



PRODUCTION OF HYDROLYTIC ENZYMES BY SOLID-STATE FERMENTATION WITH NEW FUNGAL STRAINS USING ORANGE BY-PRODUCTS

PRODUCCIÓN DE ENZIMAS HIDROLÍTICAS POR FERMENTACIÓN EN ESTADO SÓLIDO CON NUEVAS CEPAS DE HONGOS UTILIZANDO SUBPRODUCTOS DE NARANJA

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Abstract

Orange is the most-produced citrus fruit worldwide, and its residues, which are discarded, represent 50% of the fruit. These residues cause major contamination problems. Orange peels are rich in cellulose, hemicellulose and pectin, which can be used for the production of enzymes. In this study, oranges-colonizing fungi were isolated and identified by 5.8S rRNA-ITS ribosomal sequence analysis as *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1 and *Penicillium minioluteum* N3C2. These strains and *Trichoderma reesei* ATCC 26921 were used for the production of endoglucanases (EG), xylanases (XYL) and polygalacturonases (PGA) by solid-state fermentation using orange peels. *P. minioluteum* N3C2 obtained 6.5 U/g of dry peel (DP), the highest production of EG, surpassing *T. reesei* ATCC 26921 (3.3 U/g DP). *T. reesei* ATCC 26921 and *P. minioluteum* N3C2 obtained the highest production of XYL, at 14.7 and 14.5 U/g DP, respectively. The highest PGA production was obtained with *Fusarium* sp. N6C6 (16.5 U/g DP), followed by *M. racemosus* N9C1 (12.3 U/g DP), which produced less EG. *C. oxysporum* N1C1 produced EG (3.2 U/g DP) and PGA (8.0 U/g DP) but did not produce XYL. The strains studied possess great potential for the production of enzymes that could be used in the extraction of citric oils.

Keywords: citrus fruits, endoglucanases, fungi, polygalacturonases, xylanases.

Resumen

La naranja es el cítrico de mayor producción a nivel mundial, y sus residuos, que son desechados, representan el 50% del fruto. Estos residuos causan problemas importantes de contaminación. Las cáscaras de naranja son ricas en celulosa, hemicelulosa y pectina que podrían emplearse en la producción de enzimas. En este estudio se aislaron hongos de naranjas y se identificaron por análisis de la secuencia ribosomal 5.8S rRNA-ITS como *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1 y *Penicillium minioluteum* N3C2. Estas cepas y *Trichoderma reesei* ATCC 26921 se utilizaron para la producción de endoglucanasas (EG), xilanasas (XYL) y poligalacturonasas (PGA) por fermentación en estado sólido utilizando cáscara de naranja. *P. minioluteum* N3C2 alcanzó 6.5 U/g de cáscara seca (DP), la mayor producción de EG, superando a *T. reesei* ATCC 26921 (3.3 U/g DP). *T. reesei* ATCC 26921 y *P. minioluteum* N3C2 tuvieron la mayor producción de XYL, con 14.7 y 14.5 U/g DP respectivamente. La mayor producción de PGA fue obtenida con *Fusarium* sp. N6C6 (16.5 U/g DP), seguida de *M. racemosus* N9C1 (12.3 U/g DP), la cual produjo menos EG. *C. oxysporum* N1C1 produjo EG (3.2 U/g DP) y PGA (8.0 U/g DP), pero no produjo XYL. Las cepas exploradas poseen gran potencial para la producción de enzimas que podrían utilizarse en la extracción de aceites cítricos.

Palabras clave: cítricos, endoglucanasas, hongos, poligalacturonasas, xilanasas.

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1 Introduction

Around the world, the food industry annually produces large volumes of residues that cause several disposal problems. Multiple microorganisms can degrade these residues because they are rich in sugars that can be transformed into high-value compounds (Ali *et al.*, 2011). Citrus fruits are the most important fruit crops in the world, with close to 120 million tons being produced in more than 100 countries. Of these, the orange is the most produced citrus fruit (FAO, 2014); approximately 50% of its weight corresponds to the peel, which is discarded, causing contamination problems. These residues have been proposed for use as feed for cattle; to obtain citric acid, essential oils and flavonoids; and for the production of biogas, ethanol, fatty acids, microbial biomass and hydrolytic enzymes (Mamma *et al.*, 2008). Citrus peels are rich in flavonoid compounds, ascorbic acid and essential oils and are formed by soluble solids and sugars such as cellulose, hemicellulose and pectin (Hours *et al.*, 2005). The use of these agro-industrial by-products could be an alternative for the production of enzymes such as cellulase, xylanase and pectinase. Filamentous fungi constitute the main source of hydrolases because they produce multi-enzyme complexes formed by endo- and exo-enzymes that include cellulase, xylanase and pectinase, which degrade polymers such as cellulose, hemicellulose and pectin, respectively (Marquina, 1991). These enzymes have great importance in industrial settings because of their widespread application in the food and pharmaceutical branches and in the treatment of residual water or for obtaining sugars for the generation of biofuels (Sorensen *et al.*, 2011).

