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Cellulolytic Enzymes Production by Solid State Culture Using Pecan Nut Shell as Substrate and Support

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Abstract: Problem statement: Great interest in the use of lignocellulosic biomass is increasing in order to diminish the accumulation of residues, such as pecan nut shells. One of the alternatives is the fungal degradation of these residues. **Approach:** The capacity of Trichoderma (coded as T1, T2 and T3) strains to produce cellulase and xylonite was evaluated. **Results:** Pecan nut shell fibers were used as sole carbon source. The fiber characterization study showed that cellulose levels were of 0.1% while hemicellulose was up to 25 %. Three Trichoderma strains were used on solid fungal cultures using the fibers as sole carbon and inductor source for the production of cellulolytic enzymes. The behavior of the sugars liberated by the fungi showed that the strain T2 is able to accumulate more monomeric reducing sugars than the other two strains, this could be attributed at this strain has a higher sugar liberation rate and slower sugar consumption rate. This strain also expressed more cellulase and xylanase activity. The low quantity of cellulose registered in the fibers can still be used to induce cellulase activity. **Conclusion:** The T2 strain had the highest level of enzymatic activity both cellulase and xylanase.

Key words: Cellulase production, solid-state culture, maintain microbial growth, lignin limits, distilled water, cellulolytic enzymes, growth culture, tannin-degrading, trichoderma strains, tannin absence, cellulose oligomers, pecan nut shell fibers, sole carbon, enzymatic activity

INTRODUCTION

Cellulolytic enzyme production has acquired great importance due mainly to the interest in exploiting lignocellulose as energy source. Pecan nut shells (Carya illinoensis) consists on the major component of the nut and also the main residue generated in the use of this alimentary product (Medina *et al*., 2010). The strong association between cellulose, hemicellulose and lignin limits the application of these residues on certain processes, such as biomolecules and biofuels (Faria-Martins *et al*., 2008, Daoud and Alam, 2010). Work is being made to improve the use of lignocellulose by hydrolysis. The process opted for the use is enzymatic hydrolysis, where cellulases and xylanases are required for this end. Many microorganisms are able to produce cellulolytic enzymes, Trichoderma is one of the most important microorganisms used in industry, allows a relatively higher enzyme production of cellulase, consisted mainly of cellobiohidrolases, endoglucanases and β-glucosidases (Fang *et al*., 2010). Cellulases are composed by three different enzymes which are, endoglucanases, this enzymes degrade the inner regions of cellulose to disrupt the polymer chains, simultaneously cellobiohydrolases degrade the cellulose ends releasing cellulose oligomers and cellobiose and β-glucosidase, which turn the latter molecule to glucose (Sukuruman, *et al*., 2009). One of the approaches that cellulase production is solid state culture, which is defined as the growth of microorganisms on moistened solid substrate, which enough moisture is present to maintain microbial growth and metabolism, but there is no or little free moving water (Orzua *et al*., 2009). There are no reports of using pecan nut shell fibers on fungal cultures for the production of cellulolytic enzymes. The objective of this study is produce cellulolytic enzymes using this kind of substrate on solid state cultures.

MATERIALS AND METHODS

Plant fiber characterization: The procedure of hemicellulose, lignin and cellulose content were

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determined by a gravimetric technique by the acid and neutral detergent fiber method (Van Soest *et al*., 1991, Jahani-Azizabadi *et al*., 2010).

Tannin removal procedure: In a 1:4 ratio, 25 g of shells were suspended in 100 mL of distilled water and in a secuential extraction, tannins were measured until removal was achieved. The plant material was filtrated and dehydrated for later experiments.

Growth assay and culture conditions: For this study, three Trichoderma strains were used and were coded as 1, 2 and T3. A control strain of Aspergillus niger GH1 was used as a control for the growth experiments. All these strains are part of the UAdeC-DIA collection. All of the strains were propagated in PDA agar slants and the spores were harvested using Tween 80 0.01%. In petri dishes, a shells-media preparation was made using 3 g of plant material with 7 mL of sterilized liquid media which was composed as follows: (g L^{-1}) $NaNO_3$ 2.5, KH_2PO_4 1.0, KCl 0.5 and $MgSO_4$ 0.5. Growth was registered every 12 h.

Fungal cultures using Trichoderma strains: This experiments were made in a similar fashion as the latter. Samples were obtained every 24 hours until a culture completion time which was at 168 hours. Reducing sugars by Somogyi-Nelson method (Falcón *et al*., 2009) and also cellulase and xylanase activities were measured using carboxymethyl cellulose and xylanes as substrate.

