Filamentous fungi isolated from Tunisian olive mill wastes: use of solid-state fermentation for enzyme production

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Abstract

Olive mill wastewaters and olive cake are effluents generated during olive oil production process. They represent a major disposal and potentially severe pollution problem for the industry, also promising source of substances of high value. The aim of this study is the valorization of olive mill wastes (OMWW, olive cake, olive twigs and leaves) to produce enzymes with high industrial and biotechnological potential, by the solid-state fermentation technique (SSF), from isolated fungi present in olive mill wastewater and olive cake. A total of 47 strains were isolated and purified from these two residues. The metabolic potential of isolated strains was study by testing the hydrolytic enzymes activities of lipase, protease, amylase, cellulase, invertase, phytase and tannase on agar plate media containing different substrate. The monitoring of SSF has shown that the metabolic activity of these strains is extremely rapid using this technique. Our fungi collection contains a diversity of strains capable to producing a variety of enzymes of biotechnological interest.

Keywords: biotechnological applications; enzymes; fungi; olive oil wastes; solid-state fermentation (SSF); valorization

Introduction

Food industries produce a large volume of solid and liquid residues, which represent disposal and potential environmental pollution problem (Fernández-Bolaños *et al.*, 2006). Likewise, olive oil production, one of the foremost agro-food industries in Mediterranean countries such as Tunisia. The manufacturing process of olive oil production yields enormous quantities of two byproducts depending on the production system adopted: olive cake (OC) and olive mill wastewater (OMWW) (Nefzaoui, 1995). These two wastes are hazardous to the environment; mainly for their high organic load and chemical composition that renders them resistant to degradation (Cegarra *et al.*, 2006; Morillo *et al.*, 2009). Therefore, Tunisia is the most important olive-growing country in the southern Mediterranean with the average production of olive oil is over the last

10 years (2006-2016) at 176 000 tones with a minimum of 70 000 tones and a maximum of 340 000 tones (ONH, 2016). The exports of olive oil are the leading agricultural exports and represent 40% of all, in 2015 Tunisia exported 312,000 tons of olive oil out of the 340,000 produced (ONH, 2018). However, the increase in olive production and olive oil will generate much larger quantities of these by-products. In this way, the pollution increases over the years and the search for an adequate treatment system becomes imperative (Benyahia and Zein, 2003).

Several disposal methods have been proposed and mainly include physico-chemical treatments (decantation with lime and/or chemical oxidation, incineration; ultrafiltration and reverse osmosis) (Niaounakis and Halvadakis, 2006; Arvanitoyannis *et al.*, 2007), agronomic uses for fertilization-irrigation (Altieri and Esposito, 2008) and biological technologies (aerobic or anaerobic bioreactors, composting) (Ramond *et al.*, 2013). The different types of treatment currently used have been only able to partly solve this problem. Indeed, most of the proposed methods remain inefficient and even limited. Current trends should focus on the integration of various technologies to treat and valorize these effluents, but at a low cost (Hafidi *et al.*, 2005).

This would therefore, require the production of added value that will cover the extra costs generated by the application of these new technologies. Innovative biological processes, which exploit the metabolic flexibility of strains of filamentous fungi that naturally survive in olive mill waste water, and olive cake, remain par excellence the least costly processes, perfectly meeting the problem posed in the olive sector. In addition, research on filamentous fungi has already acquired great interest, mainly in the production of enzymes used in industrial processes (Maheshwari *et al.*, 2000; Hassouni *et al.*, 2006 a, b). Therefore, some authors described fungi biodiversity from oleic ecosystems and screened their enzymatic potential activities for biotechnological use (Romo-Sánchez *et al.*, 2010; Baffi *et al.*, 2012). In fact, enzymes are biological catalysts that speed up biochemical reactions in living organisms, and which can be extracted from cells and then used to catalyze a wide range of commercially important processes (Robinson, 2015). They have found application in many industries including pulp and paper, leather, detergents and textiles, pharmaceuticals, chemical, biofuels, food and animal feed, among others. In all areas of application, enzymes are used to effectively facilitate transformations or to replace traditional chemical processes making them cleaner with less impact on the environment (Adrio and Demain, 2014; Lobedanz *et al.*, 2016).

Microbial enzymes are the most requested because of their ease of production and extraction, and owing to their special characteristics such as stability in large range of temperature and pH (Nigam, 2013). Therefore, microorganisms, as enzymes source, are of much attention because they can be grown economically and are amenable to genetic modification. Researchers are looking for new microbial strains in order to produce different enzymes which fulfill the current industrial requirements (Sanchez and Demain, 2017). Among the processes used for enzyme production, Solid-state fermentation (SSF) is attractive because of its numerous advantages, particularly the possibility of using solid agricultural and agro-industrial residues as a medium for microbial growth (Pandey *et al.*, 2001; Panagiotou *et al.*, 2003; Rodrìguez Couto and Sanroman, 2006). Thus, SSF has built up credibility in recent years in biotech industries due to its potential applications in the production of biologically active secondary metabolites, apart from feed, fuel, food, industrial chemicals and pharmaceutical products and has emerged as an attractive alternative to submerged fermentation (SmF) (Virupakshi *et al.*, 2005; Roopesh *et al.*, 2006). Although SSF has several advantages over submerged fermentation which include lower wastewater output, reduced energy requirements, simpler fermentation media, easier aeration, reduced bacterial contamination etc. (Pandey *et al.*, 2000). SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free-flowing liquid (Raimbault, 1980; Sato and Sudo, 1999; Singhania *et al.*, 2010). The low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have also been used (Mitchell, 2000). The solid matrix could be either the source of nutrients or simply support impregnated by the proper nutrients that allow the development of the microorganisms (Thomas *et al.*, 2013). SSF resembles the natural habitat of

microorganisms and is, therefore, preferred choice for microorganisms to grow and produce useful value-added products. So, fungi have been considered to be the organisms most adapted to SSF because their hyphae can grow on particle surfaces and penetrate into the interparticle spaces and thereby colonizing solid substrates (Santos *et al.*, 2004).