The fungi usually used in the production of these enzymes include *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola* and *Phanerochaete* (Izarra *et al.*, 2010). In recent years studies for the production of enzymes with filamentous fungi are mainly performed by solid-state fermentation (SSF) because higher enzymatic activity per reactor volume can be obtained and the enzymes are less affected by the catabolite repression compared to submerged fermentation. The SSF could be applied to produce enzymes such as phytases, pectinases, amylases, invertases, inulinases, cellulases, xylanases, proteases, lipases, tannases, laccases, between others (Singhania *et al.*, 2010; Álvarez-Cervantes *et al.*, 2016). Some of the substrates used for the production of cellulolytic and hemicellulolytic enzymes by SSF include wheat straw, rice husks, corn cobs, sugar beet pulp, coconut

fibre, tea residues, sorghum pulp, poplar pulp and sugarcane bagasse (Pandey *et al.*, 1999). However, there are few reports in which citrus residues are used for the production of these enzymes. To identify enzymes with higher affinity towards citrus fruits' polysaccharides (pectin, cellulose and hemicellulose), in this study, oranges-colonizing fungi were isolated and identified by 5.8S rRNA-ITS ribosomal sequence analysis for the subsequent production of hydrolytic enzymes (cellulase, xylanase and pectinase) by SSF using orange peels as a substrate.

2 Materials and methods

2.1 Characterization of orange peels

The composition of orange peels was characterized by their levels of cellulose, hemicellulose, lignin pectin, ethereal extract, ash, proteins and water-extractable compounds. Cellulose, hemicellulose, lignin (fibre fraction), pectin and water-extractable compounds were determined using the method described by Aravantinos-Zafiris *et al.*, (1994). Fat (ethereal extract) and ash content were determined according to the standard method of the AOAC (AOAC, 1990) and the protein content by the Kjeldahl method (Aravantinos-Zafiris *et al.*, 1994).

2.2 Microorganism isolation

The isolation of the fungi was performed from rotten orange residues (from two municipal markets of Guadalajara, Mexico). The strains were isolated and purified using plates with potato dextrose agar (PDA) containing 10% tartaric acid as a bacterial inhibitor. Once plated, they were incubated at 25°C for 3-5 d (Cornejo, 1984). The conservation of the strains was performed by periodic re-plating and maintenance at 4°C.

2.3 Selection of hydrolytic enzyme-producing microorganisms

Hydrolase-producing strains were qualitatively selected by culturing in a medium containing (g/l): KH_2PO_4 , 1; NaNO_3 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; agar, 15; supplemented with 1% (w/v) carboxymethylcellulose (CMC), xylan or pectin (they were purchased from Sigma-Aldrich Co.) as the sole carbon source. The pH was adjusted to 6.0, and the strains were incubated at 30°C with periodic observations at 24, 48 and 72 h.

2.4 Molecular identification

Fungi strains were identified by PCR and sequencing of the region encompassing the ITS1, 5.8S rRNA and ITS2 (5.8S-ITS region). The strains were grown in 1.5 ml of yeast extract-peptone-dextrose (YPD) broth at 30 °C during 72 h. The culture was centrifuged at 10,000 rpm for 3 min for more biomass recovery. The pellet was used for total DNA extraction as described by Querol *et al.*, (1992) with chitinase instead of lyticase. The ITS region of rDNA was amplified with primers ITS1 and ITS4 (White *et al.*, 1990).

PCR amplifications were carried out according to da Silva-Filho *et al.* (2005). The PCR products were separated and analysed by 1.4 % (w/v) agarose gel (Invitrogen).

Sequencing reactions were performed with BigDye™ Terminator v3.1 Cycle Sequencing (Applied Biosystems) following the manufacturer's protocols with the same primers as those used in the PCR. Sequencing reactions were purified with AutoSeq™ G-50 column (Amersham Biosciences) with 18 µl of formamide being added. Sequences were obtained by capillary electrophoresis on an ABI-Prism 310 Genetic Analyzer (Applied Biosystems).

For the DNA sequence analyses, the sequence was edited using Mega 5.1 (Tamura *et al.*, 2011) and aligned with the sequences of Type strains that were obtained in the GenBank database at <http://www.ncbi.nlm.nih.gov/nucleotide> using the CLUSTAL method (Thompson *et al.*, 1994) available in the program MEGA5 (Tamura *et al.*, 2011).

2.5 Solid-state fermentation

To evaluate the production of endoglucanase (EG), xylanase (XYL) and polygalacturonase (PGA) under the same culture conditions, four of the oranges-isolated strains and identified were selected along with *Trichoderma reesei* ATCC 26921. The strains were incubated in PDA at 30°C for 4-5 d. The spores obtained were transferred to 5 mL of 0.1% Tween-80 sterile solution. The inoculum for the fermentation was obtained as follows: the spore suspension was mixed with 500 mL Toyama culture medium at pH 5 (previously sterilized) containing 10 g (NH₄)SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O and 0.5 g CaCl₂ per liter of solution (Mamma *et al.*, 2008). The spore concentration in the suspension was estimated by counting in a Neubauer chamber, and the initial spore amount in the fermentation was 1 × 10⁶ spores per g of peel.

