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Changes in the Content of Bioactive Polyphenolic Compounds of Olive Mill Wastewater by the Action of Exogenous Enzymes

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ABSTRACT: The aim behind the present research is to develop an enzymatic treatment for olive mill wastewater (OMW) to release high amounts of simple phenolics having high antioxidant value. OMW was hydrolyzed by a mixed enzyme preparation rich in β-glucosidase produced by *Aspergillus niger*. This research shows that *A. niger* β-glucosidase played a major role in the release of simple phenolic compounds from OMW. These compounds were recovered by ethyl acetate extraction and identified by HPLC and LC-MS. The main identified phenolic compound is hydroxytyrosol. The results of enzymatic hydrolysis of OMW under optimum conditions indicated a maximum hydroxytyrosol concentration of 2.9 g L^{-1} compared to 0.015 g L^{-1} contained in the control (test without added enzyme). The above results prove that OMW is a potential substrate for producing hydroxytyrosol through enzymatic hydrolysis of its glycosides.

KEYWORDS: olive mill wastewater, β-glucosidase, enzymatic hydrolysis, hydroxytyrosol

INTRODUCTION

The Mediterranean basin provides 97% of the total olive production of the world, the olive oil industry being an important activity, and produces 9% of the world's olive oil.[1] The manufacturing process of olive oil has undergone evolutionary changes. The traditional discontinuous pressing process was initially replaced by continuous centrifugation, using a three-phase system and, subsequently, a two-phase system. The different olive oil production methods would undoubtedly yield different waste materials.

The classic production of olive oil generates three phases and two wastes materials: olive oil (20%), solid waste (30%), and aqueous liquor (50%). The solid waste (olive oil cake) is a combination of olive pulp and stones. The aqueous liquor originates from the vegetational water and from the soft tissues of the olive fruits, with water added during the refinement process, the so-called "olive mill wastewater" (OMW). The presence of large amounts of organic substances such as oil, polyphenols, protein, and polysaccharides, which are responsible for high chemical oxygen demand (COD) values (up to 220 g L^{-1}) and mineral salts, represents a major problem for this wastewater treatment.[2] So far, the emphasis has been laid on detoxifying these waste materials prior to disposal, feeding, fertilization, or composting because they are not easily biodegradable and phytotoxic.[2] However, OMW is rich in phenolic compounds, which constitute a very important category of antioxidant phytochemicals that are useful for the pharmaceutical and cosmetic industries.[3]

The concentration of polyphenolic compounds in olive oil ranges from 50 to 1000 μg g^{-1} of oil depending on the olive variety and the extraction system. This amount of antioxidants in the olive oil is only 1−2% of the available pool of antioxidants in the olive fruit.[4] More than 30 biophenols and related compounds have been identified in OMW, and the majority of them manifest potential antioxidant, cardioprotective, and cancer preventive activities in humans as stated by Obied et al.[5] Among these prominent components are hydroxytyrosol, tyrosol, and caffeic acid.[5] The recovery of these compounds or the reuse of OMW for other products demonstrates that the recovery process is more economical and more practical.[3] Besides, recycling OMW is an alternative to diminish its impact on the environment and the ecological system in general. It would also allow the repositioning of the olive oil industry in highly competitive levels by considering its waste materials as byproducts.[2,3] By using adequate technology, the OMW can be converted into value-added products. Similarly, alternative use might be represented by many possible applications: (i) bioconversion into useful products; (ii) recovery of natural components; and (iii) enrichment of OMW in its hydroxytyrosol amount.

Hydroxytyrosol is one of the major phenolic compounds present in olive fruit, and it has been proven to be the most beneficial because of its remarkable pharmacological and antioxidant activities.[6] With regard to the beneficial effects of hydroxytyrosol, several methods have been developed to produce this compound by means of chemical synthesis and enzymatic conversion.[7,8] Other biological methods have been also developed to produce hydroxytyrosol.[9,10] Capasso et al.,[7] Briante et al.,[8] and Liebgott et al.[9] used a simple substrate (oleuropein and tyrosol) for the bioformation of hydroxytyrosol. However, Aranda et al.[10] used an ethyl acetate fraction and the corresponding aqueous exhausted fraction of dry olive mill residue as substrate for the culture of some saprobe fungi that led to the production of hydroxytyrosol.