SSF processes offer potential advantages in bioremediation and biological detoxification of hazardous and toxic compounds such us olive by-products (Kumar and Shamsher, 2012). Therefore, these by-products can be used as a substrate in SSF to produce high value-added metabolites such as enzymes, especially phytases for the feed industry, tannases for the food coffee industry, lipases for the laundry industry and detergents (D'annibale et *al.*, 2006; Leite *et al.*, 2016). The use of olive by-products in SSF processes come up with the reduction of pollution around the olive mill, contributing thereafter to the sustainable development of the olive sector. This study aims to develop an innovative process for the valorization of olive oil wastes (OMWW, OC and olive twigs and leaves (OTL). The process consists of growing isolated fungi strains in the solid substrate. For fermentation, fungus will produce several enzymes with high industrial value.

Materials and Methods

Samples origin

Olive mill wastewater (OMWW) and olive cake used in the present study were obtained from olive mills using a three-phase discontinuous process for extraction of olive oil. Samples were taken during the olive growing season (November 2015-March 2016) from different regions in Tunisia namely: Al Krib, Téboursouk, Mornag, Gaâfour, Sfax, Zarzis, Châal, and, Jammel (Figure 1). Then, they have been stored at 4 °C until analyzed.

Figure 1. Sampling regions Teboursouk, Al Karib, Gaâfour, Mornag (northest of Tunisia), Jammel (central coast) and Sfax, Châal, Zarzis (southeast of Tunisia). Data source: World Imagery exploited by Global Mapper v18.2

Fungi isolation and identification

Isolation of fungi present in OMWW was done by spreading 0.1 ml of different OMWW dilutions on Petri dishes containing 20 ml of Potato Dextrose Agar (Commercial PDA Powder Sigma-Aldrich) contain chloramphenicol (0.25 g/l) to avoid bacteria development (Botton *et al.*, 1990). For olive cake samples, three fragments were put directly at three different locations on the surface of Petri dishes. Incubation was done at 30 °C for 72 h. The obtained fungi from these two types of residues were purified. Pure cultures of fungal strains were kept in tubes with PDA medium inclined and stored at 4 °C, with periodic replications every 3 months. The purified fungi were then subject to microscopic identification basing on the morphological behavior by reference to classical keys reported in the literature (Raper and Fennell, 1965; Barnet and Hunter, 1972; Ainsworth *et al.*, 1973; von Arx, 1974). The morphological identification was followed by a molecular identification, which was performed based firstly on the extraction of the DNA followed by the amplification of the ITS region from the rDNA through polymerase chain reaction (PCR) in a thermocycler (Biometra®), using universal primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') (sense) and ITS4 (5'TCCTCCGCTTATTGATATGC3') (antisense) described by White *et al.* (1990). The reaction mixture of the PCR having a final volume of 20 µl was composed of: 1 µl of each primer, Taq buffer (× 5) with MgCl2 (25 mM), dNTP (10 mM), BSA (0.5 mg/ml), Taq polymerase (2 U/µl), 2 µl of DNA template and pure water (Attili *et al.*, 1998). The amplification program was run under the following conditions: initial denaturation step of 3 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 60 sec, annealing at 54 °C for 60 sec, and extension 72 °C for 2 min. At the end of the cycles, a final extension was performed at 72 °C for 7 min in order to complete the synthesis of unfinished DNA strands (Hassi *et al.*, 2007). Amplification efficiency was confirmed by electrophoresis on 1% agarose gel. The PCR products were then purified and sequenced using the software Chromas and were compared to those in the 2015 GenBank database of NCBI [http://www.ncbi.nlm.nih.gov/] using the Basic Local Alignment Search Tool (BLAST) algorithm and the 2015 version of the tool Classifier of the Ribosomal Database Project [http://rdp.cme.msu.edu].

Sporulation index and spore production

Sporulation index of filamentous fungi was determined as described by Roussos (1985) and expressed as the number of spores per gram of substrate utilized in the culture medium. For each isolated strain two to three drops of spore suspension were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of supercooled PDA and incubated at 30 °C for 5-7 days. The spores are recovered on the surface of the agar medium using an electromagnetic stirrer for about 30 minutes, in a Tween 80 solution (0.01%). For counting spores, a cell of Malassez was used under optical microscopy. For this purpose, a 1/100 dilution was carried out using water containing tween 80 (0.01%). The count is considered valid when the number of spores is between 10 and 30 per reading field.

Determination of apical growth

The determination of apical growth for each strain was evaluated by measuring the elongation of the mycelium in millimeters per hour (Roussos and Raimbault, 1982) from the center of the agar surface of the Petri dish (point of inoculation) to the periphery. This elongation is measured daily using a graduated ruler during 7 days. The apical growth expressed by average speed on mm/hour was calculated following the formula:

$$\text{Average speed} = \frac{D\,max/2}{T}$$

D_{max}: the diameter in mm of the last measurement (mm).

T: time of the last day (hour).

Screening of the enzyme-producing strains

For tested the potential enzymatic activities, the isolated strains were cultured on agar synthetic culture media containing different substrates (Table 1): casein (protease activity), Tween 80 (lipase activity), starch

(amylase activity), sucrose (invertase activity), carboxyl methyl cellulose (CMC) (cellulase activity), tannic acid (Tannase activity), and phytic acid (Phytase activity).

Table1. Compositions of culture media containing different carbon sources in g/l H_2O

Culture media	Composition of culture media (g/ l H_2O)													
	$CaCl_2$	$MgSO_4$	KH_2PO_4	NaCl	$(NH_4)_2SO_4$	Agar	Tween	Casein	glucose	Sucrose	Starch	CMC	TA	PA
Tween	0.48	0.38	2.47	0.32	6.6	20	20							
Casein	0.48	0.38	2.47	0.32	6.6	20		10						
Glucose	0.48	0.38	2.47	0.32	6.6	20			10					
Sucrose	0.48	0.38	2.47	0.32	6.6	20				10				
Starch	0.48	0.38	2.47	0.32	6.6	20					10			
CMC	0.48	0.38	2.47	0.32	6.6	20						10		
TA	0.48	0.38	2.47	0.32	6.6	20							4	
PA	0.48	0.38	2.47	0.32	6.6	20								1

All nutritional media undergoing the same treatment except the tannic acid medium that undergoes sterilization by filtration. (CMC: carboxyl methyl cellulose, TA: tannic acid, PA: phytic acid).