All experiments were carried out in triplicate and were analised in Microsoft Excel®.

RESULTS

Plant material fiber characterization: The hemicellulose values obtained are not as low as cellulose and it is among other values reported for different plants. Being an objective of our work the fungal cultures using PNS and the production of cellulolytic enzymes, fungal solid cultures were made to assay the invasion capacity of Trichoderma strains. The Trichoderma strains were able to grow in the culture system (data not shown), but no difference in how fast they grew was detected, so all strains are usable for the programmed experiments.

Growth assay and enzyme production: A growth culture was made using Aspergillus niger GH1 to compare another fungi with the Trichoderma strains and we observed that A. niger GH1 was unable to grow on those culture conditions. The fact that a tannins removal process was made on the PNS, could be the reason of why the latter strain was inhibited. For the enzyme production three Trichoderma strains were used coded as 1, 2 and T3. A growth assay showed that all strains were able to use pecan nut shells as sole carbon source; hence they can synthesize the enzymes required for the degradation of the shell components as showed the cellulase and xylanase assays with positive results. Latifian *et al*. (2007) and Al-Taweil *et al*. (2009), reports cellulase production using a Trichoderma strain where cultures were made in a similar way as the present work. After the solid fungal cultures were finished, the behavior on the total and reducing sugars profile was analyzed, showing different values of sugars liberated by fungal degradation as can be seen in Fig. 1-2, shows that the T2 strain is able to liberate a higher quantity of sugars beginning at 24 h steadily until the end of fermentation time at 168 h reaching a maximum of 0.39 g L^{-1}.

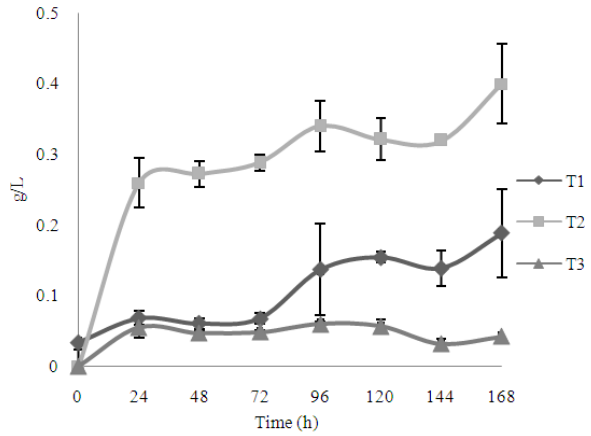

Fig. 1: Reducing sugars behavior during culture time by Trichoderma. T1, T2 and T3

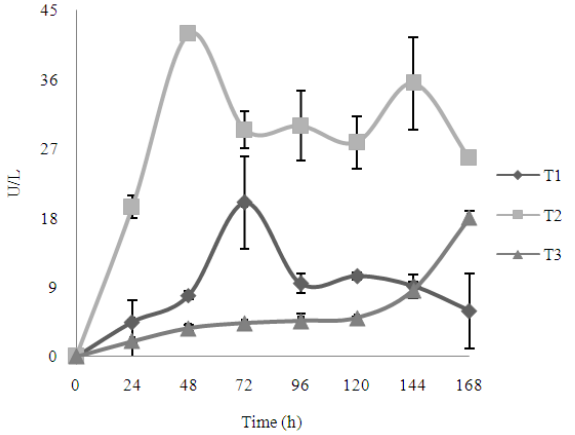

Fig. 2: Cellulase activity registered on the fermentative process

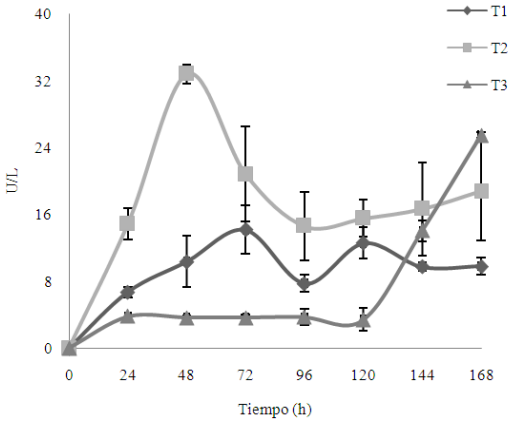

Fig. 3: Xylonite activity registered in the fermentative process

The strains 1 and T3 showed lesser values. The T1 strain liberated at highest 0.18 g/L and T3 0.059 g L^{-1}. In Fig. 2-3 is shown that both xylanase and cellulase activities were at their peak at 48 h of culture having 32.8 U/L and 41.9 U/L of xylanase and cellulase activities. The registered activities on both fungi (T1 and T3) had their peak of maximum activity in later hours and less quantity. T2 strain, according to the Fig. 2, produces more active enzymes than the other two strains. The T2 strain apparently has a slower consumption, due to the accumulation of sugars which is shown in Fig. 2, this behavior was different on the two other strains where the levels liberated were lower.