In this work, we attempted the use of raw OMW as a natural source of hydroxytyrosol, which is abundant contrary to leaf extract or synthetic oleuropein. Indeed, hydroxytyrosol is present in OMW in two forms, free and associated with other molecules such as oleuropein, demethyloleuropein, verbascoside, and hydroxytyrosol glucosides.[11−13] Conjugated hydroxytyrosol cannot be recovered by membrane technology or solvent extraction or by

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endogenous hydrolytic enzymes. Moreover, chemical hydrolysis of OMW needs a high quantity of chemicals (acids, bases) for acidification to pH 2 followed by neutralization. In this last case, the label "BIO" would disappear. Therefore, this research aims at finding methodology to hydrolyze OMW by a β-glucosidase-enriched enzyme preparation to get maximum hydroxytyrosol recovery. The main operational variables governing the enzymatic hydrolysis process (temperature, time, pH, agitation) are studied. The proportion between the enzyme and the substrate has also been evaluated. The proposed enzymatic reaction has been applied to two different kinds of OMW; the first is generated from the traditional discontinuous pressing process (MSP), and the second is generated from a continuous centrifugation using a three-phase system (MCC).

MATERIALS AND METHODS

Materials. Fresh OMW was sampled from an olive mill using traditional discontinuous pressing processs and a continuous centrifugation process, located in Sfax, Tunisia. Samples were obtained from 'Chemlali' olives harvested in the same period in November 2007 and were immediately processed and stored at −20 °C.

Ethyl acetate, acetonitrile, and orthophosphoric acid were HPLC-grade solvents and purchased from Prolabo (France). Hydroxytyrosol, used as a standard, was purified from OMW in our laboratory.[14] 4-Nitrophenyl-β-D-glucopyranoside (PNPG), *p*-nitrophenyl acetate (PNPA), oat spelt xylan, and Folin−Ciocalteu phenol reagent were purchased from Sigma-Aldrich Chemie, Switzerland.

Aspergillus niger (CTM 10099) as a biological material was obtained from the Collection Tunisienne de Microorganismes, Centre de Biotechnologies de Sfax (Sfax, Tunisia). The strain was maintained on potato dextrose agar (PDA) slants.

Fungal Culture Technique. A spore stock suspension was obtained by growing the fungus on a PDA slant at 30 °C for 5 days. The conidia were harvested from the surface by adding sterile water containing 2% (v/v) Tween 80 and scraping the surface with a sterile spatula. The spore concentration obtained was stored in 25% (v/v) glycerol at −20 °C.

Culture broths of *A. niger* were obtained after having grown on wheat bran as the sole carbon source in 500 mL Erlenmeyer flasks containing 100 mL of medium for the fungal growth. The medium was adjusted to pH 4.0 and sterilized by steam autoclaving (20 min, 121 °C). Erlenmeyers were inoculated to give 10^6 spores mL^{-1} and incubated at 30 °C for 5 days in an orbital incubator shaker operated at 160 rpm.

Preparation of Enzyme Solution. After 5 days of incubation, culture broths of *A. niger* were recovered and used as a mixed-enzyme preparation for the hydrolysis reaction of OMW. These preparations were filtered and then centrifuged (6000 rpm). The clarified enzyme solutions were kept at −20 °C until their use for biotransformation and/or enzyme assays.

The enzymatic preparation contained various enzyme activities, which were β-glucosidase (4600 IU mL^{-1}), esterase (200 IU mL^{-1}), α-amylase (92.22 IU mL^{-1}), xylanase (5.40 IU mL^{-1}), CMCase (0.60 IU mL^{-1}), and filter paper (0.144 IU mL^{-1}).

Enzymatic Assays. The β-glucosidase activity was determined according to the method of Norkrans[15] but using a 5 mM substrate instead of the 1 mM concentration. A 0.1 mL volume of enzymatic sample was mixed with 1 mL of 5 mM PNPG in 50 mM sodium citrate buffer (pH 4.8) and kept at 50 °C for 10 min. The reaction was stopped by the addition of 2 mL of 1 M Na_2CO_3 followed by 10 mL of water, and the absorbance was measured at 405 nm. In the control, 0.1 mL of water was used instead of the enzymatic sample. The activities were measured in triplicates and were expressed in International Units per milliliter (IU mL^{-1}). One IU is defined as the amount of enzyme that catalyzes the release of 1 μmol of *p*-nitrophenol per minute.

The esterase activity was carried out according to the method of Mackness et al.[16] The reaction mixture contained 50 μL of crude enzyme, 50 μL of 150 mM PNPA in ethanol, and 2.9 mL of Tris-HCl buffer (9.2 mM, pH 7.5) to give a final concentration of 2.5 mM PNPA in a total volume of 3 mL. The reaction was initiated by the addition of enzyme extract and incubated at 25 °C during 4 min. Absorbance was determined at 405 nm. A standard curve was prepared in the range of 0−220 nmol of *p*-nitrophenol.