Revelation of enzymatic activities in solid medium

Tannase and phytase producing strains were determined by a clear and visible halo on a black background (Brahmbhatt and Modi, 2015). For the protease the colonies with a clear zone formed by the hydrolysis of casein (Ozturkoglu-Budak *et al.*, 2016). The lipolytic activity is indicated by the appearance in agar medium an orange fluorescence halo around the colonies (Geoffry and Achur, 2018). Thus, the presence of the halo marks the hydrolysis zone of the appropriate substrate. For the other enzymes, they were highlighted by the appearance of clear zones around the colonies (Madhav *et al.*, 2011; Ozturkoglu-Budak *et al.*, 2016).

Production of enzymes under solid-state fermentation

For the production of enzymes under SSF, we selected the five best strains: *Trichodrema virens*, *Fusarium oxysporum*, *Aspergillus niger*, *Talaromyces assiutensis* and *Penicillium toxicarium*. Olive cake (OC), olive twigs and leaves (OTL) and OMWW were used as substrate for SSF cultures. The OC and OTL were dried in solar greenhouse with 10% (w/w) of moisture retained and then was sieved to provide particle sizes between 0.8 and 2 mm, washed three times with distilled water, adjusted to 50% moisture with distilled water. The individual ingredients of the substrate were mixed thoroughly by mechanical means to achieve a homogenous mixture and sterilized at 121 °C for 20 min. The column bioreactor used (Figure 2) was composed by small glass columns, with 4 cm of diameter and 20 cm of length, and an effective reaction volume of 250 ml. 100 g of SSF support (30% OMWW, 40% OTL and 30% olive cake) and 100 ml of nutritive solution (KH_2PO_4: 1 g, $NaNO_3$: 1 g, $MgSO_4$: 0.5 g, pH=6.) were mixed with 100 ml of 2.107 spores/g of initial dry matter (Roussos and Raimbault, 1982). The final humidity was about 75%. The column bioreactor was packed with 30 g of solid culture medium and incubated in a water bath set at temperature of 30 °C for 5 days. Five columns are used for each strain and were sampled at 0, 8, 16, 24, 32 and 40 h of fermentation for kinetics determination. Aeration of columns was performed by injecting humid air at a flow rate of 25-30 ml/min in each column. Non inoculated solid medium incubated in the same conditions was used as a control (without spores).

Measure of dry matter

The dry matter was determined after drying two grams of fermented product at 105 °C for 24 h, before and after SSF.
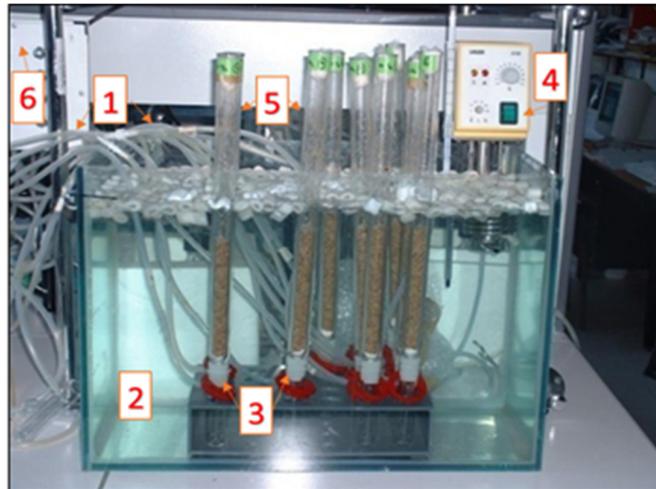
Figure 2. Fermenter used for monitoring the solid-state fermentation
1. Air inlet; 2. Thermoregulated water bath; 3. Humidifier; 4. Heating element; 5. Fermentation columns; 6. Aeration manifold

Enzyme extraction

The enzymes were extracted by mixing 5 g of fermented product with 100 ml of distilled water in an Ultra-Turrax IKA T25 homogenizer (19,000 rpm, 10 min, 4 °C). This extract was centrifuged (5000 * g, 5 min, 4 °C) then filtered through a 0.45 μm membrane filter (Millipore). The cell-free supernatant was used for enzymes activities assay.

Estimation of reducing sugar

The reducing sugar (glucose) levels in samples solution were analyzed by 3, 5-dinitrosalicylic acid (DNS) assay using glucose as standard, following the method described by Miller (1959).

Cellulase assay

Filter paper assay was used to estimate total cellulase activity in the crude enzyme preparation. A rolled Whatman no. 1 filter paper strip of dimension 1.0× 6 cm (50 mg) was placed into each assay tube. The filter paper strip was saturated with 0.5 ml of Na-citrate buffer (0.05 M, pH 4.8) and was equilibrated for 10 min at 50 °C in a water bath. A half milliliter of an appropriately diluted (in Na–citrate buffer, 0.05M; pH 4.8) enzyme was added to the tube and incubated at 50 °C for 60 min. Appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 ml of 3,5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The reaction mixture was diluted appropriately and was measured against a reagent blank at 575 nm in a UV-VIS spectrophotometer (Ghose, 1987). The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of cellulase activity was defined as the amount of enzyme required for liberating 1 μmole of reducing sugar as glucose per minute per ml of fermented juice, under the assay conditions (U/ml/min).

