

Solid State Fermentation of Mexican Oregano (*Lippia Berlandieri* Schauer) Waste

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Abstract: Problem statement: Mexican oregano is recognized for their aromatic characteristics and flavor quality. Principal products obtained from the plant and marketing are the leaves and essential oil; however the extraction of the essential oil generates large amounts of agro industrial wastes; that can be used as support-substrates in Solid-State Fermentations (SSF). **Approach:** In this study a fungal bioprocess, as solid state fermentation using Mexican oregano wastes as support, for the use of these residues to obtain adds value products and/or molecules were developed. The fungal strain was selected by its adaptability to the support. The aqueous and non polar extracts were obtained kinetically until 120 h and then it was partially characterized (hydrolysable tannins, total sugar and proteins contents, antioxidant activity, thymol and carvacrol concentration). **Results:** Solid state fermentation of oregano wastes, with *Aspergillus niger* PSH, allowed the accumulation of a phenolic compound with catechin similar characteristics and could be responsible of the biotransformation of small amounts of carvacrol to thymol. **Conclusion:** These results could give an add value to Mexican oregano wastes and with more investigation the obtained products can be used in several industries.

Key words: Solid-State Fermentations (SSF), agroindustrial wastes, fungal biotransformation, phenolic compounds, hydrolysable tannins, support-substrates, Thin Layer Chromatography (TLC), results above described

INTRODUCTION

Lippia berlandieri Schauer or *Lippia graveolens* H.B.K. is the most distributed oregano specie in Mexico. Principal products obtained from the plant and marketing are the leaves and the essential oil; however the essential oil extraction generates large amounts of agro industrial wastes. These wastes have been used as animal feed, but the consumed amount is lower than the produced. To be friendlier with the environment, researchers had inverted efforts to reduce the amount of wastes by finding alternative uses (Orzua *et al.*, 2009). One possibility is to use the agro industrial residues as support-substrates in Solid-State Fermentations (SSF).

SSF is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source (Holker *et al.*, 2004). The fermentation takes place in the absence or near absence of free water (Pandey *et al.*, 2000);

however, the substrate must possess enough moisture to support growth and metabolism of the microorganism (Singhania *et al.*, 2009).

This activity has been done by other investigation groups and the molecules that have been released are enzymes, organic acids, polymers, flavors, antibiotics and aroma (Couto, 2008; Swe *et al.*, 2009) or bioconversions of crops and crop-residues (Holker *et al.*, 2004; Medina-Morales *et al.*, 2011). In this study we report a methodology development to find Mexican oregano wastes applications.

MATERIALS AND METHODS

The Mexican oregano waste was obtained from the rural industry "Oreganeros of central-south of Chihuahua" (in Chihuahua, Mexico). Was placed in black bags and transported to the Microbiology Laboratory of the Food Research Department, where the material was dried at 60°C during 48 h, pulverized in a

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blender until fine powder, and stored at room temperature in black bags.

Fungal strain tested were *Aspergillus niger* GH1, *A. niger* PSH, *A. niger* ESH, *A. terricola* PSS, *Penicillium pinophilum* EH2 and *P. pinophilum* EH3 (culture collection of the DIA-UADEC, Saltillo, Coahuila, Mexico) because of its high potential to degrade polyphenols (Cruz-Hernandez *et al.*, 2005). Strains were propagated in Sabouraud broth at 30°C for 3 days. Wastes and Pontecorvo Medium with the following components (g L^{-1}): $\text{C}_6\text{H}_{12}\text{O}_6$ (30), KH_2PO_4 (2.47), $(\text{NH}_4)_2\text{SO}_4$ (6.6), CaCl_2 (0.48), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.38), NaCl (0.32), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.124), yeast extract (0.05) and oligo-elements solution ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, H_3BO_3 , $\text{MnCl} \cdot 4\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (1.0 m L^{-1}) were sterilized, 15 Pa for 15 min. Wastes and medium were mixed and subsequently weighed out 10 g in Petri's dishes (70% of humidity). 10 μL of the strains were inoculated at the center of the plates. Mexican oregano plants were evaluated too as support-substrate following the same methodology for Mexican oregano wastes. Radial growth was kinetically monitored until 120 h at 30°C. All these assays were in triplicate and the growth rate was expressed in mm.

