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Improvement of protein content and decrease of anti-nutritional factors in olive cake by solid-state fermentation: A way to valorize this industrial by-product in animal feed

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The present work investigates the bioconversion of the olive cake (OC) generated by olive oil industries in Morocco through solid-state fermentation using selected filamentous fungi to increase its nutritional values for subsequent valorization as ruminants feed. The fungi, namely *Beauveria bassiana*, *Fusarium flocciferum, Rhizodiscina cf. lignyota*, and *Aspergillus niger* were cultured on OC for 15 days. Chemical composition as well as enzymes activities were determined. Results showed (i) an increase in protein content of up to 94% for treated OC and (ii) significant ($P < 0.05$) decreases of phenolic compounds, up to 43%, 70% and 42% for total phenolic content, total flavonoids content, and total condensed tannins, respectively. Moreover, the RP-HPLC analysis of fermented OC confirmed the degradation of individual phenolic compounds by the strains. These findings demonstrate that *F. flocciferum* and *Rhizodiscina cf. lignyota* are efficient enzymes producers leading to a nutritive enhancement of this by-product.

[**Key words:** Olive cake; Solid-state fermentation; Filamentous fungi; Phenolic compounds; Protein content]

All over the world, large quantities of organic wastes are constantly generated as by-products from agro-food industries. These residues are often difficult to dispose of and most of them cause negative environmental impacts. Therefore, the management of these by-products not only proves necessary, but also may even present an economic and ecological benefit.

In Morocco, the most significant produced waste is released by olive oil mills. The cultivated area of olive trees in 2016 was 1,000,000 ha (1), and there are over 345 olive milling factories and 16,000 traditional units that produce more than 160,000 tons of olive oil (2). It is not surprising that the production of such amounts of olive oil generates huge amounts of by-products.

Olive cake (OC) is one of the by-products that remain after oil extraction; it contains the olive kernel shell crushed into small fragments, the crushed pulp and the skin. Between 300 kg and 800 kg of OC are generated per ton of olives crashed, depending on the extraction system and the mill type (3); these large amounts of OC are deposited in the immediate environment of the mills, occupying an important space and posing environmental problems such as the emission of noxious odors during storage or combustion, as well as soil and ground water contamination as factories use organic solvents to extract residual oil from OC. Despite its phytotoxicity and other negative effects, this by-product contains insufficiently exploited valuable resources that could be recycled and valorized. One such recycling solution could be the valorization of this by-product as ruminants feed.

It is well known that the ruminant feeding is based on the coverage of the nutritional needs for animal production. Its effectiveness is determined by respecting nutritional balances, mainly energy intake, protein and mineral inputs.

Indeed, proteins play an important role in livestock farming. They are among the main constituents of cells and have many structural and functional roles in the organism. Among the cattle feed used nowadays, soybean meal is an ideal source of protein and occupies therefore an important place in world imports of livestock feed. In 2011–2012, Morocco was the 22nd biggest importer of soybean meal in the world. During this year, the total cost of oilseed meal imports exceeded USD 320 million, of which soybean meal represents about 85% of the import bill. According to predictions from several studies, by 2025, the total cost of oilseed meal imports may increase by up to 90% from its level in 2011 (4). This confirms the use of olive by-product as ruminant feed will be beneficial to both the environment and therefore ruminant farms while reducing Morocco's dependency on imported cattle feeds.

However, OC contains a large proportion of cell wall constituents, has low protein content (PC) and contains a considerable percentage of phenolic compounds. In fact, during the olive-oil extraction process, only 2% of phenols from the olive fruit are transferred to the oil and 98% remain in the olive mill wastes (5,6). These phenolic compounds, particularly tannins, render OC

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unpalatable and poorly digestible for ruminants due, among other mechanisms, to their inhibitory action on the extracellular enzymes secreted by the ruminal microflora (7,8). In order to allow OC to be used as a cattle feed source, a treatment method allowing phenolic compounds removal or degradation must be developed.

To improve the nutritional value of OC, biological processes such as solid-state fermentation (SSF) attract more attention than chemical or physical processes due to their low cost, simplicity and higher yields (9). SSF is known for being able to reproduce culture conditions similar to that of the natural environment of the microorganisms (10). SSF was also proved to be particularly suitable for the production of enzymes by employing filamentous fungi (11).

Increasing the efficiency of enzyme secretion during fermentation was shown to improve the feed digestion ratio in poultry (12), and thus enhances the value of animal feed.