Phytase assay

Enzyme activity was assessed according to the method, described by Engelen *et al.* (2001), using sodium phytate as a substrate. The measure of phytase activity was performed by reacting the enzyme extract with the substrate at 45 °C for 30 minutes. The reaction was stopped by adding 2.5 ml of trichloroacetic acid (TCA 20% w/v). For the purpose of measuring the concentration of phosphorus, 5 ml of the colored reagent were added to the reaction mixture. Then, the absorbance was measured by UV-VIS Spectrophotometer at 700 nm. One

unit of phytase is defined as the amount of enzyme capable of releasing one μmole of phosphorus per min and per ml of fermented extract.

Tannase assay
Tannase activity was estimated following the method reported by Sharma *et al.* (2000) and using gallic acid as standard. The absorbance of the pink color developed has been read at 520 nm. The enzyme activity was calculated from the change in absorbance. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions.

Lipase assay
Lipase activity was measured using p-nitrophenyl octanoate as a substrate (Castilho *et al.*, 2000). This activity was carried out by measuring the increase in the absorbance at 412 nm due to the release of p-nitrophenol during the hydrolysis of 0.5 mM p-nitrophenyl propionate at pH=7 and 30 °C. To initialize the reaction, 0.1 ml of lipase solution was added to 4.4 ml of substrate solution. One unit of lipase activity was defined as the amount of enzyme liberating one μmol of p-nitrophenol per minute per ml of fermented juice, under the assay conditions.

All enzymes studied are expressed as units per gram dry substrate (U/gds), and all the experiments were carried out in four repetitions and standard error was calculated.

Statistical analysis
All experiments were conducted in four repetitions and the results were expressed as media ± standard error. ANOVA was used for statistical comparisons with p value fixed at 0.05.

Results and Discussion

Fungal isolates
During the 2015-2016 olive oil production campaign, 40 olive mills waste water and olive cake samples were taken from different olive mills. A total of 47 strains of filamentous fungi were isolated and purified from these two residues. Table 2 shows the identified fungus isolated from the various sites; results showed the presence of a very interesting fungal biodiversity. The samples of OMWW and olive cake from the southern region (Zarzis, Sfax and Chaal) contain a higher number of fungi than the northern and Sahel regions. For OMWW, despite their toxic composition, we found a very important number of strains which exceeds the olive cake in all regions. Moreover, the acidic pH of olive mill wastes may be advantageous for this microbial group to inhibit the growth of bacteria. *Aspergillus* and *Penicillium* spp were the most prevalent fungi in OMWW samples and for all studied sites, whereas for olive cake samples a greater heterogeneity was observed, the same results were fund by (Roussos *et al.*, 2006; Aissam *et al.*, 2007). Therefore, the members of the fungal genera *Acremonium, Alternaria, Aspergillus, Chalara, Fusarium, Lecythophora, Paecilomyces, Penicillium, Phoma, Phycomyces, Rhinocladiella,* and *Scopulariopsis* have been identified in OMWW disposal ponds, possessing the ability to detoxify olive mill effluents (Millan *et al.*, 2000).

Apical growth and sporulation index of the strains
The measurement of apical growth indicates the capacity of filamentous fungi for superficial colonization on PDA culture medium. The values provide information's about the kinetics of biomass production and helps better understanding the growth strategy of fungi in their natural habitat. The data of the average apical growth and sporulation index of isolated strains are presented in Table 3. The results obtained indicate that the isolated strains showed a variable apical growth rate and sporulation index at a constant incubation temperature. The comparison of apical growth and sporulation index averages for

different species revealed an interval ranging from 0.05 to 0.80 mm/h and $2.4 \cdot 10^8$ to $2.35 \cdot 10^{10}$ spores/g carbon dry matter respectively. *Trichoderma virens, Fusarium oxysporum, Talaromyces trachyspermus, Rhizopus oryzae* showed the highest apical growth. *Talaromyces trachyspermus, Penicillium griseofulvum, Trichoderma virens, Purpureocillium lilacinum, Aspergillus welwitschiae* showed the highest sporulation index.

Table 2. Different genera and species of filamentous fungi isolated from olive mill wastewater and olive cake from different regions of Tunisia

Region/ Origin	OMWW	Olive cake
Mornag	*Penicillium crustosum* *Geotrichum candidum* *Mucor fragilis*	*Cladosporium cladosporioides* *Penicillium expansum*
Teboursouk	*Penicillium polonicum* *Penicillium commune* *Aspergillus tubingensis*	*Aspergillus pseudoglaucus* *Mucor circinelloides*
Gaâfour	*Trichoderma virens* *Penicillium commune* *Penicillium crustosum*	*Alternaria infectoria* *Fusarium oxysporum*
Al Karib	*Penicillium expansum* *Aspergillus awamori* *Aspergillus welwitschiae*	*Talaromyces trachyspermus* *Aureobasidium melanogenum*
Zarzis	*Aspergillus proliferans* *Curvularia spicifera* *Aspergillus ustus* *Aspergillus hiratsukae* *Penicillium griseofulvum*	*Talaromyces assiutensis* *Aspergillus niger* *Cladosporium cladosporioides*
Sfax	*Aspergillus niger* *Penicillium polonicum* *Purpureocillium lilacinum* *Aspergillus tubingensis*	*Geotrichum candidum*
Châal	*Penicillium citreonigrum* *Aspergillus niger* *Aspergillus hiratsukae* *Aspergillus awamori* *Penicillium toxicarium* *Myceliophthora verrucosa* *Penicillium commune*	*Aspergillus awamori* *Talaromyces trachyspermus*
Jemmal	*Alternaria alternata* *Rhizopus oryzae* *Aspergillus niger*	*Alternaria infectoria* *Aspergillus welwitschiae*

Table 3. Fungal colony apical growth on PDA culture medium and sporulation index of different strains incubated at 30 °C during 7 days

Strains	Apical growth	Sporulation index
Geotrichum candidum	0.27±0.041	$5.2 \cdot 10^9$
Aspergillus tubingensis	0.19±0.019	$9.6 \cdot 10^9$
Mucor fragilis	0.36±0.041	$6.2 \cdot 10^9$
Penicillium crustosum	0.15±0.0353	$7.4 \cdot 10^{10}$
Alternaria infectoria	0.15±0.050	$1.85 \cdot 10^9$
Fusarium oxysporum	0.62±0.110	$2.4 \cdot 10^9$
Talaromyces assiutensis	0.36±0.038	$6.2 \cdot 10^9$
Aspergillus pseudoglaucus	0.29±0.027	$5.6 \cdot 10^9$