Erlenmeyer flasks containing 3 g of oregano waste, 7 m L^{-1} of Pontecorvo's culture medium and inoculated with 1×10^7 spores per gram of dry oregano waste powder were incubated at 30°C. Samples were monitored kinetically during 120 h; of each sample was obtained an aqueous and a non polar extract. For aqueous extract the content of one flask was re-suspended in 15 m L^{-1} of distilled deionized water, filtered and stored in bottles under light protection at -20°C. For the non polar extract the vegetal material, of each flask, were dried and re-suspended in 10 mL of hexane by 8 h at 200 rpm, filtered and stored at 4°C until its analysis. Consumption of total sugars (Dubois *et al.*, 1956), hydrolyzable tannins (Makkar *et al.*, 1993), proteins (Bradford, 1976) and antioxidant activity (Re *et al.*, 1999) were evaluated in all the samples by triplicate.

The aqueous extracts was filtered by 0.45 μm nylon-membrane and injected into HPLC (Varian Pro Star, Palo Alto, CA, USA) using a photodiode array detector (PDA Pro Star 330) at 254 nm. Separation was carried out with a Prodigy ODS column (5 μm ; 250 \times 4.6 mm, Phenomenex) and temperature of 30°C. A gradient profile of mobile phase, consisting of acetonitrile (solvent A) and 0.3% acetic acid in water (v/v; solvent B), 7-20 A (0-7 min), 20-30 A (7-12 min), 30 A (12-18 min), 30-60 A (18-20 min), 60-100 A (20-23 min), 100 A (23-30 min), 7 A (30-31 min), and 7 min for baseline stabilization was applied at a flow rate of 0.7

$\text{m L}^{-1}\text{min}$. The sample and standards (gallic acid, ellagic acid and catequin) injection was of 10 μL .

Thin Layer Chromatography (TLC) was done only with the 120 h extract, following the methodology proposed by Sharma *et al.* (1998). Mobile phase used was chloroform-ethyl acetate-acetic acid (50:50:1), 2 μL of samples and standards (gallic acid and catequin) were tested and chloride ferric solving in methanol was used to detect the spots.

The non polar extract was filtered by 0.45 μm nylon-membrane and injected into GC-MS (Perkin-Elmer AutoSytmen XL 610N1052906-Perkin-Elmer Turbomax Gold 647N1050901) with ionized electronic detector. Separation was carried out with Perkin Elmer column (30 m length, 0.25 mm diameter, 0.25 μm thickness). Condition works was temperature injector of 220°C, helium as carrier gas and flow rate of 1.0 $\text{m L}^{-1}\text{min}$. The sample and standards (thymol and carvacrol) injection was 1 μL . The oven program was 120°C for 1 min at 1 $\text{m L}^{-1}\text{min}$, 180°C for 7 min at 1 $\text{m L}^{-1}\text{min}$ and 180°C for 11 min.

RESULTS

Tested fungal strains did not growth in the Mexican oregano plants. However all the fungal strains growth in Mexican oregano wastes, but the lag phase duration and the radial growth rate was different for each (Fig. 1).

A. niger PSH grew faster, 16 h earlier, than the others strains and had the greatest invasion capacity, average of 16 mm. *A. niger* GH1 was the second in importance but its lag phase (32 h) is significant different to *A. niger* PSH. The rest of the strains had little growth, from 11-2.4 mm. Based on the adaptability to support-substrate (phase lag duration) and the invasion capacity of the wastes, selected strain for the fermentation process was *A. niger* PSH.

In the chemical partial-characterization of extracts the results showed that proteins, hydrolysable tannins and total sugar amount had similar behavior. The maximum value is at 20 h of fermentation time (1.05, 4.5 and 169.54 mg g^{-1} dry matter respectably). Minimum value for proteins and total sugar was at 60 h of fermentation time (0.73 and 72.04 mg g^{-1} dry matter respectably), while hydrolysable tannins was at 40 h of fermentation time (2.65 mg g^{-1} dry matter). However, after this, the values rise again. On the other hand, the antioxidant activity was higher at the beginning of the kinetics (73.2%) and it totally decreased at 80 h of fermentation time.