In order to identify novel strains with a diverse range of enzymatic activities, fungi were isolated from OC and decayed wood. Fifty strains were preselected by using qualitative tests (data to be published) based on their diverse enzymatic production. Special focus was given on hydrolytic and oxidative enzymes involved in the degradation of major components of lignocellulosic biomass into low molecular weight compounds which can be assimilated by fungi (13). From the 50 preselected strains, four were utilized for SSF tests.

The aim of the present study was to assess (i) the enzymatic activities of selected fungi during SSF, and (ii) the ability of these fungi to reduce the anti-nutritional components in OC, especially phenolic compounds, while increasing its PCs.

MATERIALS AND METHODS

Substrates preparation Fresh OC was obtained from a factory within Meknes region, located in northern central Morocco. The OC was ground (SM 300, Retsch) and sieved ($\leq$5 mm) to obtain a homogenized substrate, then it was stored at 4 °C.

Fermentative organisms *Fusarium flocciferum* and *Beauveria bassiana*, were isolated from OC, while *Rhizodiscina cf. lignyota* was isolated from decayed wood collected from Ifrane forest (Morocco). *Aspergillus niger* (MUCL 28820) was purchased from Belgian Co-ordinated Collections of Micro-organisms (BCCM).

The strains were grown on potato dextrose agar (PDA, VWR, Fontenay-sous-Bois, France) and incubated at 25 °C for 96 h.

Preparation of spore suspension Ten mL of sterilized water with 0.1% Tween-80 solution were added to 5-day-old culture dishes and agitated thoroughly to suspend the spores. The spores concentration was adjusted to 10^6 spores/mL and was used as an inoculum throughout the study.

Solid state fermentation One mL aliquot (v/v) of the inoculum (10^6 spores/mL) was used to inoculate 500 mL Erlenmeyer flasks each containing 10 g of OC dry matter previously sterilized (20 min at 121 °C) and moistened to 60% (v/w) with one of the four different solutions (0.2% yeast extract solution, 0.1% Glucose solution, distilled water or salts solution: KH_2PO_4 (0.5%) $(NH_4)_2SO_4$ and $MgSO_4\cdot7H_2O$ (0.2%)). The inoculated flasks were incubated for 2 weeks at 25 °C in a static incubator. Each fermentation condition was done in triplicate.

Analytical determination Proteins content and phenolic compounds were analyzed before inoculation and at the end of the incubation period. All measures were performed in triplicate.

Determination of PC PC was determined on aqueous extracts according to Bradford method using bovine serum albumin (BSA) as a standard (14). The absorbance was measured at 595 nm.

Determination of total phenolic compounds Phenolic compounds were extracted in 80/20 v/v methanol/water.

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu method (15), using gallic acid as a standard. Briefly, 100 μL of sample were added to 1.6 mL of H_2O and 100 μL of Folin-Ciocalteu reagent. The mixture was incubated at 25 °C for 10 min. Then 600 μL of a 20% solution of Na_2CO_3 were added and the sample was incubated at 40 °C for 20 min, with intermittent shaking. The absorbance was measured at 760 nm. Concentration of sample was obtained by comparing the absorbance value with a standard curve of gallic acid and the TPC content in the extract was expressed as gallic acid equivalents (GAE) in mg/g dry weight (DW) of OC.

Determination of total flavonoid content Total flavonoid content (TFC) was measured by using a colorimetric assay according to Dewanto et al. (16). For this, 250 μL of the extract were mixed with 1.25 mL distilled water in a test tube followed by addition of 75 μL of a 5% $NaNO_2$ solution. After 6 min at 25 °C, 150 μL of a 10% $AlCl_3(6H_2O)$ solution were added and incubated at 25 °C for another 5 min before 500 μL of 1 M NaOH was added. The mixture was brought to 2.5 mL with distilled water. The absorbance was measured immediately at 510 nm, and the TFC was determined using a calibration curve of (+)-catechin. The total flavonoid content is expressed as milligrams of catechin per gram of dry weight OC.

Determination of total condensed tannins Total condensed tannins (proanthocyanidins) (TCT) were determined according to the method of Sun et al. (17). To 50 μL of diluted sample, 3 mL of 4% vanillin solution in methanol and 1.5 mL of concentrated HCl were added. The samples were incubated at 25 °C for 15min. Absorbance variation was monitored at 500 nm. The amount of total condensed tannins is expressed as mg (+)-catechin/g DW.

Chromatographic analysis OC solvent extracts were analyzed by a Thermo Fischer Scientific HPLC system (Ultimate 300, Thermo Fischer Scientific, Waltham, MA, USA) equipped with diode array and multiple-wavelength detectors. Separation was achieved on a 5 μm C-18 reversed-phase column (250 mm−4.6 mm) (Thermo Fischer Scientific). Elution was performed at a flow rate of 0.5 mL/min, using as mobile phase: solvent A (100% distilled water), solvent B (100% acetonitrile) and solvent C (0.1% formic acid). The samples were eluted by the following gradient: 50% A and 50% C as initial conditions during 10 min, 40% A, 10% B and 50% C for 10 min, 35% A, 15% B and 50% C for 40 min, and finally 20% A, 30% B and 50% C for 10 min. The total running time was 70 min.