Penicillium polonicum	0.05±0.012	$2.4 \ 10^9$
Talaromyces trachyspermus	0.50±0.106	$2.35 \ 10^{10}$
Curvularia spicifera	0.23±0.025	$2.4 \ 10^8$
Aspergillus ustus	0.07±0.014	$7.6 \ 10^8$
Aspergillus hiratsukae	0.37±0.033	$1.6 \ 10^{10}$
Aspergillus niger	0.37±0.054	$6.2 \ 10^9$
Alternaria alternata	0.21±0.014	$3.6 \ 10^8$
Rhizopus oryzae	0.40±0.019	$6.8 \ 10^9$
Cladosporium cladosporioides	0.14±0.013	$5.8 \ 10^9$
Penicillium citreonigrum	0.12±0.014	$2.64 \ 10^9$
Penicillium commune	0.12±0.017	$1.6 \ 10^{10}$
Penicillium toxicarium	0.10±0.01	$3.4 \ 10^9$
Myceliophthora verrucosa	0.13±0.01	$3.2 \ 10^9$
Aureobasidium melanogenum	0.29±0.046	$3.6 \ 10^9$
Penicillium expansum	0.24±0.022	$2.56 \ 10^9$
Aspergillus awamori	0.33±0.052	$1.2 \ 10^9$
Aspergillus proliferans	0.16±0.014	$2.4 \ 10^9$
Penicillium griseofulvum	0.30±0.056	$2.3 \ 10^{10}$
Trichoderma virens	0.775±0.109	$1.6 \ 10^{10}$
Aspergillus welwitschiae	0.60±0.127	$1.5 \ 10^{10}$
Purpureocillium lilacinum	0.30±0.024	$1.9 \ 10^{10}$
Mucor circinelloides	0.25±0.036	$2.4 \ 10^9$

Data are mean (n = 4)

Screening of the enzyme-producing strains

The screening of the hydrolytic enzyme activities on agar media of the isolated strain was performed by the nutritional study. Thus, this nutrient study reflects the ability of these strains to develop on a specified substrate as well as the involvement of the enzymatic system on the degradation and hydrolysis of these substrates. Therefore, growth on a starch-based medium as the unique carbon source involves the secretion of amylases, for the sucrose, phytic acid, tannic acid, Tween 80 and carboxyl methyl cellulose (CMC), the enzymes involved are invertases, phytases, tannase, lipases (or esterase) and cellulases respectively. Generally, microorganism screening techniques for the production of enzymes are performed using shaken liquid media. Therefore; the implementation of these techniques is cumbersome considering the large number of strains to be tested. Hence, agar medium culture was first used for esterase detection by Hankin and Anagnostakis, (1975). Figure 3 shows the percentage of strains degrading the different substrates. According to the results obtained, we can be noted that starch, sucrose, glucose, casein and carboxyl methyl cellulose have been used by all the strains, while tannic acid is the least used by the strains with a percentage of 53%.

The measurement of the apical growth of different species according to different carbon sources (Table 4) showed that all fungi can degrade a variety of substrates at 30 °C. On average all strains use glucose, sucrose, casein and starch, suggesting that these species can produce the extracellular proteases and amylases. According to Calvo *et al.* (2002), the simple compounds such as glucose, fructose, sucrose and sorbitol as the unique carbon source generally allow good growth and sporulation. Our results showed that *T. virens* can be considered the best strain in view of the good apical growth on all mediums, so it is able to use all substrates. It can be noted also that the apical growth varies according to the species and even according to the strain, the same results was fund by *Lamrani et al.* (2006) which have shown that the use of different substrates depends on the production of the enzyme degrading the corresponding substrate. The results obtained showed that the isolated strains are able to hydrolyze and assimilate several carbon sources due to their own enzyme production such as amylase, phytase, protease, cellulase on agar plate synthetic culture media at 30 °C
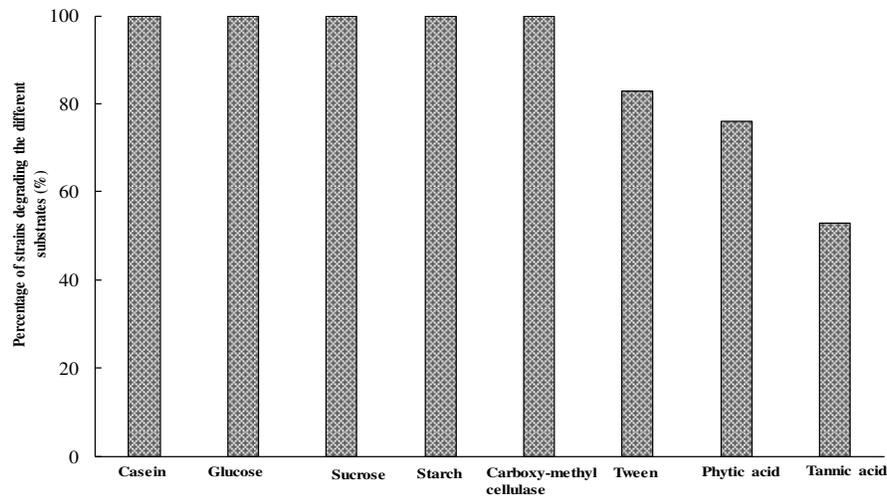
Figure 3. Percentage of selected strains able to grow on different carbon sources

Table 4. The apical growth (mm/h) of the fungal colonies cultivated on different culture media and incubated at 30 °C during 7 days

