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Fungal Culture Systems for Production of Antioxidant Phenolics Using Pecan Nut Shells as Sole Carbon Source

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Abstract: Problem statement: Many agro-industrial wastes have little or none utilization, when these materials could be a very rich source of several value-added compounds, such as: the pecan nut shells, which contain Antioxidant Phenolic (AP) molecules like tannins. **Approach:** In this study, a bioprocess for the liberation of AP from Pecan Nut Shells (PNS) was described. A chemical characterization of raw material was evaluated to determine polyphenolic content of PNS, among other components. Several fungal culture systems were evaluated at 96 h fermentation processes and using PNS as sole carbon source. Solid (SSC) and Submerged (SmC) fermentations were carried out using three strains of *Aspergillus niger*. Culture medium was composed of a solid (10-50%) and a liquid (90-50%) part in order to complete the 100% of the medium. **Results:** A high concentration (19%) of tannins was found in PNS on a dry basis, from which condensed tannins and their monomers were found as the main fraction (14%) of tannins of PNS, also on a dry basis. In the strain selection step, *Aspergillus niger* GH1 showed better growth on pecan nut shells compared to *A. niger* PSH strain, therefore, *A. niger* GH1 was used for later experiments. The highest concentration of AP was obtained with SSC inoculated with spores of *A. niger* GH1 on the 40% of solids system and in 20% solids system where, among the other systems both presented 114 mg TP g^{-1} of phenolics liberation at 24 h of fermentation. PNS is an important source of catechin, being this kind of compound which can be liberated in higher proportion compared to other phenolics compounds. **Conclusion:** This study demonstrated that fermenting PNS represent a good alternative to both use residues and obtaining AP.

Key words: Pecan nut shells, fungal culture systems, antioxidant phenolics, Solid and submerged fermentation, *Aspergillus niger* GH1and PSH

INTRODUCTION

In North America, among the template weather zones of México and USA, the pecan nut tree grows producing nuts which have great demand in the food industry (Orzua *et al*., 2009). The main left-over of nut processing are the shells, which have in its composition many phenolics derived from tannins. There are four main groups of tannins, gallotannins, ellagitannins, condensed tannins and complex tannins (Khanbabaee and van Ree, 2001) According to other report, pecan nuts contain antioxidant phenolics and PNS contain higher amounts of these molecules, mostly condensed tannins (Villarreal-Lozoya *et al*., 2007). These compounds have many biological activities, such as antimicrobial, antimutagenic and antioxidant (Ascacio-Valdes *et al*., 2010; Abdul Rahim *et al*., 2010). The recalcitrancy of these molecules are due to the strong protein binding activity, so microbes tend to inhibit their growth in presence of these compounds. However, there are few microorganisms able to degrade them. Filamentous fungi have been studied for biodegradation of tannins and liberation of phenolics (Aguilar *et al*., 2004; Mata-Gomez *et al*., 2009). According to these kind of experiments, filamentous fungi are capable of metabolize tannins and in a given moment, liberate tannin-monomers, which can be isolated. Solid state cultures are systems which have received praise due to the higher yields of compounds of interest than submerged cultures and also being a low cost

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technology (Alam *et al*., 2005). The objective of this work was to determine the capability of three *Aspergillus niger* strains to use pecan nut shells to liberate antioxidant phenolics under solid and submerged state culture and to detect and quantify the amounts of tannin derived AP resulting of these processes.

MATERIALS AND METHODS

Microorganism: For this study, three strains of *Aspergillus niger (*GH1, PSH and Aa-20), belonging to the DIA-UAdeC collection previously isolated, conserved and characterized by Cruz-Hernandez *et al*. (2005), were used.

Plant materials: The pecan nut shells were obtained from the Southern region of Coahuila State, Mexico, during the summer season of 2009 and transported to the Microbiology Laboratory of our institute. PNS were dried at sun light and stored in a black bag.

Content characterization: The chemical assays (protein, fat, ashes, crude fiber and nitrogen free extract) carried out were performed by previously established methods (Williams, 1984).

Strain selection for the bioprocesses: Once PNS were dehydrated, we proceeded to make a strain selection step. Three *Aspergillus niger* strains were used for this purpose, Aa-20 (control strain), PSH and GH1, fungal strains were growth on Petri dishes containing PNS and culture broth (Pontecorvo medium) in a 30:70 relation, in order to evaluate their adaptability on PNS. Once the system was ready, 50 µL of spore suspension from the *A. niger* strains was placed in the center of the Petri dishes and mycelia growth was measured every 12 h.