Orange peels were collected from local markets in Guadalajara, Mexico. The endocarp and seed were removed, and the exocarp was washed twice with distilled water at 50°C for 45 min to eliminate sugars (Mamma *et al.*, 2008). Then, it was oven-dried at 70°C for 24 h and cut into irregular pieces measuring 1.5 ± 0.5 cm². The equipment used for the fermentation consisted of columns of 25 cm length and 4 cm diameter (Durand, 2003), which were sterilized at 121°C for 15 min. In Erlenmeyer flask, 20 g of dry orange peels were mixed with 7 g of ring-shaped plastic pieces of 5 mm diameter to allow the flow of air and avoid clogging and then sterilized at the same conditions. The filled flask were inoculated with the spores prepared as previously described, mixing to guarantee homogeneity. The mixture was placed into the column and installed in a water bath at 30°C. At the base of the column, a vessel with sterile water was placed to humidify the air. The air supplied during fermentation to the columns with the inoculated peels was filtered with 0.22 µm membranes, to maintain the peels humidity at 70%. The flow rate was 0.12 l/h-g substrate.

For the quantification of enzymes, a sample was taken every 24 h. For this step, the column was removed and its contents placed in a flask with Toyama medium at a 10:1 (v/w) ratio. The medium was shaken at 150 rpm for 60 min at 30°C. The mixture was filtered through a synthetic mesh and clarified by centrifugation at 12,000 × g at 4°C for 15 min. The supernatant was used for the determination of the enzymatic activity (Mamma *et al.*, 2008).

2.6 Determination of the enzymatic activity

The supernatant (0.5 mL) obtained from fermentation was mixed with 1.5 mL of the solution with the substrate. The EG, XYL or PGA activity was determined with 1% solutions of carboxymethylcellulose, wood xylan or citrus pectin (they were purchased from Sigma-Aldrich Co.), respectively, in citrate-phosphate buffer at pH 4.8 for EG and XYL at 5.8 for PGA. The mixture was held at 50°C for 30 min. The release of reducing sugars was determined by 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). One unit (U) of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of reducing sugars per min. The results are expressed as units per g of dry peel (U/g DP).

3 Results and discussion

3.1 Characterization of orange peels

The chemical composition of orange peels to be used in the SSF was determined. Table 1 shows the results obtained. It can be observed similarities between the results obtained in this study and those reported by Mamma *et al.* (2008) for orange peels, such as hot water-extractable compounds (46.9%), pectin (17.4%) and cellulose (14.9%). The composition of lignin (3.9%), ethereal extract (1.9%) and ashes (3.5%) differs slightly compared to that report. These differences in the composition of the peels can be due to several factors such as the orange species, maturity level, region where they were grown (type of soil and components), season of the year in which the fruit was harvested and sample treatment, among others. The cellulose, hemicellulose and lignin content of orange peels was found to be lower than those of agro-industrial residues (sugarcane, corn stubble, wheat straw and sweet sorghum), which contain 16-23% lignin, 16-24.5% hemicellulose and 32-39% cellulose (Prinsen, 2010). Lignin is a complex molecular structure present in plant cell walls that confers structural support and resistance against microbial attacks (Kumar *et al.*, 2009). The lignin present in the polymer matrix covers and protects the cellulose microfibrils (Ramírez-Carmona and Muñoz-Blandón, 2016). Therefore, high lignin content limits the access of microorganisms to cellulose and hemicellulose, which could reduce the production of cellulase and hemicellulase. In contrast, the lowest content of lignin in the orange peels could favour the enzyme production because the tissue structure is more flexible, allowed the access of the microorganism to

the cellulose and hemicellulose.

It has been demonstrated that the production of cellulase and hemicellulase is inducible and is affected by the nature of the substrate used in the fermentation (Kang *et al.*, 2004). It is expected that the cellulose, hemicellulose and pectin of the orange peels serve as inducers of the enzymes for a high production of these.

3.2 Microorganism isolation, selection and identification

To find microorganisms that produce enzymes with higher affinity for the citrus fruits' components, 16 strains fungi were isolated from rotten oranges and purified. Four strains were selected for their ability to grow on cellulose, xylan and pectin suggesting that they could produce the enzymes of interest, as has been reported by other authors (Mikán and Castellanos, 2004). The selected strains were molecular identified as *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1 and *Penicillium minioluteum* N3C2.

3.3 Production of endoglucanases

The production of EG (endoglucanase) by SSF was evaluated with strains *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1, *Penicillium minioluteum* N3C2 and *Trichoderma reesei* ATCC 26921. *Trichoderma reesei* is one of the microorganisms most commonly used since its high production of lignocellulosic enzymes, in different substrates and fermentation conditions (Domingues *et al.*, 2000; Martins *et al.*, 2008; Olsson *et al.*, 2003; Neagu *et al.*, 2012; Liming and Xueliang, 2004).

Table 1 Orange peels chemical composition.

| Compound | % (w/w dry basis) | |
|-------------------------|-------------------|-------------------------|
| | This study | Mamma <i>et al.</i> (3) |
| Water soluble materials | 46.9 | 41.1 |
| Pectin | 17.4 | 14.4 |
| Fibres fraction | 35.7 | |
| Hemicellulose | 7.6 | 13.8 |
| Cellulose | 14.9 | 16.2 |
| Lignin | 3.9 | 1.0 |
| Protein | 3.9 | 7.9 |
| Crude fat | 1.9 | 3.9 |
| Ash | 3.5 | 1.7 |

Trichoderma reesei ATCC 26921 is known for its high production of cellulolytic enzymes (Tengerdy and Szakacs, 2003); it is a mutant strain of *Trichoderma viride* ATCC 24449, which is used in the production of cellulase in the commercial product Celluclast (Novozymes Corp.). Therefore, its enzyme production was also evaluated in this study to compare it with the strains isolated from citrus fruits under the same culture conditions (pH, temperature, substrate amount and humidity).