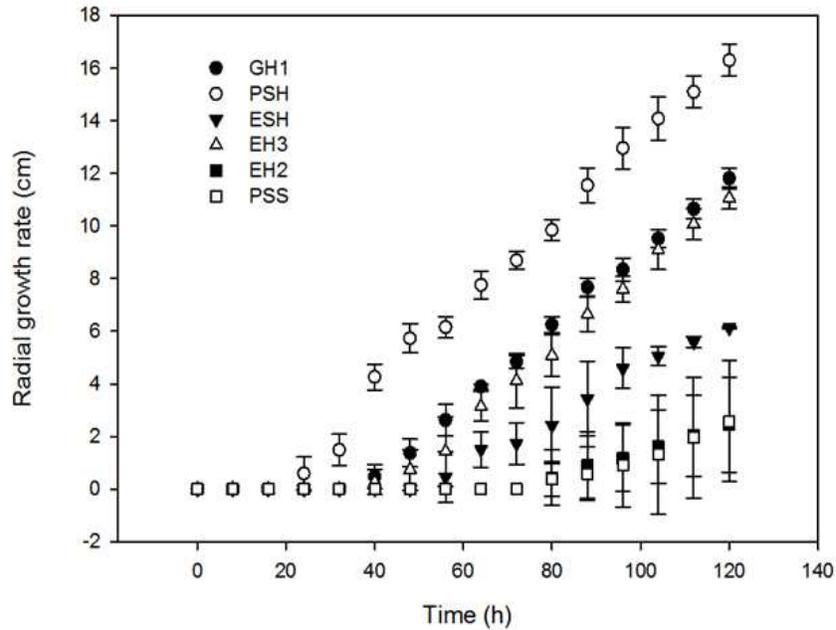


Fig. 1: Kinetical growth of six fungal strains above Mexican oregano wastes

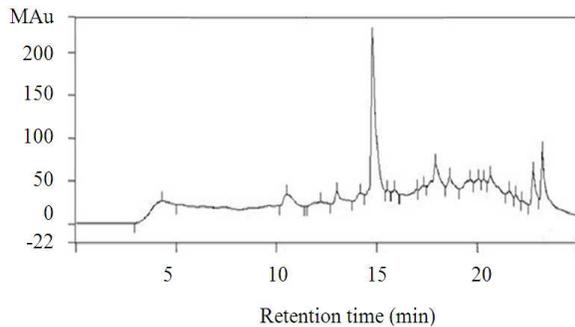


Fig. 2: HPLC-Chromatographic profile of aqueous extract at 120 h solid state fermentation with *A. niger* PSH used as support-substrate Mexican oregano wastes

In HPLC profiles is possible to see a release compound since time 60-120 h, it has a singular peak with retention time of 14.5 min (Fig. 2). This compound agrees on time where proteins, total sugar and hydrolysable tannins had its lower values; however the retention time of the compound did not match with any standard (13.3 min for catechin, 7.15 min for gallic acid and 18.34 min for ellagic acid). As result in TLC, only one compound could be observed and its chemical properties seem to catechin; the R_f of the standards were 0.12 for gallic acid and 0.75 for catechin, while in the samples were 0.76.

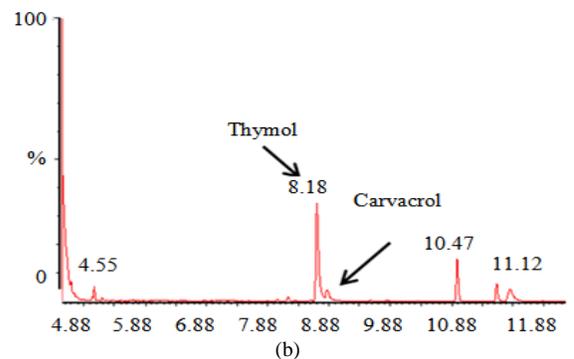
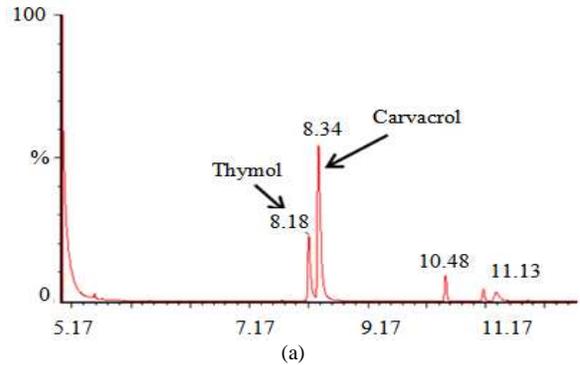