Peak detection was carried out at 285 nm. The individual phenolic compounds were identified by comparison with relative retention times of standard compounds: 3,4-dihydroxybenzoic acid (97%), *trans-p*-coumaric acid (98%), caffeic acid (98%), *trans*-ferulic acid (99%), vanillic acid (97%), vanillin (99%), (+) catechin (98%), *trans*-cinnamic acid (99%), sinapinic acid (98%), gallic acid (99%), 4-hydroxybenzoic acid (99%), 4-hydroxyphenylethanol (tyrosol) (99.5%) (Sigma−Aldrich, St-Quentin-Fallavier, France).

Enzyme extraction Every 48 h, through the entire incubation period, the enzymes were extracted with a sodium citrate buffer (0.05 M, pH 5.0; 10 mL buffer/g substrate), while shaking at 180 rpm for 1 h at room temperature. The extract was filtered through Whatman No. 1 filter paper and the filtrate was centrifuged for 20 min at 5000 rpm (18).

Xylanase assay Xylanase activity was measured according to Bailey et al. (19) using 900 μL of 1% birchwood xylan as substrate (prepared in 50 mM Na-citrate buffer, pH 5.3) with 200 μL of suitably diluted enzyme and an incubation period of 5 min at 50 °C. The reaction was stopped by the addition of 3 mL of 3,5-dinitrosalicylic acid (DNS) and the content was boiled for 5 min (20). After cooling, absorbance was measured at 540 nm. The amount of liberated reducing sugars was quantified using xylose as a standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 μmol of xylose equivalents per minute under the assay conditions.

Laccase activity Laccase activity was measured with 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in a 0.1 M phosphate-citrate buffer at pH 4. Oxidation of ABTS was determined measuring the absorbance at 420 nm ($\varepsilon420 = 36$ mM/cm) (21). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min.

Lignin peroxidase Lignin peroxidase activity was assayed by the azure B method. The reaction mixture contained 1 mL of 50 mM sodium tartarte buffer (pH 4.5), 0.1 mM H_2O_2, 32 μM azure B. The reaction was initiated by adding hydrogen peroxide. One unit of enzyme activity was expressed as an optical density (OD) decrease of 0.1 unit per minute per mL of the culture filtrate (22).

Manganese peroxidase Manganese peroxidase (MnP) activity was measured as described by Fujian et al. (23). The reaction mixture contained 1.70 mL of citrate-phosphate buffer (pH 3; 0.1 M), 50 μL of $MnSO_4$ (0.40 M), 200 μL of enzyme and 50 μL of H_2O_2 (0.016 M). One unit of MnP activity was defined as 10% of A240 nm increase/min.

Cellulase assay The endo-cellulase (endo-1,4-β-glucanase) activity was analyzed using the enzymatic kitCellG5-4V 02/17 (Megazyme, Bray, Ireland). One unit of activity was defined as the amount of enzyme, in the presence of excess thermostable β-glucosidase, required to release 1 μmol of 4-nitrophenol from CellG5 in one minute under the defined assay conditions (24).

Strain identification Molecular identification of the strains was undertaken by the laboratory of mycology, Mycothèque de l'Université Catholique de Louvain (MUCL), Belgium.

Statistical analysis The results are expressed as mean values and standard deviation (SD). Mean values were tested for significant differences ($P < 0.05$) using one-way analysis of variance ANOVA followed by Tukey's test for all pairwise comparisons and Dunnett test for comparisons to the control. Significant differences are indicated by different letters.

RESULTS

Analytical determination The crude OC used in this study was characterized by low PC (42.65 mg/g DW) and relatively high TPC (7.04 mg/g DW). All tested strains grew on OC and had an effect

TABLE 1. Proteins, total phenolic, total flavonoid and total condensed tannins content of OC moistened with distilled water after SSF.