Out of the five strains examined, *Penicillium minioluteum* N3C2 exhibited the maximum EG activity, with 6.5 U/g DP (units per g of dry peel). Analysis of variance revealed a statistically significant difference ($P < 0.05$) for this strain compared to the rest, and this strain reached the maximum activity at 72 h (Fig. 1-a). In other study *Penicillium decumbens* by SSF using orange peels as a substrate reached the maximum activity after 10 d (Mamma et al., 2008). *Penicillium minioluteum* N3C2 produced almost twice of EG than *T. reesei* ATCC 26921. It has been reported that the sugars released by the enzyme produced during fermentation inhibit the production of cellulase, so it is possible that in this study, the production of EG was stopped by the presence of sugars released by the peel hydrolysis. *T. reesei* ATCC 26921 achieved 2.7 U EG/g DP after 72 h (Fig. 1-b), and the maximum activity (3.3 U/g DP) was reached after 120 h. Filamentous fungi are the more prolific source of commercial cellulase. Among the biomass-degrading fungi, the strains of *T. reesei* are considered undisputed leaders in the industrial production of these enzymes. However, recent publications have demonstrated that *Penicillium* species exhibit higher cellulolytic activity compared to *T. reesei* under the same culture conditions, with values up to 80 times higher for β -glycosidase activity in *Penicillium* (Gusakov and Sinitsyn, 2012; Krogh et al., 2004). Furthermore, significant differences have been found in the induction and regulation of cellulase by the substrate in *Trichoderma* and *Penicillium*. It has been reported that some substrates are more favorable for some fungal genera than for others, even across species of the same genus (Gusakov and Sinitsyn, 2012). In this study, *P. minioluteum* N3C2 was isolated from orange peels, but *T. reesei* ATCC 26921 is a mutant strain that has been used with lignocellulosic residues as a substrate. This fact could explain the higher cellulase production in *P. minioluteum* N3C2 because the specificity for the substrate is a key condition for the production of lignocellulosic enzymes (Tengerdy and Szakacs, 2003).

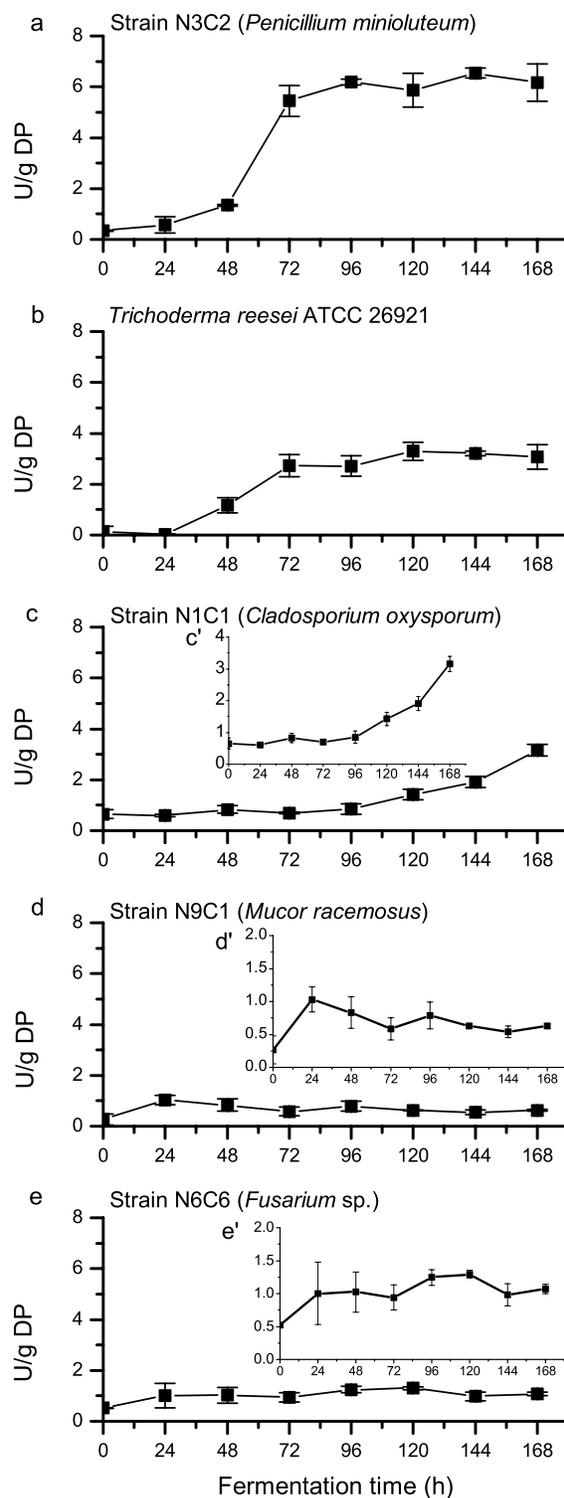


Fig. 1 Profiles of endoglucanase production by solid state fermentation from orange peels with strains isolated from citrus and *T. reesei* ATCC 26921.