Strains	Tween	Casein	Glucose	Sucrose	Starch	Carboxy-methyl cellulose	Phytic acid	Tannic acid
Penicillium expansum	0.34 ±0.013	0.29±0.008	0.33±0.017	0.27±0.017	0.13±0.017	0.2±0.005	0	0
Geotrichum candidum	0.31±0.037	0.35±0.022	0.37±0.017	0.33±0.005	0.17±0.017	0.54±0.053	0.23±0.022	0
Aspergillus niger	0.17±0.008	0.23±0.017	0.33±0.008	0.34±0.017	0.20±0.017	1.6±0.163	0.27±0.026	0.31±0.033
Penicillium crustosum	0.36±0.013	0.31±0.010	0.25±0.026	0.31±0.017	0.14±0.017	1.15±0.125	0	0
Alternaria infectoria	0.16±0.016	0.26±0.022	0.22±0.016	0.24±0.016	0.19±0.016	0.4±0.007	0	0.27±0.016
Mucor fragilis	0.20±0.008	0.32±0.017	0.30±0.008	0.36±0.017	0.32±0.026	0.75±0.085	0.34±0.022	0
Aspergillus pseudoglaucus	0.17±0.016	0.22±0.016	0.19±0.005	0.22±0.017	0.17±0.013	0.7±0.129	0.18±0.017	0
Talaromyces assiutensis	0.18±0.016	0.28±0.016	0.33±0.016	0.34±0.016	0.12±0.016	0.55±0.112	0.15±0.022	0.35±0.036
Curvularia spicifera	0	0.25±0.016	0.18±0.008	0.22±0.007	0.12±0.014	0.24±0.016	0	0
Aspergillus ustus	0	0.25±0.014	0.25±0.022	0.25±0.025	0.16±0.007	1.3±0.158	0	0.26±0.022
Trichoderma virens	0.41±0.036	0.41±0.032	0.41±0.007	0.48±0.016	0.20±0.007	1.9±0.158	0.19±0.05	0.25±0.022
Aspergillus awamori	0.26±0.007	0.15±0.016	0.16±0.016	0.15±0.007	0.26±0.016	0.4±0.064	0.52±0.022	0
Alternaria alternata	0.17±0.014	0.11±0.016	0.13±0.007	0.12±0.016	0.18±0.026	0.61±0.026	0.28±0.026	0
Rhizopus oryzae	0	0.15±0.007	0.3±0.007	0.32±0.016	0.12±0.016	0.81±0.064	0	0.26±0.032
Cladosporium cladosporioides	0	0.25±0.007	0.21±0.016	0.23±0.016	0.13±0.016	0.2±0.07	0	0.24±0.016
Penicillium citreonigrum	0.41±0.014	0.25±0.016	0.25±0.016	0.21±0.016	0.25±0.016	0.6±0.072	0.34±0.038	0.27±0.032
Penicillium commune	0.18±0.022	0.41±0.041	0.19±0.007	0.19±0.016	0.14±0.007	0.45±0.041	0.15±0.028	0.52±0.058
Fusarium oxysporum	0.2±0.016	0.3±0.021	0.19±0.025	0.21±0.007	0.15±0.016	0.7±0.125	0.25±0.029	0.16±0.051
Myceliophthora verrucosa	0.17±0.025	0.12±0.017	0.12±0.017	0.11±0.017	0.19±0.005	0.57±0.112	0.17±0.042	0
Aspergillus tubingensis	0.11±0.007	0.25±0.016	0.18±0.007	0.22±0.016	0.11±0.007	0.4±0.007	0.10±0.038	0
Penicillium polonicum	0.22±0.016	0.16±0.007	0.16±0.016	0.16±0.016	0.19±0.025	0.5±0.079	0.2±0.038	0
Talaromyces trachyspermus	0.11±0.007	0.09±0.016	0.11±0.016	0.01±0.007	0.14±0.016	0.4±0.03	0.13±0.016	0.08±0.016
Asperillus hiratsukae	0.41±0.025	0.25±0.016	0.25±0.025	0.21±0.016	0.25±0.016	1.25±0.112	0.41±0.016	0.27±0.016
Penicillium toxicarium	0.18±0.007	0.45±0.038	0.33±0.016	0.18±0.007	0.20±0.016	1.08±0.166	0.21±0.016	0.41±0.015
Aureobasidium melanogenum	0.20±0.0016	0.45±0.029	0.22±0.016	0.15±0.007	0.23±0.007	1.1±0.158	0.17±0.016	0.41±0.035
Aspergillus proliferans	0.15±0.016	0.25±0.016	0.28±0.016	0.28±0.014	0.14±0.014	0.76±0.032	0.18±0.016	0.25±0.029
Penicillium griseofulvum	0.11±0.007	0.25±0.035	0.18±0.014	0.22±0.014	0.11±0.007	0.6±0.079	0.10±0.007	0.13±0.016
Aspergillus welwitschiae	0.22±0.016	0.28±0.013	0.29±0.009	0.25±0.005	0.18±0.008	0.42±0.017	0.21±0.013	0.31±0.022
Purpureocillium lilacinum	0.20±0.022	0.45±0.057	0.22±0.016	0.15±0.016	0.23±0.016	1.1±0.158	0.17±0.025	0.41±0.014
Mucor circinelloides	0	0.26±0.022	0.19±0.014	0.21±0.016	0.14±0.007	0.35±0.079	0.22±0.016	0

Data are mean (n = 4).

Enzymes activity under solid-state fermentation

After screening the strains grown on agar plate media containing different substrates, the five best strains were selected for the SSF study: *Trichoderma virens, Fusarium oxysporum, Aspergillus niger, Talaromyces assiutensis,* and *Penicillium toxicarium.* The choice of these strains is due to their high sporulation index and apical growth on all the media's which implies they are able to hydrolyze and assimilate the different substrates. According to Roussos (1985) and Saucedo-Castaneda (1991) the inoculum in solid-state fermentation processes it is very important in order to select strains with the highest apical growth rates and sporulation index. Based on these screening results we have studied the highlighting of the production of cellulase, phytase, tannase and lipases in solid-state fermentation by these selected strains. Thus, the research concerning the

production of enzymes in solid-state fermentation remains very limited compared to other fermentation processes, in particular that in liquid medium.