Solid and Submerged State Cultures (SSC and SmC): The culture processes were carried out using 5 different shell concentrations (10, 20, 30, 40 and 50 g) adjusted with Pontecorvo culture broth (90, 80, 70, 60 and 50 mL, respectively). Culture broth composition was (g L^{-1}) $NaNO_3$ (0.6), KH_2PO_4 (0.152), KCl (0.052), $MgSO_4.7H_2O$ (0.052), $ZnSO_4$, (0.0001). Other components of the broth were yeast extract at 0.05% and 1 mL of trace metals solution, with a composition as follow (mg.L^{-1}) $Na_2B_4O_7$ (100), $Mn_2Cl.{}_4H_2O$ (50), $NaMoO_4.2H_2O$ (50) and $CuSO_4.2H_2O$ (250), the broth pH was adjusted to 6.2 with 0.02M KOH. Pecan nut shells were the only carbon source in the systems and were inoculated with 2×10^7 spores of *Aspergillus niger* GH1 and incubated at 30°C 48 h^{-1}. Samples were obtained every 12 h and stored at -4°C in an ethanol-water mixture (1:1) until further analysis.

Total hydrolysable phenols (HT) and Gallic acid determination: The first determination was carried out using the Folin-Ciocalteu method (Makkar *et al*., 1995), the other one with the Sharma *et al*. (2000) method, both standard curves were prepared with gallic acid.

Condensed Tannins (CT): The method utilized was the reported by Schofield *et al*. (2001) using a catechin standard curve.

Ellagic acid determination: This analysis was carried out by HPLC using a gradient method and the conditions were: 1 mL min^{-1} flow, using a Triphasic pump (Varian model 230), autosampler (Varian 410), a photo diode array detector UV-Visible (Varian 330) and a Prodigy ODS 5 µ (Phenomenex). The analysis conditions were as follow: A: Methanol, B: Acetonitrile and C: 0.3% Acetic acid at 0-5 min 7% B-93% C, 12-13 min 60% B-40% C and 15-25 min 7% B-93% C.

Total Phenols (TP) during fungal culture: This parameter was quantified by the sum of the results of the Folin-Ciocalteu and the Schofield methods which were Gallic acid and catechin equivalents respectively.

RESULTS

Chemical characterization: The results which can stand out of the chemical characterization (Table 1) are the fiber content of 62.88 and 19.22% of tannin equivalents of both hydrolysable and condensed tannins, from which shells have 14.46% of condensed tannins and 5.43% of hydrolysable tannins. These compounds were molecules that matter the most to our work for the subsequent experiments.

Strain selection: Results of this study showed the capacity of three strains of using PNS as a sole carbon source. *Aspergillus niger* GH1 and Aa-20 could grow on PNS until 72 h, where the experiment was stopped and *A. niger* PSH started and stopped its growth very quickly at 24 and 36 h respectively. The GH1 and Aa-20 strains showed good adaptation capacity, so both strains were capable to degrade PNS components; many of those components are tannins. Narrowing the analysis on the 12 h of growth, it was observed that GH1 could grow even more rapidly than Aa-20, having a more stable growth (Fig. 1), even though, Aa-20 grew on the shells 12 h earlier than GH1. Past 12 h, GH1 grows faster than the control strain, so GH1 must have a more developed tannin degrading enzymatic system than Aa-20.

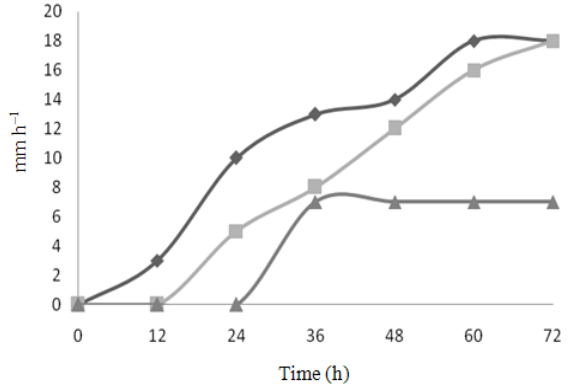

Fig. 1: Growth kinetic of *Aspergillus niger* GH1 (□), *Aspergillus niger* PSH (Δ) and *Aspergillus niger* Aa-20 (◊) strains on pecan nut shells