Fusarium sp. N6C6 achieved the maximum activity of 1.3 U/g DP after 120 h (Fig. 1-e). This activity comes down after 144 h to slightly increase again after 168 h to 1.1 U/g DP (Fig. 1-e).

Cladosporium oxysporum N1C1 exhibited an increasing of EG activity after 96 h reaching the maximum activity (3.2 U/g DP, Fig. 1-c) at 168 h, but the EG activity does not reach a maximum that remains constant. It is possible that with longer fermentation time, a higher production of these enzymes could be achieved. Nevertheless, EG activity is similar to that obtained with *T. reesei* ATCC 26921. Zhao et al. (2009) produced and purified a new β -glycosidase (cellulase) with *Cladosporium fulvum* isolated from tomato. There are few reports on the production of cellulase with genus *Cladosporium*, so it would be interesting to continue the study of enzymes from this genus.

The maximum EG activity obtained with *Mucor racemosus* N9C1 occurred at 24 h (1.0 U/g DP, Fig. 1-d), declining at 48 h, then slightly increasing again (0.8 U/g DP) after 96 h and finally decreasing again at 120 h (Fig. 1-d'). This behaviour suggests the enzymes can be degraded by proteases during the fermentation, which is observed with the decrease in activity. The enzymatic activity obtained with *Mucor racemosus* N9C1 represents the 30% of the *T. reesei* ATCC 26921, but in lower time. Saha (2004) used the strain *Mucor circinelloides* for the production of cellulase (EG) using lactose and corn liquor as substrates. For the *Mucor* genus as well, there are few reports on the production of cellulase. This loss of activity could be due to the presence of proteases or the culture medium's acidity (Haab et al., 1990). There are few reports regarding the production of cellulase with *Fusarium* species from orange peels. Panagiotou et al. (2003) optimized the cellulolytic activity of a *Fusarium oxysporum* strain by SSF. In this study, the conditions were kept constant and no optimization was performed, so it would be interesting to continue this work with *Fusarium* sp. N6C6.

It is worth noting that it is not possible to compare enzymatic activity for strains used in this study with those reported in the literature because in some cases, the enzymes are purified or different substrates are used, and although some microorganisms belong to the same genus, the species are different, so the enzymes could show a maximum activity at different conditions of temperature, pH, buffer type, etc.

3.4 Xylanase production

Aspergillus and *Trichoderma* genera have been widely studied among the XYL producer fungi at industrial scale. However, in recent years, a considerable amount of information regarding the production of XYL by fungi from the *Penicillium* genus has been accumulated. Krogh et al. (2004) tested different *Penicillium* strains, observing differences in the production of β -xylosidase and endoxylanase. Moreover, several studies have been performed to test the effect of different culture conditions in the production of XYL by *Penicillium canescens*, *Penicillium janthinellum*, *Penicillium oxalicum* and *Penicillium decumbens* (Bakri et al. 2003, Oliveira et al. 2006 and Li et al. 2007, Mamma et al., 2008). For the production of XYL, the same strains mentioned for the production of cellulase were used under the same culture conditions. *P. minioluteum* N3C2 and *T. reesei* ATCC 26921 strains exhibited the highest xylanolytic activity compared to the other strains tested (Fig. 2-a and 2-b), and the values were similar: 14.5 and 14.7 U/g DP, respectively. The variance analysis for a confidence level of 95% indicated that there is no significant difference among them, but there is a difference when they are compared to the rest of the strains. However, *P. minioluteum* N3C2 achieved the maximum activity after 72 h, and *T. reesei* ATCC 26921 reached an activity of 13 U/g DP after 72 h, and after 144 h achieved the maximum activity (14.5 U/g DP). The products of the degradation of cellulose can be different substrates, suggesting that after 72 h, *T. reesei* ATCC 26921 reorganizes its molecular components, synthesizes new enzymes and represses the synthesis of others based on the available nutrients (Shuler and Kargi, 1992). This finding can be explained by the regulation of the enzyme expression because of the availability of the carbon source (Aro et al., 2003).

The XYL production of the strains tested, with the exception of *C. oxysporum*, indicates an initial XYL activity of 1-3 U/g DP (Fig. 2). This is probably due to the production of XYL by the fungi in the inoculum; when transferred to the culture medium with the orange peels, there is a high initial enzymatic activity. It can also be inferred that the XYL detected at the beginning of the fermentation are constitutive because the culture medium of the inoculum contained dextrose as substrate. This effect is not so notable with *P. minioluteum* N3C2 and *T. reesei* ATCC 26921 as in the XYL production profiles of *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1 and *Mucor*

racemosus N9C1 because the production of XYL is relatively low with the latter.

It had been reported xylanase production of XYL by *Cladosporium cladosporioides* and *Cladosporium herbarum* species (Hong et al. 2011; Abdel-Sater and El-Said 2001). In this study, *Cladosporium oxysporum* N1C1 XYL profile (Fig. 2-c') shows that the activity was constant and near 1.0 U/g DP throughout the fermentation. This behaviour suggests that the enzyme is not produced since they remain constant for a long time and the xylanase activity is less than 1 U/g DP most of the time.