DISCUSSION

As can be seen in the Table 1 the cellulose percentage in our material is very low compared to the values reported by other authors where the minimum quantity is 7% on grape seeds (Couto and Sanroman 2005; Zhang *et al*., 2010). This strain is reported as tannin-degrading (Mata-Gomez *et al*., 2009). In a previous study (Medina *et al*., 2010), using the Aspergillus strain, solid cultures were made and the fungi could proliferate on the system which was tannin-rich. In the case of the actual cultures of this study, the tannin absence, possibly affects the growth of another strains, such as Aspergillus.

Concerning the fiber composition of the PNS with the capacity of the Trichoderma strains, these are able to use to their advantage the components of the shells. Working on solid state systems has several advantages. The moisture levels are low, just enough for the microorganism to proliferate on the substrate. This system emulates the natural conditions where many microorganisms were isolated from and high adaptability is expected. Also sterility is not necessary.

Table 1: Percentages of cellulose, hemicellulose and lignin content in the shell with and without tannins

Sample	Lignin (%)	Hemicellulose (%)	Cellulose (%)
S(T)	17.61 +/- 3.91	10.39 +/- 0.08	1.0 +/- 0.29
S(TF)	5.44 +/- 0.08	24.62 +/- 0.08	0.9 +/- 0.06

S(T): Shells with tannins; S(TF): Shells tannin-free

High levels of substrate, in the form of agroindustrial wastes can be used (Singhania *et al*., 2009). A high accumulation of sugars can be the result of high enzyme activity of the enzymes produced by the fungi T2, in other words, the fungi has a low sugar consumption rate which promotes higher levels of sugars without consumption tendency, also biomass is forming, so metabolism is active. There is another phenomenon related, the enzymatic hydrolysis rate. The high levels of sugars liberated by T2, compared to the other strains, besides of apparent slow consumption, can be attributed to high cellulolytic and xylanolytic activity. The enzymes cleave the polysaccharides releasing monosaccharides faster than they are being consumed by the fungi.

The fact that cellulase activity is present although there is a minimum amount of cellulose present in PNS available for the fungi to degrade. Lu *et al*. (2010), mentions in their research that many hydrolytic enzymes can be expressed by a fungi by degrading certain substrates. Xylose degradation in fungal metabolism can manifest the production of many proteins such as arabinase and cellulase enzymes, probably due to the fact that xylose, as part of hemicellulose, is naturally bound to many types of sugars or polysaccharides. The fungi, while detecting the presence of xylose, can produce the enzymes responsible for the degradation of the compounds that could be bound to the xylose, such as arabinanes, mannanes, xylans and cellulose, hence the production of those enzymes are present in the fungal extracellular proteome.

The ability to degrade lignocellulose efficiently is thought to be associated with mycelial growth habit that allows the fungus to transport scarce nutrients such as nitrogen and iron, to a distance into the nutrient-poor lignocellulosic substrate that constitutes its carbon source (Taqhizadeh and Zabihollah, 2008). The fungal degradation occurs exocellularly, either in association with outer cell envelope layer or extracellularly, because of the insolubility of lignin, cellulose and hemicellulose. Many research groups are in progress in the production of cellulases and more specifically, using lignocellulolytic residues, where higher cellulose levels were used such as sawdust (Levin *et al*., 2007; Bhanaruddin *et al*., 2010), rice residues (Liu *et al*., 2006; Monte *et al*., 2010), corn stover (Faria-Martins *et al*., 2008; Daoud and Alam, 2010) to mention a few, their main goal is to achieve a more economically process attractive to improve costs on cellulase production.

CONCLUSION

Enzyme production processes, such as cellulase production can be made using pecan nut shells as carbon source. Fungal strains, such as the ones used in this study; which able to proliferate on this kind of substrate has high potential on the use of enzyme production or biodegradation of similar plant materials which have low cellulose content, but still enough to generate a response in the fungi to produce cellulase enzymes.

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