	PC (mg/g DW)	TPC (mg/g DW)	TFC (mg/g DW)	TCT (mg/g DW)
B. bassiana	53.53 ± 3.57[b*]	5.78 ± 0.56[b]	1.43 ± 0.13[b]	3.99 ± 0.26[b]
Rhizodiscina cf. lignyota	63.64 ± 2.67[c**]	4.16 ± 0.56[a]	1.07 ± 0.04[a]	3.8 ± 0.21[a,b]
F. flocciferum	64.79 ± 2.69[c]	4.30 ± 0.21[a]	1.24 ± 0.05[a]	3.23 ± 0.21[a]
A. niger	57.57 ± 2.27[b]	6.18 ± 0.39[b,c]	2.83 ± 0.1[c]	4.21 ± 0.25[b,c]
Control	42.65 ± 1.16[a]	7.04 ± 0.32[c]	3.01 ± 0.07[C]	4.78 ± 0.41[c]

DW, dry weight.
* Mean value of three replicates ±standard deviation.
** The statistically significant difference between the treatments was discerned by the procedure of the honest significant difference of Tukey's (HSD); the differences are expressed with letters in increasing order.

on PC and the degradation of anti-nutritional compounds varying with inoculated strain and moistening solution.

Using distilled water to moisten the OC (Table 1), *F. flocciferum* and *Rhizodiscina cf. lignyota* exhibited similar performances regarding increases of PC (around 52%) and decreases of TPC (40%), TFC (64%) and TCT (32%).

With the salt moistening solution (Table 2), the maximum increase of PC ranged between 41.6% (*A. niger*) and 79.5% (*F. flocciferum*) of the initial PC. *B. bassiana*, *Rhizodiscina cf. lignyota* and *F. flocciferum* showed a good reducing power for TPC, while *A. niger* exhibited a lower ability for the degradation of TPC (14.8%). On the other hand, the maximum decrease of TFC was observed for *Rhizodiscina cf. lignyota* (70.1%) whereas *F. flocciferum* decreased TCT most efficiently (42.5%).

The increase in PC with the glucose solution (Table 3) was similar (about 15%) for all strains. The maximum TPC removal was recorded with *Rhizodiscina cf. lignyota* and *F. flocciferum* with 28.4% and 27.8%, respectively. For TFC and TCT, the lowest decrease was recorded with *A. niger*.

Finally, when using yeast extract as a moistening solution, *F. flocciferum* exhibited a significant increase in protein concentration (94.2%) compared to the other strains (Table 4). For TPC, this is *Rhizodiscina cf. lignyota* that provided the maximum reduction (43.6%), followed by *F. flocciferum* (40.9%) then *B. bassiana* (30.4%) and *A. niger* (12.9%). Maximum TFC removal ranged between 17.6% and 65.5% of the initial TFC content, and for TCT the maximum removal was 41.6% by *F. flocciferum*.

Chromatographic analysis OC extracts were analyzed with reversed-phase HPLC in order to assess the degradation of individual phenolic compounds (Table 5). In every fermentation conditions, the total number of compounds (peaks number), including unknown compounds, were reduced compared to the control (32 peaks). This total number was reduced to 29 peaks by *A. niger*, 23 peaks by *B. bassiana* and even further reduced to 13 peaks by *Rhizodiscina cf. lignyota* and to 8 peaks by *F. flocciferum*.

Among the 12 phenolic standards used, seven compounds were present in OC. Chromatographic analysis showed that the strains *Rhizodiscina cf. lignyota* and *F. flocciferum* were able to remove completely tyrosol, vanillic acid, caffeic acid, (+) catechin and *trans*-ferulic acid. The other two strains, *B. bassiana* and *A. niger* completely removed at least three compounds, and partially removed 39% of *trans*-ferulic acid (*A. niger*) and 88% of vanillin (*B. bassiana*). It should be noted that the maximum reduction of these compounds was recorded in samples moistened with salts and yeast extract solutions.

Enzymes production The reduction of OC phenolic content through the SSF process, using the four *Ascomycota* strains, is not surprising considering that these fungi possess an efficient lingo-hemicellulolytic system. As shown in Figs. 1 and 2, all the strains tested show a moderate to important enzymatic production.

The onset of laccase production occurred after 4–6 days of incubation for *Rhizodiscina cf. lignyota*, and the activity peaked after 15 days averaging 4 UI/mL.

A very weak cellulase and no xylanase activity were detected for *Rhizodiscina cf. lignyota* and *B. bassiana*. On the contrary, there was a more important production of these enzymes by *F. flocciferum* and *A. niger* about 14 UI/mL of xylanase and 2 UI/mL (*F. flocciferum)*, 4 UI/mL (*A. niger)* of cellulase, It is noteworthy to mention that low

TABLE 2. Proteins, total phenolic, total flavonoid and total condensed tannins content of OC moistened with salts solution after SSF.