The strain identified as *Mucor racemosus* N9C1 exhibited increased enzymatic activity after 48 h (Fig. 2-d and 2-d'), reaching its maximum activity at 96 h (4.6 U/g DP) and decreasing at the end of the fermentation (3.0 U/g DP). This decrease could be due to enzyme degradation by proteases. Grajek (1987) obtained XYL production by *Mucor pusillus*. To the best of our knowledge, there are no reports of the productions of these enzymes with *Mucor racemosus*.

Fusarium sp. N6C6 maintained XYL activity from 2.9 to 4.6 U/g DP, reaching its maximum level at 96 h (Fig. 2-e). This strain also exhibits a maximum production and a slight decrease in the enzymatic activity at the end of fermentation, which is attributed to the degradation of the enzymes by protease. The production of XYL has been reported with *Fusarium oxysporum* (Arabi et al., 2011; Gupta et al., 2009; Panagiotou et al., 2003; Xiros et al., 2008) at different conditions and substrates. The production of XIL with other species of *Fusarium* has not been reported.

Although *C. oxysporum*, *Fusarium* sp. and *M. racemosus* produced a smaller amount of enzymes than *T. reesei* ATCC 26921, they have potential for their use because they produce them in a shorter time.

3.5 Pectinase production

Fusarium sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1 and *Penicillium minioluteum* N3C2 strains produced PGA in SSF under the same culture conditions. Fig. 3 shows the production profiles of these enzymes.

Fusarium sp. N6C6 exhibited the highest PGA activity (16.5 U/g DP). The analysis of variance, for a confidence level of 95%, indicated that the PGA production for this strain is significantly different from that of other strains. After 48 h, the enzymatic activity was constant (3-e). It was not observed enzyme degradation since the activity remained constant for 168 h.

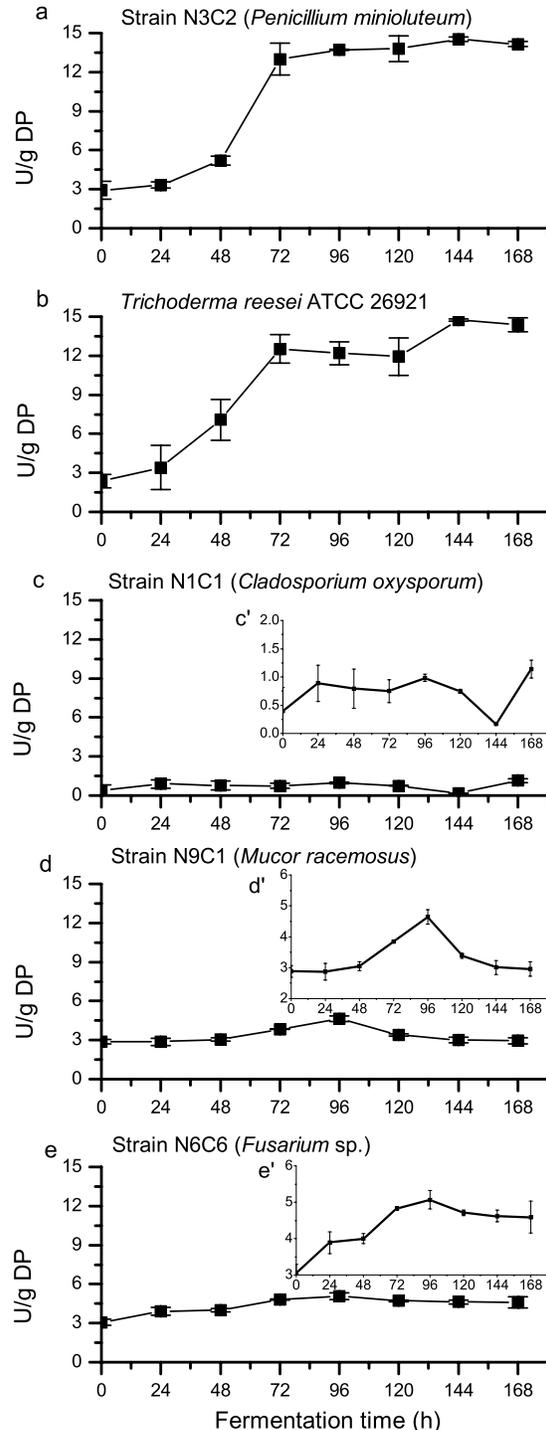


Fig. 2 Profiles of xylanase production by solid state fermentation from orange peels with strains isolated from citrus and *T. reesei* ATCC 26921.

There have been different studies in which the production of these enzymes with *Fusarium* species

with varied substrates (Gupta *et al.*, 2009; Mamma *et al.*, 2008; Panagiotou *et al.*, 2003; Xiros *et al.*, 2008). It is possible that the presence of cellulase produced by the microorganism released glucose from the peel's cellulose and consequently the glucose repressed the production of pectinase (Jayani *et al.*, 2005). The pectinase activity of *Fusarium* was 2-fold higher than *T. reesei* ATCC 26921. *Mucor racemosus* N9C1 showed PGA activity of 12.3 U/g DP. However, the production began until 96 h (Fig. 3-d). Saad *et al.* (2007) produced a polygalacturonase from *Mucor rouxii* NRRL 1894 using citrus pectin as an inducer. *T. reesei* ATCC 26921 exhibited a maximum PGA activity (8.2 U/g DP, Fig. 3-b) at 72 h, lower than that obtained by *Fusarium* sp. N6C6 and *Mucor racemosus* N9C1. This low production is consistent with reports by other authors, in which cellulase production and the subsequent release of glucose was found to repress the expression of pectinase in *T. reesei* (Olsson *et al.*, 2003). However, it can be observed that pectin from orange peels induces PGA production.