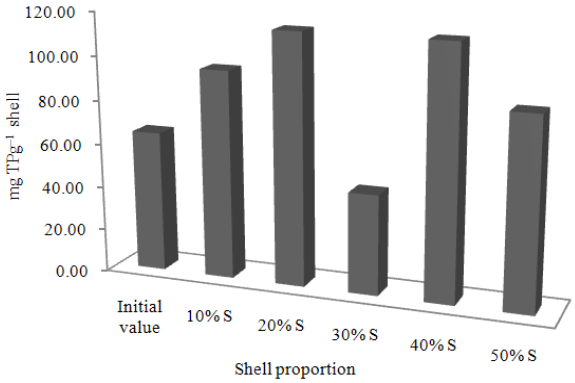

Fig. 2: Total phenolics liberated by *Aspergillus niger* GH1 from fermenting pecan nut shell at 12 h of culture

Table 1: Chemical content of the pecan nut shells

Parameters	Content (%)
Dry matter	91.90
Humidity	8.10
Ashes	2.28
Protein	4.29
Fat	1.36
Fiber	62.88
Nitrogen free extracts	29.19
Total sugars	2.83
Reducing sugars	0.03
Hydrolyzable tannins (GAe)	5.43
Condensed tannins (Ce)	14.46
Total phenols	19.12

AP liberation: In almost every culture systems, the AP level was at its highest point at 12 h. It was observed that in both 20 and 40% of PNS, the highest phenolic liberation was observed. The behavior of condensed tannins liberation (Fig. 3) showed high catechin concentration in the culture systems, while hydrolysable tannins had lower amounts.

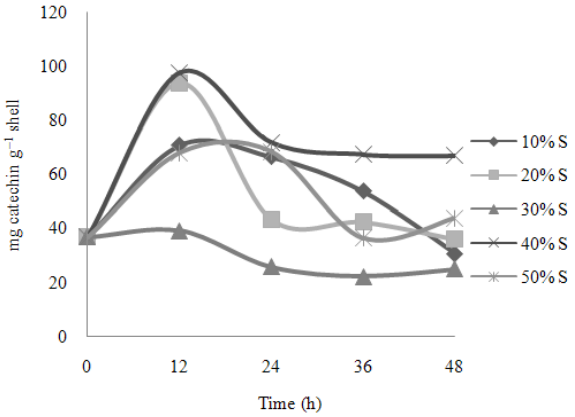

Fig. 3: Catechin equivalents liberated by *Aspergillus niger* GH1 al and from fermenting pecan nut shell

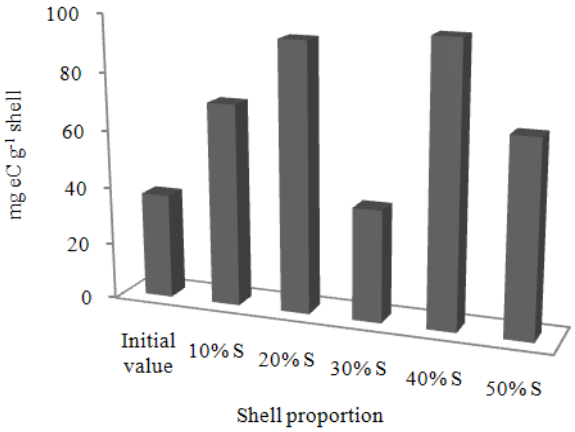

Fig. 4: Catechin equivalents at 12 h of culture

Having the sum of these compounds, in the Fig. 2 the TP pattern during the culture demonstrate that there is a phenolics liberation; consequence of the microbial hydrolysis of phenolics. In all systems, except 30% shell, there's a steady liberation of TP where were registered in the 20 and 40% shells values up to 114.48 and 114.17 mg TP g^{-1}, followed by 10% shell with 95.31 mg g^{-1}. All systems had an increase in both CT and HT at 12 h (Fig. 4). In this work, by the Folin-Ciocalteu assay, we could quantify up to 24.6 mg g^{-1} of HT in shells in the 10% shell system, in our process we obtained 114.48 mg g^{-1} of total phenolics by culture on the 20% PNS system. According to the chemical content, most of the phenolics present are CT so a major liberation of CT monomers could be expected.