	PC (mg/g DW)	TPC (mg/g DW)	TFC (mg/g DW)	TCT (mg/g DW)
B. bassiana	65.42 ± 1.98[b,c*]	4.89 ± 0.46[a]	1.38 ± 0.04[c]	3.52 ± 0.31[a,b]
Rhizodiscina cf. lignyota	68.78 ± 2.06[c]	4.10 ± 0.44[a]	0.90 ± 0.04[a]	3.17 ± 0.46[a,b]
F. flocciferum	76.57 ± 3.84[d]	4.00 ± 0.10[a]	1.05 ± 0.05[b]	2.75 ± 0.35[a]
A. niger	60.38 ± 2.8[b]	6.00 ± 0.41[b]	2.45 ± 0.09[d]	3.89 ± 0.31[b]
Control	42.65 ± 1.16[a]	7.04 ± 0.32[c]	3.01 ± 0.07[e]	4.78 ± 0.41[c]

* The statistically significant difference between the treatments was discerned by the procedure of the honest significant difference of Tukey's (HSD); the differences are expressed with letters in increasing order.

TABLE 3. Proteins, total phenolic, total flavonoid and total condensed tannins content of OC moistened with glucose solution after SSF.

	PC (mg/g DW)	TPC (mg/g DW)	TFC (mg/g DW)	TCT (mg/g DW)
B. bassiana	49.43 ± 4.11[b*]	5.99 ± 0.44[a,b]	1.45 ± 0.03[c]	4.23 ± 0.27[b]
Rhizodiscina cf. lignyota	49.03 ± 2.63[b]	5.04 ± 0.61[a]	1.06 ± 0.03[a]	3.91 ± 0.10[b]
F. flocciferum	49.34 ± 2.33[b]	5.08 ± 0.06[a]	1.25 ± 0.00[b]	3.22 ± 0.15[a]
A. niger	48.92 ± 2.93[b]	6.32 ± 0.33[b,c]	2.88 ± 0.06[d]	4.29 ± 0.18[b,c]
Control	42.65 ± 1.16[a]	7.04 ± 0.32[c]	3.01 ± 0.07[e]	4.78 ± 0.41[c]

* The statistically significant difference between the treatments was discerned by the procedure of the honest significant difference of Tukey's (HSD); the differences are expressed with letters in increasing order.

TABLE 4. Proteins, total phenolic, total flavonoid and total condensed tannins content of OC moistened with yeast extract solution after SSF.

	PC (mg/g DW)	TPC (mg/g DW)	TFC (mg/g DW)	TCT (mg/g DW)
B. bassiana	62.95 ± 4.09[b,c*]	4.90 ± 0.5[b]	1.40 ± 0.06[b]	3.79 ± 0.31[b]
Rhizodiscina cf. lignyota	67.22 ± 1.83[c]	3.97 ± 0.49[a]	1.04 ± 0.02[a]	3.18 ± 0.47[a,b]
F. flocciferum	82.83 ± 3.85[d]	4.16 ± 0.51[a,b]	1.09 ± 0.01[a]	2.79 ± 0.44[a]
A. niger	60.03 ± 3.65[b]	6.13 ± 0.35[c]	2.48 ± 0.12[C]	3.99 ± 0.21[b,c]
Control	42.65 ± 1.16[a]	7.04 ± 0.32[c]	3.01 ± 0.07[d]	4.78 ± 0.41[c]

* The statistically significant difference between the treatments was discerned by the procedure of the honest significant difference of Tukey's (HSD); the differences are expressed with letters in increasing order.

TABLE 5. The removal (%) of phenolic compounds by the four strains compared to control after 15 days of incubation at 25 °C.

	B. bassiana	*Rhizodiscina cf. lignyota*	*F. flocciferum*	*A. niger*
3,4-Dihydroxybenzoic acid	78	100	84	55
4-Hydroxyphenylethanol (tyrosol)	100	100	100	100
Vanillin	88	88	78	62
Vanillic acid	100	100	100	100
Caffeic acid	42	100	100	30
(+) Catechin	100	100	100	100
trans-Ferulic acid	100	100	100	39