Cellulase activity

The results of cellulase activity produced by selected strains are presented in Figure 4 a. The cellulase concentration was calculated from the standard curve as mg of cellulase per ml of test samples.

Figure 4a showed that all the strains showed maximum cellulase activity at 24 h of culture and after the cellulosic activity gradually disappears. Therefore, during the experimentation, the strains tested showed varied cellulase activity. This concentration ranged between 3.83 and 14.55 U/gds. Our study demonstrated the ability to produce enzyme activity varied from strain to strain. Among the five strains, *Trichodrema virens* and *Aspergillus niger* have a significantly better ability to produce cellulase activity during the experimentation with a concentration of 14.55 and 9.92 U/gds respectively at 24 h. Our results are in agreement with those found by Berne *et al.* (2013) who showed that cellulase activity for *Trichoderma* sp at SSF that varied from 11 to 50 U/gds. Reports in the literature suggest that *Trichoderma reesei* has the strongest cellulose-degrading activity (Penttila *et al.*, 1986; Tomme *et al.*, 1988). Meanwhile, a wide range of *Aspergillus* sps and *Penicillium* sps have been identified to possess all components of cellulases complex (de Vries and Visser, 2001; Jahangeer *et al.*, 2005). In addition, Mirzaakhmedov *et al.* (2007) have shown that the most active producer of cellulolytic enzymes was *Aspergillus terreus* compared to *Penicillium tigrinus, Penicillium ostreatus.*

Lipase activity

Lipases activity of the selected strains were presented in Figure 4b. According to the results found, the strains showed appreciable differences in their lipolytic activity. This concentration ranged between 19.21 and 33.56 U/gds. We have noticed that the maximum of lipase activity has been recorded for all fungi after 24 h of culture. Once the maximum lipase production has been reached, the lipase activity decreases gradually. This could be explained either by the presence of proteases in the medium capable of hydrolyzing the lipases or by the thermal instability of the lipolytic enzymes. Therefore, *Trichoderma virens* showed the highest lipase activity than other strains at 24 h (33.56 U/gds) suggesting a good adaptation of this strain to the experimental conditions. This is in accordance with Toscano *et al.* (2013) who reported that *Trichoderma harzianum* exhibited elevated extracellular lipase activity (71.3 U/gds) in SSF. However, *Aspergillus niger* (24.55 U/gds) and *Penicillium toxicarium* (22.69 U/gds) have shown a moderate lipase activity, while *Talaromyces assiutensis* (19.21 U/g gds) and *Fusarium oxysporum* (20.10 U/gds) have lowest lipase activity. In addition, our results are in agreement with those of Gombert *et al.* (1999) and Falcony *et al.* (2006), who reported that lipase enzyme produced by fungi, could be enhanced under SSF. Thus, several lipase producing fungal strains have been used for lipase production by SSF which include *Penicillium restrictum* (Gombert *et al.,* 1999), *Aspergillus niger* (Kamini *et al.*, 1998; Mahadik *et al.*, 2002), and *Rhizopus oligosporus* (ul-Haq *et al.,* 2002), *Rhizopus delemer* (Christen *et al.,* 1998) and *Aspergillus oryzae* (Kumar and Ray, 2014).

Phytases activity

Figure 4.c illustrate the phytase activity of selected strain. According to the results, all strains showed significant phytase activity which were ranged between 45.90 U/gds and 32.55 U/gds for *Trichoderma virens* and *Aspergillus niger* respectively. The phytase activity for all strains began after 8 h of incubation to reach their maximum after 24 h, then they disappear gradually. Therefore, the phytase activity produced by all the strains is generally attributed to the optimization of initial parameters of the culture in SSF (Initial humidity, pH, aeration rate, etc..). In the literature, it is reported that several filamentous as well thermophilic fungi (*Thermoascus aurantiacus, Aspergillus fumigatus, Myceliophthora thermophila,* etc...) than mesophilic (*A. niger, A. ficuum,* etc.) are phytase producers (Mitchell *et al.*, 1997; Liu *et al.*, 1999; Nampoothiri *et al.*, 2004). According to Hassouni *et al.* (2006 a), it is very important to optimize SSF culture parameters for high-activity phytase production.
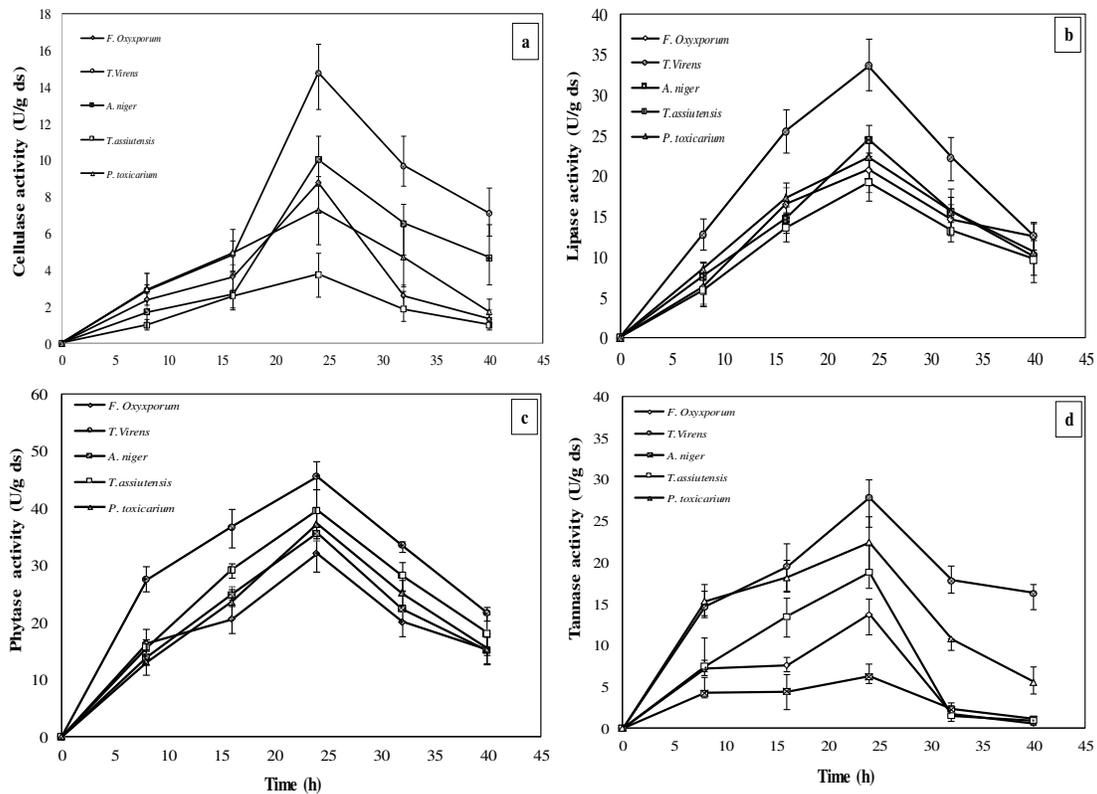
Figure 4. Time course of cellulase (a), lipase (b), phytase (c) and tannase (d) activities from selected strain
Data are mean (n = 4)