Cladosporium oxysporum N1C1 revealed an increase in the production of pectinase after 48 h (Fig. 3-c), reaching a maximum at 144 h with 8.0 U/g DP. For this microorganism, the information regarding the production of pectinase is scarce because this fungus does not produce large quantities of these enzymes. Skare *et al.* (1975) tested a strain of *Cladosporium cucumerinum* to produce pectinase from cucumber, but for the purpose of relating their pathogenicity mechanism towards cucumber cultivars and not to produce these enzymes for other applications. *C. oxysporum* N1C1 produced a similar amount of pectinases than *T. reesei* ATCC 26921.

Penicillium minioluteum N3C2 exhibited low pectinase production compared with the other strains. The PGA production began from 24 h reaching the maximum (6.2 U/g DP, Fig. 3-a) after 168 h of fermentation, although not much lower than that obtained by the other strains. Mamma *et al.* (2008) obtained polygalacturonase and pectate lyase with a *P. decumbens* strain. Additionally, Silva *et al.* (2005) performed SSF using a *Penicillium viridicatum* strain and obtained endo-polygalacturonase, exo-polygalacturonase and pectin lyase.

3.6 Multi-enzyme fungi

All of the strains tested, (*Fusarium* sp. N6C6, *C. oxysporum* N1C1, *M. racemosus* N9C1, *P.*

minioluteum N3C2 and *T. reesei* ATCC 26921),

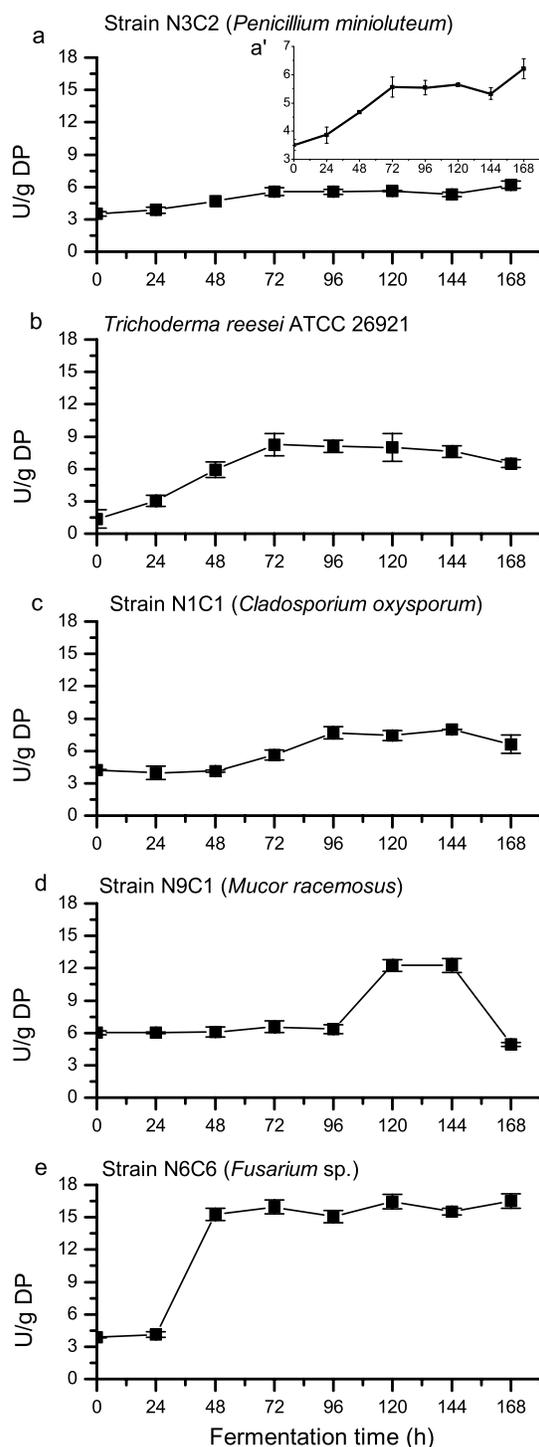


Fig. 3 Profiles of polygalacturonase production by solid state fermentation from orange peels with strains isolated from citrus and *T. reesei* ATCC 26921.

