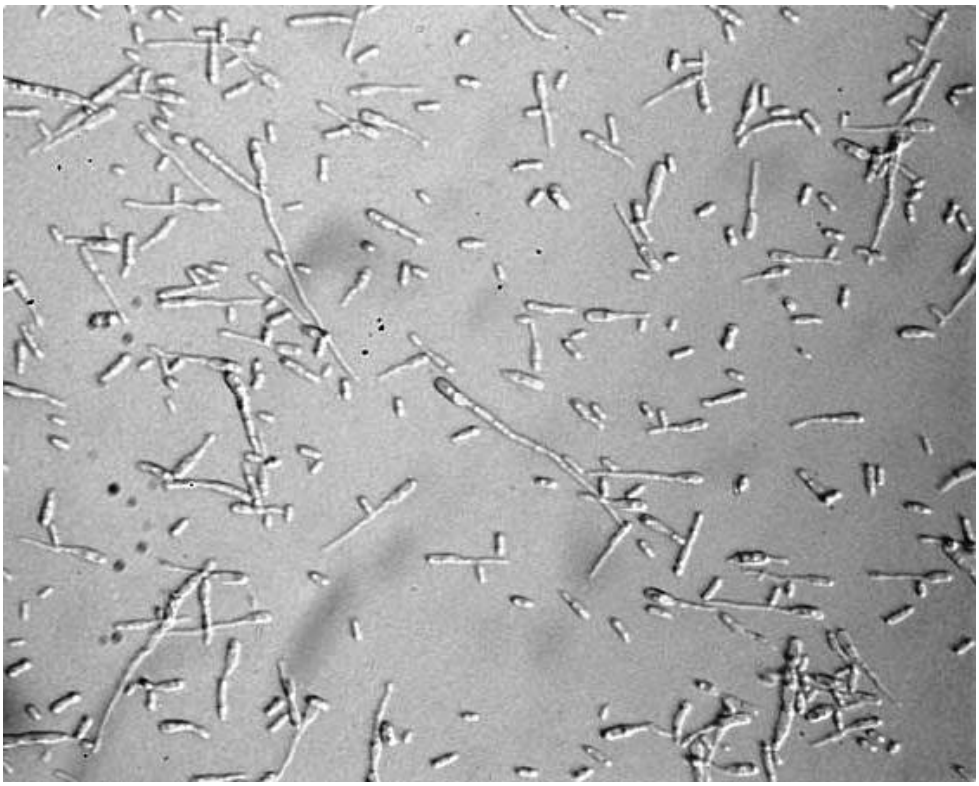

Figure 6. Morphology of *Acremonium strictum* (AAJ6 strain) grown on SERVACEL® during exponential growth phase. Two distinct cellular forms can be observed (120X): pseudo-mycelia and predominantly unicellular form (yeast-like).

enzymatic hydrolysis for production of bioenergy.

Conclusion

From a total of 390 previously isolated wild yeast strains, 16 strains showed a halo of hydrolyzed substrate in the solid medium, and five stood out for having high levels of enzyme activity. Considering the screening assays in liquid media, only one strain (AAJ6 - isolated from Cerrado) among the five pre-selected on solid medium was selected as a potential producer of cellulase, which showed the highest hydrolytic activity on carboxymethyl-cellulose and filter paper. The microorganism was molecularly identified as *Acremonium strictum*, a dimorphic microorganism which will be subjected to further studies involving cloning and heterologous expression, focusing on the production of bioethanol from lignocellulolytic material.

ACKNOWLEDGEMENTS

We are grateful to the FAPESP (The State of São Paulo Research Foundation) for the PhD scholarship and for the project funding. We acknowledge the Brazilian National Synchrotron Light Laboratory (LNLS) for the use of MegaBACE equipment (GE Healthcare) necessary for the molecular identification of the microorganism studied.

REFERENCES

Anagnostakis SL, Hankin L (1975). Use of selective media to detect enzyme production by microorganisms in food products. J. Milk Food Technol., 38: 570-572.

Almeida MN (2009). Cellulases and Hemicellulases from Species of *Acremonium Endophytes*. M.S. Thesis. Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Brazil, p. 70.

Atala DIP, Costa AC, Maciel Filho R, Maugeri Filho F (2001). Kinetics of Ethanol Fermentation with High Biomass Concentration Considering the Effect of Temperature. Appl. Biochem. Biotechnol., 91-93: 353-366.

Castro AM, Pereira NJ (2010). Production, Properties and Application of Cellulases in the Hydrolysis of Agroindustrial Wastes. Quim. Nova., 33(1): 181-188.