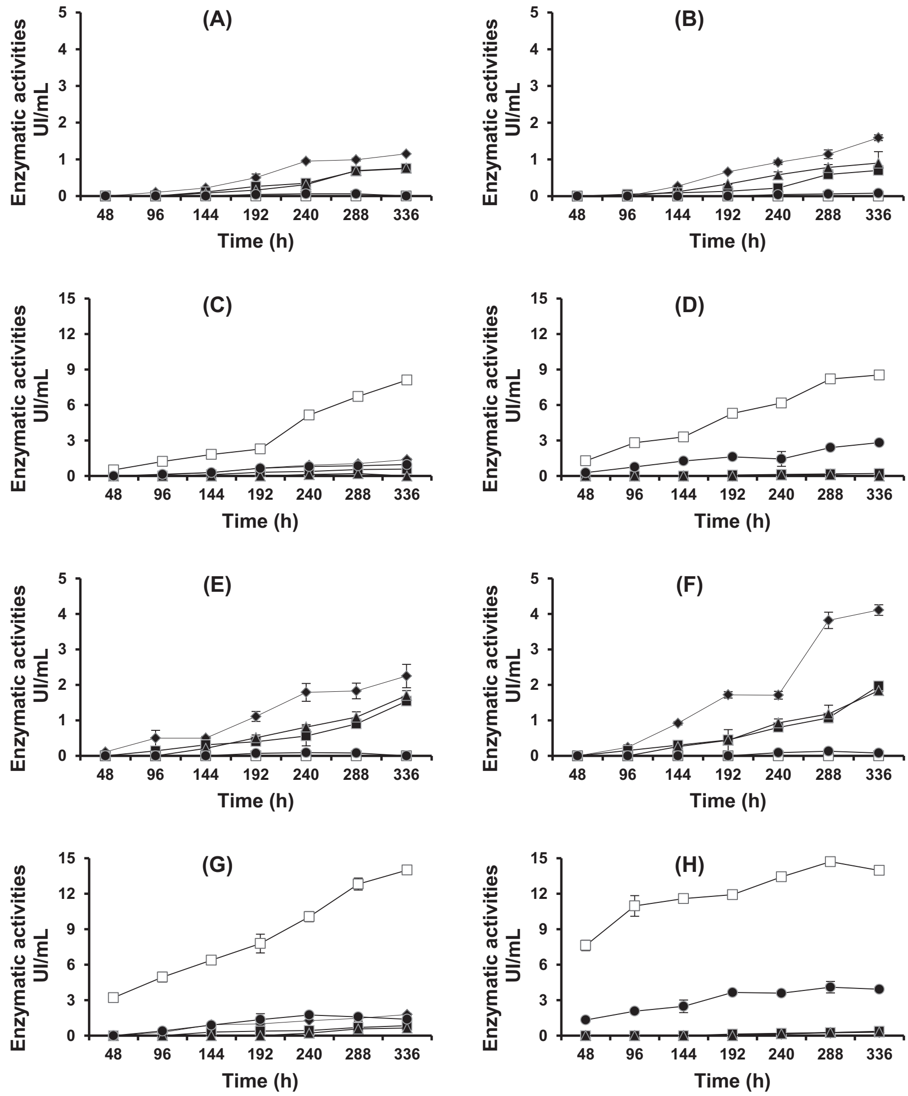

FIG. 1. Enzymatic activities of (A, E) *B. bassiana*, (B, F) *Rhizodiscina cf. lignyota*, (C, G) *F. flocciferum*, and (D, H) *A. niger*, cultered on OC for 15 days at 25 °C, using distilled water (A–D) and salts (E–H) as moisetining solutions. Closed diamonds, closed squares, closed triangles, open squares, and closed circles represent the laccase, MnP, LiP, xylanase, and cellulase production, respectively. Vertical bars indicate standard deviation (SD) from three independent experiments.

titers of MnP and lignin peroxidase (LiP) were recorded for these two strains (less than 1 UI/mL).

For all the strains, enzymatic production was more prominent for the samples moistened with yeast extract and salt solution. The glucose solution used does not seem to have a notable effect since the enzymatic production rate recorded was roughly similar to that obtained with distilled water.

DISCUSSION

Through this study, one can conclude that it is possible to improve the nutritional value of OC by SSF. Three locally isolated strains, which, to our knowledge, have never been used in SSF, and the commercially available strain *A. niger* MUCL 28820, known for its enzymatic production (25–27) were used in this work in order to study the impact of these fungi on the chemical composition and anti-nutritional compounds of OC. Indeed, the use of selected strains leads to an increase of PC and a concomitant decrease of phenolic content after 15 days of culture.

All tested strains were able to grow on OC under SSF even without the need of nutrient supplementation nor sample pretreatment. PC increased up to 94% in OC inoculated with *F. flocciferum*, using yeast extract as moistening solution, compared to the untreated control. This PC increase could be the result of increased fungal biomass (28). Arguably, it could also be due to the secretion of some extracellular fungal enzymes into the substrate in order to convert the fiber materials into monosaccharides (29,30). Similar results had been reported by Iyayi and Losel (31), using different fungi to enrich cassava peel and pulp in protein, and also by Aderemi and Nworgu (32), who demonstrated the ability of *A. niger* to enrich PC of cassava root sieviate and peels.