Table 2 Maximum enzyme activities and volumetric productivity.

| Microorganism | Endoglucanase | | Xylanase | | Polygalacturonase | |
|--------------------------------------|---------------|----------------|-------------|---------------|-------------------|---------------|
| | $Y_{E/S}$ | r_E | $Y_{X/S}$ | r_X | $Y_{P/S}$ | r_P |
| <i>Fusarium</i> sp. N6C6 | 1.3 ± 0.06 | 0.006 ± 0.0002 | 5.1 ± 0.26 | 0.027 ± 0.003 | 16.5 ± 0.68 | 0.098 ± 0.012 |
| <i>Cladosporium oxysporum</i> N1C1 | 3.2 ± 0.24 | 0.019 ± 0.005 | - | - | 8.0 ± 0.00 | 0.040 ± 0.005 |
| <i>Mucor racemosus</i> N9C1 | 1.0 ± 0.19 | 0.004 ± 0.0008 | 4.6 ± 0.23 | 0.018 ± 0.002 | 12.3 ± 0.63 | 0.030 ± 0.009 |
| <i>Penicillium minioluteum</i> N3C2 | 6.5 ± 0.20 | 0.037 ± 0.004 | 14.5 ± 0.19 | 0.084 ± 0.03 | 6.2 ± 0.35 | 0.037 ± 0.010 |
| <i>Trichoderma reesei</i> ATCC 26921 | 3.3 ± 0.36 | 0.018 ± 0.007 | 14.7 ± 0.10 | 0.086 ± 0.02 | 8.2 ± 1.03 | 0.039 ± 0.015 |

exhibited the ability to produce EG, XYL and PGA simultaneously. The volumetric productivity (q_P) was calculated based on the enzymatic activity reported over the fermentation time. Table 2 summarizes the maximum enzymatic activities and the enzymatic formation rate for the three enzymes for all five strains, in which it can be observed that the EG activity was the lowest for all strains.

On average, the EG activity was seven times lower than the XYL activity. *Fusarium* sp. N6C6 and *Mucor racemosus* N9C1 produced a XYL/PGA ratio of 1.15, in contrast to Mamma *et al.*, (2008), who obtained a XYL/PGA ratio of 0.14 with *F. oxysporum* using orange peels as a substrate and while PGA/EG enzymatic activity ratio were near 10. This finding can be attributed to the fact that the strains in this study were isolated from citrus, with lower cellulose content than that found in lignocellulosic residues. On the other hand, *P. minioluteum* N3C2 and *T. reesei* ATCC 26921 produced between four and five times more XYL than PGA. *P. minioluteum* N3C2 also exhibited a different behaviour compared to *Penicillium decumbens* using orange peels as a substrate, because this strain exhibited EG and XYL activities with similar ratios, and PGA enzymes had the highest activity, at least twice as high as that of XYL and EG (Mamma *et al.*, 2008). Regarding the volumetric productivity (q_P), the XYL were produced at higher rates and EG were produced at the lowest rates.

The strains with the highest EG and XYL production were *T. reesei* ATCC 26921 and *P. minioluteum* N3C2. The highest EG enzymatic formation rate (q_P) was obtained with strain *P. minioluteum* N3C2, with a q_P of 0.04 U/g-h. The productivity of *P. minioluteum* N3C2 was twice as high as that obtained with *T. reesei* ATCC 26921. Moreover, the highest XYL formation rate was obtained with *T. reesei* ATCC 26921 and *P. minioluteum* N3C2, with a q_P of 0.09 and 0.08 U/g-h. *Fusarium* sp. N6C6 exhibited the highest PGA activity and q_P , with the production rate being twice as high as the rest of the strains. Mikán and Catellanos

(2004) isolated microorganisms for the degradation of cellulose and hemicellulose, identifying *Fusarium oxysporum* as one of the fungi that exhibited the capacity to degrade cellulose, xylan and pectin in synthetic culture media containing the above-mentioned substrates as the only carbon source.

Mucor racemosus N9C1 also exhibited a considerable PGA activity (the highest after *Fusarium*) but an EG activity six fold lower (the lowest of the strains produced). This result contrasts with other reports because the genus *Mucor* has been used to produce cellulase such as β -glycosidase (primarily) in addition to endoglucanase and cellobiohydrolase (Saha, 2004; Borgia and Mehnert, 1982; Krisch *et al.*, 2010). For this genus, the production of pectinase (Saad *et al.*, 2007; Geetha *et al.*, 2012) and, in a few cases, xylanase (Grajek, 1987; Yadav, 2011) has also been reported, although there is only slight production of the latter. However, the production of EG, XYL and PGA has not been evaluated simultaneously as in this study.

Cladosporium oxysporum N1C1 exhibited EG and PGA activities similar to those of *T. reesei* ATCC 26921 but also a tendency to increase EG production after 168 h of fermentation; therefore, it is able to produce more EG with higher fermentation time. The genus *Cladosporium* has only minimally been used for the production of enzymes despite its confirmed capacity to produce pectinolytic and ligninolytic enzymes (Hankin and Anagnostakis, 1975; Geetha *et al.*, 2012), although not simultaneously as is demonstrated in this study.

Conclusion

The strains isolated from oranges produce EG, XYL and PGA simultaneously. To our knowledge, this is the first report of the simultaneous production of these enzymes with *Mucor racemosus* and *Cladosporium oxysporum*. The strains evaluated exhibited enzymatic activities similar or superior to those produced by *Trichoderma reesei* ATCC 26921. *P. minioluteum*

N3C2 reach the maximum activity in a shorter time than *T. reesei* ATCC 26921 suggesting that it could have greater affinity for the components of the orange peels. The enzymatic activity ratio of EG/XYL/PGA was influenced by the isolation origin of the strains, because with the orange peels, the production of EG was lower than that of XYL and PGA. Future studies with these microorganisms will be focused on optimizing culture conditions and purifying enzymes to characterize the different types of enzymes present, as well as increasing the production of enzymes.

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