Fig. 3: GC-Chromatogram profile of non polar extract at (A) 0 h and (B) 120 h solid-state fermentation with *A. niger* PSH used as support-substrate Mexican oregano wastes

In the no polar extract we found that main terpenes (thymol and carvacrol) not were extracted totally in essential oil. Chromatograms showed that the carvacrol signal was lower with increasing fermentation time, while the thymol signal was higher (Fig. 3).

DISCUSSION

Oregano plants are not possible using as support-substrate in SSF because the main antimicrobial compounds (thymol and carvacrol) (Portillo-Ruiz *et al.*, 2005) may not allow fungal growth and by consequence the bioprocess; instead, in the Mexican oregano wastes all the fungal strains grew because the thymol and carvacrol concentrations was minimal and culture media provide the necessary nutrients to the fungal metabolism.

Fungal strains, especially filamentous organisms, with a potential enzymatic machine to degrade chemical complex compounds are one of the most useful microorganisms in solid state fermentation (Banerjee *et al.*, 2005, Medina *et al.*, 2010). *A. niger* PSH had been used to produce tannases and release gallic and elagic acids from tar bush, creosote bush (Ventura *et al.*, 2008) and pomegranate residues (Robledo *et al.*, 2008) as others uses of agroindustrial wastes to obtain compounds with industrial applications; these reports support the strain selection to the bioprocess in Mexican oregano wastes.

Proteins, total sugar and hydrolyzed tannins consumption is major at the beginning of the fermentation because the microorganism needs them to start its reproduction phase. After several days, when the principal nutrients were consumed, the microorganism releases enzymes to degraded complex molecules, obtaining by this way sugars and other nutrients. If the metabolism of the microorganism is able, can be accumulated other molecules with industrial applications and it should be purified at downstream process. Antioxidant activity in the samples can be attributed to hydrolysable tannins present in earlier fermentation kinetics times.

With the results of HPLC and TLC is able to think that the main compound present in the aqueous extract of fermented oregano waste is a phenolic compound with catechin in its molecule or a catechin derivate. This can be supported because the major amount groups of molecules that can be found in oregano plants and essential oil are monoterpenes and phenolics acids (Silva-Vazquez *et al.*, 2008) but flavonoids (Arcila-Lozano *et al.*, 2004) are present too. Bhat *et al.* (1998) mentioned that the distribution of tannins, based on their structure is hydrolysable and condensed;

condensed tannins are composed by flavonoid units, however there is an intermediate position group formed by catechin tannins. We can propose that as consequence of fungal invasion, condensed or catechin tannins are formed with the components of the oregano wastes and this is the reason of the observed HPLC chromatogram profile.

On the other hand, thymol and carvacrol molecules are isomers, so we made the hypothesis that *A. niger* PSH makes a bioconversion (Bicas *et al.*, 2009; Leutou *et al.*, 2009; Ramachandran *et al.*, 2008; Shimoda *et al.*, 2006) during fermentation process and for these reason the thymol content was higher in later fermentation times than earlier. The isomerization from carvacrol to thymol can be a new method to purify this compound and then it can be used in pharmacy, food or perfumes industries (Carrera *et al.*, 2005; Lambert *et al.*, 2001; Tsimogiannis *et al.*, 2006).

CONCLUSION

With the results above described, we propose that the compound released or/and accumulated at 120 h of SSF did not had antioxidant activity because it has tannins condensed structure, more similar to catechin than other molecules, as flavonoids. Also we have the hypothesis that *A. niger* PSH makes possible monoterpene isomerization of carvacrol to thymol. This possibilities gives an add value to Mexican oregano wastes, but is necessary to do more investigation about that.

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