The present study also revealed that total phenolic compounds, that are the main obstacles towards the use of OC as animal feed, decreased up to 43% when treated with *Rhizodiscina cf. lignyota* and *F. flocciferum* using yeast extract or salts solutions as moistening agent. This decrease in phenolics might be due to the phenol oxidases (laccase) and peroxidases (LiP, MnP) that are produced by fungi. Indeed, the highest recorded TPC decrease was obtained with

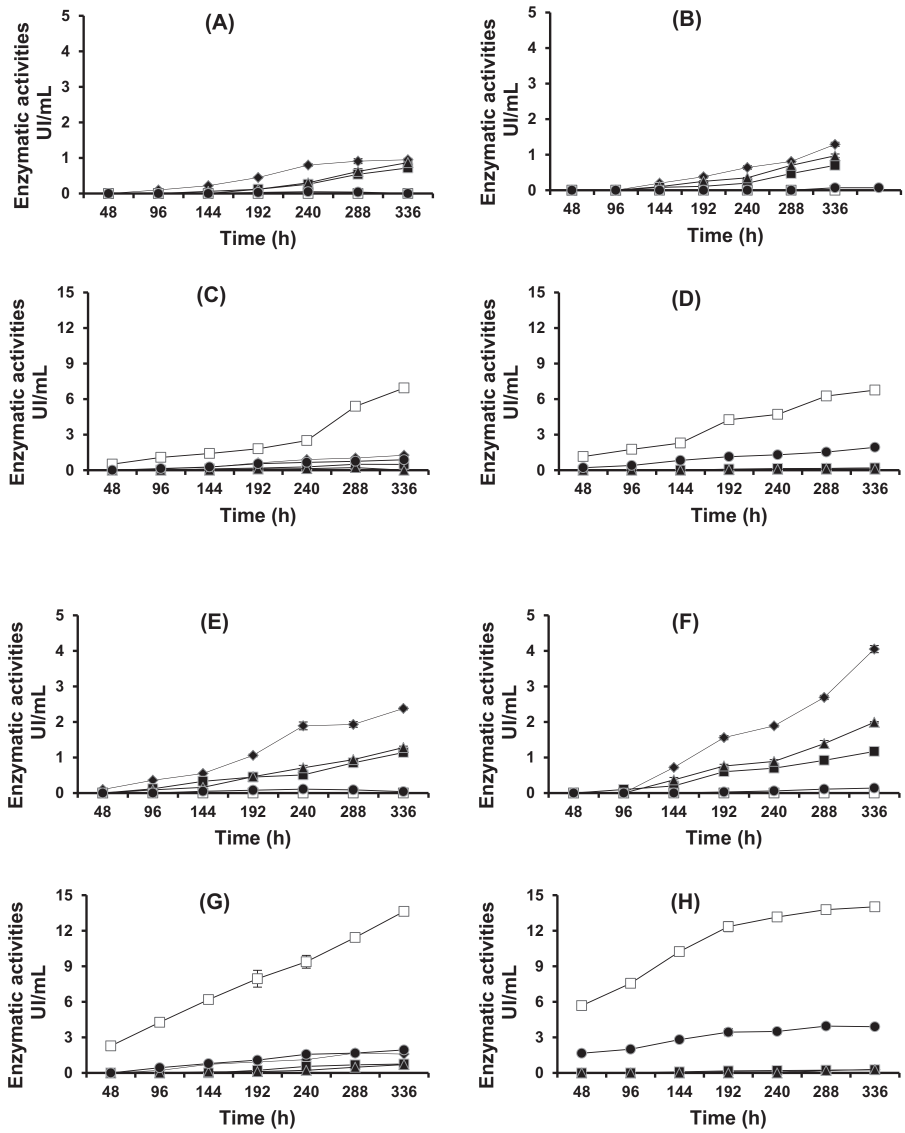

FIG. 2. Enzymatic activities of (A, E) *B. bassiana*, (B, F) *Rhizodiscina cf. lignyota*, (C, G) *F. flocciferum*, and (D, H) *A. niger*, cultered on OC for 15 days at 25 °C, using glucose (A–D) and yeast extract (E– H) as moisetining solutions. Closed diamonds, closed squares, closed triangles, open squares, and closed circles represent the laccase, MnP, LiP, xylanase, and cellulase production, respectively.

two of the strains (*Rhizodiscina cf. lignyota* and *F. flocciferum)* that produced the highest amount of these enzymes.