These authors showed that, for the same strain, the phytases activity produced is 3.5 times higher after optimization of the SSF culture parameters than that obtained before optimization, and 2.5 times for the reference strain (*A. ficuum*). Thus, the best condition obtained, was the parameters used in our study which are: pH=6, an initial humidity of 75% and aeration rate 20-25 ml/min/column. In addition, the initial pH is a key parameter for the production of phytases and an initial pH = 6 is clearly favorable for high phytase production. According to this study, the phytase activity for *T. virens* reaches 45.90 U/gds at pH = 6. This is in agreement with the result of Hassouni *et al.* (2006 a) who tested phytase activity in *M. thermophila* at different pH values and showed that the strain produced a high phytases activity (180 U/gds) after 36 hours of SSF at pH=6. Gargova and Sariyska (2003) and Kim *et al.* (1999) also, found that the initial pH of the culture medium influences phytase production in *Aspergillus spp.* Additionally, the nature of the carbon and the nitrogen sources influence the production of phytases (Ramachandran *et al.*, 2005; Roopesh *et al.*, 2006). So, the glucose in the nutrient solution used by the strains as a carbon source for the SSF culture makes it possible to have a maximum enzymatic activity. Glucose is a simple source of carbon that is, easily, assimilated by microorganisms. It allows having a lot of biomass needed for the dephosphorylation of phytic acid. Accordingly; glucose, starch or sucrose are widely used for the production of phytases, especially, in *M. thermophila* (Mitchell *et al.*, 1997) and *Rhizopus oligosporus* (Ramachandran *et al.*, 2005).

Tannase activity
    The results of Tannase activity produced by selected strains are presented in Figure 4d. According to the obtained results, the tannase activity showed the same appearance as the other enzymatic activity (cellulase, lipase and phytase). Thus, the activity of tannase for all the strains start at 8h to reach is maximum at 24 h and after decreased gradually. According to Kar *et al.* (1999) this decrease in production of enzyme activity may be

due to accumulation of end product which hampers tannase production or may be due to accumulation of toxic metabolites secreted during fermentation. Our results showed that the tannase activity varied between strain, some being much more efficient than others. Among the five strains, *Trichoderma virens* have a significantly better ability to produce cellulase activity with a concentration of 27.93 U/gds at 24 h while *Aspergillus niger* (6.22 U/gds) have lowest lipase activity. Moreover, the SSF monitoring showed that the tannase activity of these strains is extremely fast using this culture technique compared with other techniques. Lamrani *et al.* (2006) tested the tannase activity in three Moroccan strains (*Rh pusillus*, *A. fumigatus*, *A. fumigatus*) and a Mexican strain (*A. fumigatus*) which has been used as a reference strain. So, the four strains produced very similar amounts ranging from 16.89 to 18.7 U/gds for the reference strain after 24 hours of incubation. Comparative studies of Lekha and Lonsane (1997) on the production of tannases by *A. niger* reveal that SSF production is 2.5 to 4.8 times higher than in liquid-state fermentation. The fermentation time to reach maximum production in SSF is 3 days whereas it is 6 days in liquid-state fermentation. In addition, the enzyme is totally extracellular in SSF and partially intracellular in liquid-state fermentation. Finally, the tannase produced in SSF is much more thermostable compared to that produced in liquid-state fermentation.

Conclusions

In the present study, the five strains tested showed high activity of tannase, cellulases, phytase and lipases. However, *T. virens* showed the highest enzymatic activities compared to the other strains which confirmed the results found of screening on agar plate synthetic culture media containing different substrate. Moreover, the monitoring of SSF has shown that the metabolic activity of these strains is extremely rapid using olive by-products as substrate. Thus, for the five strains, the biosynthesis of the enzymes starts from 8 hours of SSF culture, and once the maximum production of these enzymes has been reached after 24 hours of culture, these enzymatic activities gradually decrease. This study showed that SSF has many advantages for the production of enzymes useful in biotechnology. Thus, a large number of commercial enzymes (cellulases, tannase, phytase, lipases) are produced by SSF and they have all been successful in the world market. From the results found, our strains collection is very interesting and contains a variety of strains capable to produce a variety of enzyme. The strains tested after their culture in SSF present promising results which will have to be better exploited. In particular, by optimizing the production conditions in SSF in a more advanced research work in order to transfer to the production of enzymes on a pilot scale. This study also opens new perspectives for the use of strain in SSF, this also for the production of enzymes, by the useful fungi, usable for the valorization of the agricultural by-products.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by ZH, and MS. The first draft of the manuscript was written by ZH; Writing - review and editing: MS; Conceptualization: RS; Supervision: AR and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Biotechnology and Chemometrics Team at Aix Marseille University for their technical support during this study.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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