Ceska M (1971). Enzyme catalysis of solidified media. Eur. J. Biochem., 71(22): 186-192.

Chen PJ, Wei TC, Chang YT, Lin LP (2004). Purification and characterization of carboxymethylcellulase from *Sinorhizobium fredii*. Bot. Bull. Acad. Sin., 45: 111-118.

Das S, Saha R, Dar SA, Ramachandran VG (2010). Acremonium Species: A Review of the Etiological Agents of Emerging Hyalohyphomycosis. Mycopathologia, 170(6): 361-375.

Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999). Identification of yeast by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol., 49: 329-337.

Henry RJ, Cannon DC, Winkelman J (1974). Clinical chemistry principles and techniques. 2ª (Ed). Harper and Row Publishers Inc, New York, USA.

Hernalsteens S (2006). Isolation, identification and characterization of oligosaccharide producing microorganisms collected from different regions of Brazil. Ph.D Thesis. Faculty of Food Engineering, University of Campinas, Brazil, p. 186.

Hernalsteens S, Maugeri F (2007). Screening of yeast strains for transfructosylating activity. J. Mol. Catal., B Enzym., 49: 43-49.

Hinrikson HP, Hurst SF, Lott TJ, Warnock DW, Morrison CJ (2005). Assessment of ribosomal large-subunit D1–D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. J. Clin. Microbiol., 43: 2092-2103.

Hurtado CAR, Rachubinski RA (2002). Isolation and Characterization of *YlBEM1*, a Gene Required for Cell Polarization and Differentiation in the Dimorphic Yeast *Yarrowia lipolytica.* Eukaryotic Cell., 4: 526-537.

Kitagawa T, Kohda K, Tokuhiro K, Hoshida H, Akada R, Takahashi H, Imaeda T (2011). Identification of genes that enhance cellulase protein production in yeast. J. Biotechnol., 151(2): 194-203.

Kurtzman CP, Robnett CJ (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol., 35: 1216-1223.

Leaw SN, Chang HC, Sun HF, Barton R, Bouchara JP, Chang TC (2006). Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. J. Clin. Microbiol., 44: 693-699.

Lee SJ, Lee KS, Kim SR, Gui ZZ, Kim YS, Yoon HJ, Kim I, Kang PD, Sohn HD, Jin BR (2005). A novel cellulase gene from the mulberry longicorn beetle, *Apriona germari*: Gene structure, expression, and enzymatic activity. Comp. Biochem. Physiol., B Biochem. Mol. Biol., 140(4): 551-560.

Lopes F, Motta F, Andrade CCP, Rodrigues MI, Maugeri-Filho F (2011). Thermo-Stable Xylanases from Non Conventional Yeasts. J. Microbial. Biochem. Technol., 3(3): 36-42.

Mandels M, Sternberg D (1976). Recent advances in cellulase technology. J. Ferment. Technol., 54: 267-286.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Ogawa K, Toyama H, Toyama N (1982). Native cellulose hydrolyzing cellulase of *Trichoderma reesei*. J. Ferment. Technol., 60: 349-355.

Oliveira NA, Oliveira LA, Andrade JS, Chagas Júnior AF (2006). Extracellular hydrolytic enzymes in indigenous strains of rhizobia in Central Amazonia, Amazonas, Brazil. Ciênc. Tecnol. Aliment. 26: 853-860.

Pelczar JRMJ, Chan ECS, Krieg NR (1997). Microbiology: Concepts and Applications. 2ª (Ed). Makron Books Publishers Inc, São Paulo, Brazil.

Peixoto AB (2006). Study of the production of enzymes and gums by wild yeasts collected in various regions of Brazil. M.S. Thesis. Faculty of Food Engineering, University of Campinas, Brazil, p. 84.

Ruegger MJS, Tauk-Tornisielo SM (2004). Cellulase activity of fungi isolated from soil of Juréia-Itatins, São Paulo, Brazil. Revista Brasil Bot., 27(2): 205-211.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. 2ª (Ed). Cold Spring Harbor Publishers Inc, New York, USA.

Suh SO, Blackwell M, Kurtzman CP, Lachance MA (2006). Phylogenetics of Saccharomycetales, the ascomycete yeasts. Mycologia, 98(6): 1006-1017.

Silva MMP, Rocha V, Maeda RN, Pereira N JR (2009). Evaluation of cellulase production by *Trichoderma harzianum* in submerged fermentation. In: Proceedings of 17th National Symposium Bioprocesses, Natal, Brazil.

Zhou J, Wang Y, Chu J, Zhuang Y, Zhang S, Yin P (2008). Identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride* T 100-14. Bioresour. Technol., 99: 6826-6833.