Laccases are copper glycosyl oxidoreductases that utilize O_2 to oxidize various aromatic and nonaromatic compounds through a radical-catalyzed mechanism. They couple the reduction of O_2 into two water molecules with the oxidation of many substrates, such as phenols, arylamines, anilines, thiols and lignins. In fact, many studies have involved laccases for their capacities to oxidize and depolymerize lignin, to delignify wood pulp and also for their capacities to detoxify phenolic pollutants (33–35). LiP and MnP from white rot fungus were reported to be effective in removing phenolics from olive mill water (36,37), and decolorizing kraft pulp mill effluents (38). In another study (39), these enzymes, produced by *Fusarium moniliforme*, were effective in the delignification of rice straw under SSF and caused relatively higher lignin degradation in comparison *to Phanerochaete chrysosporium*. Of the four strains used in this study *Rhizodiscina cf. lignyota*, *B. bassiana* and *F. flocciferum* produced larger quantities of the enzymes mentioned above, in comparison with *A. niger*. Moreover, these three strains were the ones that proved to be effective in decreasing the TPC, thus confirming what has been reported above.

A. niger and *F. flocciferum* have been characterized by a high production of xylanase and cellulase. Almost same findings have been reported in other studies that investigate the cellulase and xylanase production by *A. niger* (27,40–42) and others *Aspergilli* species (22,40) under SSF, and higher enzymes activities than those obtained in our study were recorded, depending on the substrate utilized, the SSF and the extraction conditions. Likewise, many *Fusarium* species have been described to be good cellulase and xylanase producing fungi (44,45).

Besides providing a source of protein in OC, the hydrolytic activity of both enzymes improves the accessibility to cellulosic material, which increases nutrient absorption and the digestibility ratio (46). In addition, the use of these enzymes was also shown to lead to weight gain in chickens and piglets (47,48).

The OC phenolics profile depends on several factors, namely the cultivar, climatic conditions, fruit maturation, and the olive oil extraction procedure (49). In our study the detected phenolic compounds were 3,4-dihydroxybenzoic acid, tyrosol, vanillin, vanillic acid, caffeic acid, catechin, and ferulic acid. The results reported herein are in agreement with those reported in previous works that showed the presence of the same compounds in OC extracts (5,50,51).

After SSF, *Rhizodiscina cf. lignyota* and *F. flocciferum* were found to be more efficient to completely remove the majority of phenolic compounds than the other tested strains. This is consistent with a previous study where *F. flocciferum* was shown to degrade phenolic compounds such as gallic, protocatechuic, vanillic, caffeic, and ferulic acids as well as syringic aldehyde and reduce them below detection limit in liquid medium (52). Other studies also showed that *F. flocciferum* is able to grow on phenol, catechol and resorcinol at concentrations up to 1 g/L (53,54).

Considering all the screening parameters studied, almost all the strains showed the highest performance for treatment when yeast extract or salts was the moistening agent. This result is congruous with the findings of other authors who found that the addition of a nitrogen source (i.e., yeast extract, ammonium sulfate) increased the production of exoglucanase by *Aspergillus fumigatus* (43) and laccase by *Streptomyces psammoticus* (55), it has also been reported that the addition of ammonium sulfate improved PC in orange waste when fermented with *Chaetomium* spp. and *A. niger* (56).

Based on all results obtained, a possible scale-up of the SSF could be envisaged. Although SSF processes are rather easy to set up, there are various important factors to be considered for the development of a successful bioprocess. The main obstacles are the low amenability of the process to regulation, and the heterogeneous fermentation conditions (e.g., heat, mass transfer) (57,58). In our case, other parameters related to the strains development have to be studied. As in most cases, SSF processes are conducted under semi-sterile conditions, and even though the water activity is quite low, a development of contaminating bacteria and yeasts may occur as the fungi growth is slower. That's why a study on the type of inoculation have to be realized so as to determine if a mycelium inoculum (in comparison with spore inoculum) can improve the development of the fungi. Despite all these bottlenecks, there are numerous advantages in the scaling-up of the process. For instance, SSF limits water consumption as well as effluent water (57). In addition, the use of OC as carbon source reduces the operational cost; moreover, many other inexpensive nitrogen sources could be utilized (e.g., urea, soymeal) to enrich the substrate. Another important advantage in using SSF for converting the OC into animal feed is that the final product requires a simple drying, thus making downstream processes, which is almost always associated with high costs, unnecessary.

The biodegradation of OC through SSF using the four *Ascomycota* resulted in an increase of crude protein up to 94% and a decrease of phenolic compounds between 10% and 43%. *F. flocciferum* and *Rhizodiscina cf. lignyota* appeared to be able to remove a significant part of the phenolic compounds due to the concerted action of oxidative and lysing enzymes produced by their mycelium. This suggests that SSF with these strains is a suitable system for the conversion of solid waste from olive processing industry into a higher value added animal feed. The next step is to evaluate the effectiveness of these fermented products on the cattle nutrition via *in vitro* digestibility tests.

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