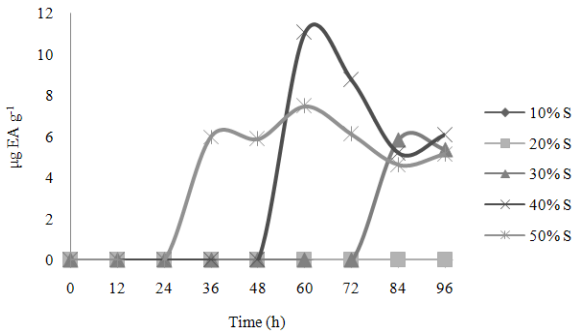

Fig. 5: Ellagic acid liberated by *Aspergillus niger* GH1 at from fermenting pecan nut shell

The gallic and ellagic acids contents were very small. Using the Sharma method, it was determined that only in the 10% shell system was possible to obtain gallic acid at 12 h of culture (data not shown) (Fig. 5). It was liberated 13.98 mg g^{-1} shell and in the other systems was obtained less than 1 mg g^{-1}. In the 10% system, it was observed a gallic acid production, but, while the other systems did not showed positive results. The ellagic presence measured by HPLC analysis, showed that only the quantity obtained were in µg g^{-1} shell and only in SSC, this behavior could be attributed to the insolubility of EA in water.

DISCUSSION

Results of this study showed that PNS can be used for fungal culture processes by *Aspergillus niger* strains, due to the fact that condensed tannins is the mayor component of the shells and the possibility of our strains being capable of degrade hydrolysable and condensed tannins (Aguilar *et al*., 2004). All used strains in the strain selection step, are reported as tannin degrading fungi (Cruz-Hernandez *et al*., 2005) and these same strains, were used in other similar growing experiments on tannin rich materials, where all of them were capable of growing on them (Orzua *et al*., 2009). Those experiments confirm the tannin degrading potential of these *Aspergillus niger* strains according to reports in literature (Bhat *et al*., 1998). As most of the tannins are of the condensed type, GH1 must have a more efficient mechanism for CT degradation and the enzymes required for that purpose, such as monooxygenases that cleave C-C bonds. *Aspergillus niger* GH1 must have also the necessary enzymes for the degradation of HT, which also are present in the PNS which is for example the tannin acyl hydrolase among others. The fungus is able to degrade tannins from the PNS after observing both growth and the results of the polyphenolic quantification. There are reports of condensed tannins degradation with fungal strains such as *Penicillium expansum* where taxifolin could be obtained as an intermediate in the condensed tannins degradation (Contreras-Dominguez *et al*., 2006), its liberation is due to monooxygenases produced by *Aspergillus fumigatus* that cleave the bonds between catechin units (Ramirez-Coronel *et al*., 2004), in this work no taxifolin was detected, but there is condensed tannins degradation. According to Bhat *et al*. (1998), some *Aspergillus* and *Penicillium* strains are capable of degrading both, hydrolysable and condensed tannins. Our results confirm the tannin degrading activity of *Aspergillus niger* GH1 over the tannins present in PNS, being this strain isolated from tannin rich materials, it was expected that this strain would be useful for AP liberation.

Aspergillus niger GH1 must synthesize the necessary enzymes for degradation those PNS components releasing phenolic monomers that form part of the polymeric structure of HT and CT; such enzymes are tannin-acyl hydrolase (Mingshu *et al*., 2006) and valonea tannase (Shi *et al*., 2005) or β-glucosidase (Vattem and Shetty, 2002) which have hydrolytic activity on ellagitannins and monooxygenases and dioxygenases responsible for the initial degradation steps of CT (Contreras-Dominguez *et al*., 2006). Lewis and Starkey (1969) mentioned that some microorganisms isolated from soils are capable of degrading tannins from rich sources of these compounds, adding the fact that, there are studies where fungal strains can produce enzymes for CT degradation (Contreras-Dominguez *et al*., 2006) and others where *Aspergillus niger* GH1 was grown on a liquid culture with catechin (Aguilar *et al*., 2004), being this molecule the monomer unit of CT and tannic acid as sole carbon source (Mata-Gomez *et al*., 2009), so it can be said that this fungus can produce enzymes to degrade these kind of compounds and generate its metabolism energy even though presence of sugars, while limited, are not the only carbon source that could be used by the microorganism.

Some tannin rich materials have been used for AP liberation such as: *Punica granatum* (Robledo *et al*., 2008), coffee pulp (Pandey *et al*., 2000), *Larrea tridentate* (Mercado *et al*., 2007) and *Fluorencia cernua* (Ventura-Sobrevilla *et al*., 2008) most of the phenolics released in both cases were catechin equivalents, like in this case, most of the compounds liberation also were CT as catechin equivalents.

CONCLUSION

The pecan nut shells are an important source of antioxidant phenolics such as catechin and because it is

a great agroindustrial residue, so an alternative biotechnological use has been developed exploiting the tannin degrading potential of *Aspergillus niger* GH1, which is capable of producing the enzymes responsible of the hydrolysis of CT and both, the filamentous fungi and the pecan nut shells have great possibilities for many other purposes that involves food and pharmaceutical industries.

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