Carboxymethyl cellulase (CMCase) assay for endo-β-1,4-glucanase was carried out according to this method: Add 0.5 mL of enzyme, diluted in citrate buffer, to a test tube of at least 25 mL volume. Add 0.5 mL of substrate solution, mix well, and incubate at 50 °C for 30 min. Add 3.0 mL of 3,5-dinitrosalicylic acid (DNS), mix, and boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks, glucose standards, and the spectro zero should be boiled together. After boiling, transfer immediately to a cold water bath. Add 20 mL of deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion. Measure the color formed against the spectro zero at 540 nm.

Assays for crude xylanase were performed using 0.5% soluble oat spelt xylan in 50 mM sodium phosphate buffer, pH 7.0. The reaction mixture was composed of 1.8 mL of substrate and 0.2 mL of crude enzyme. The mixture was incubated in a water bath at 60 °C for 15 min. The released reducing sugar was measured by the DNS method in which the reaction was stopped by adding 3 mL of DNS reagent. A reddish brown color developed after the reaction tubes had been placed in a boiling water bath for 5 min. After the reaction tubes had cooled to room temperature, the optic density was measured at 575 nm with xylose as the standard, where 1 IU of xylanase activity is defined as the amount of enzyme that releases 1 μmol of xylose per minute per milliliter under the above-mentioned conditions.

α-Amylase activity was evaluated by using 1.0 mL of crude enzyme extract prepared as above added to 1.0 mL of substrate (0.15% potato starch) incubated for 10 min at room temperature, and the reaction was quenched by the addition of 3 mL of iodine reagent (0.6% iodine, 6% KI in water). One milliliter of this was diluted to 50 mL with 0.5 N HCl. The absorbance at 620 nm of the reaction mixture (1.0 mL of enzyme added to a mixture of 1.0 mL of substrate and 3 mL of iodine reagent) was read against the blank (containing no substrate). The total activity was estimated against a standard and expressed as micrograms of substrate degraded per minute per milliliter of enzyme.

Filter paper assay for saccharifying cellulase (FPU assay) was carried out according this method: Add 1.0 mL of 0.05 M sodium citrate, pH 4.8. Add 0.5 mL of enzyme, diluted in citrate buffer. At least two dilutions must be made of each enzyme sample investigated. Temper to 50 °C, add one filter paper strip, and mix (no. 1 filter paper strip, 1.0 × 6.0 cm). Then incubate at 50 °C during 60 min. Add 3.0 mL of DNS and follow the steps as for the CMCase assay. After cooling, add 20 mL of deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion. When the "pulp" has settled well, the color formed is measured against the spectro zero at 540 nm.

Biotransformation Reaction. The bioconversion reactions for the determination of optimum conditions were conducted in Erlenmeyer flasks. For the optimization of pH and temperature and for the choice of OMW source, bioconversion reactions were carried out at a β-glucosidase concentration of 900 IU mL^{-1} of reaction medium. The effects of temperature on β-glucosidase activity and biotransformation efficiency were determined by assaying the enzyme reaction, respectively, during 10 min and 2 h at pH 4.8 in static condition.

The effect of pH on β-glucosidase activity was determined by assaying the enzyme activity in static condition at 50 °C and varying the pH from

2.0 to 8.0. Citrate−phosphate buffer solutions (50 mM) were prepared under different pH conditions (from 2.0 to 8.0) in such a way as to assess the enzyme behavior even in conditions very different from those of OMW bioconversions. The effect of this parameter on biotransformation efficiency was determined by assaying the reaction at 50 °C and varying the pH of the reaction medium.

For the agitation parameter, enzymatic reactions at optimum conditions (pH, temperature, and enzyme concentration) were monitored with (400 rpm) and without agitation.

Total Phenol Content Determination. Concentration of total phenols was measured as gallic acid equivalents.[17] One milliliter of diluted sample was transferred to a test tube, and 1 mL of 95% ethanol, 5 mL of distilled water, and 0.5 mL of Folin−Ciocalteu phenol reagent were added. After an incubation period of 5 min, 1 mL of 5% Na_2CO_3 was added, and the mixture was mixed well and kept in the dark for an hour. Subsequently, the samples were vortexed, and the absorbance was measured at 725 nm using a UV spectrophotometer.

HPLC Analysis. The presence and amount of phenolic compounds in the OMW extract were investigated by reversed-phase HPLC analysis using a binary gradient elution. HPLC was performed on a Shimadzu C-R6A liquid chromatograph. The separation was carried out in a Supelco C18 column (250 mm × 4.6 mm; Waters Chromatography). Compounds were eluted with a gradient of acetonitrile (70%)−H_3PO_4 (0.1%), where the acetonitrile concentration was varied as follows: 0 min, 10%; 0−20 min, increased to 50%; 20−25 min, 50%; and 25−30 min, decreased to 10%. The column temperature was maintained at 40 °C, and the flow rate was 0.5 mL min^{-1}. Sample detection was achieved at 280 nm with a Shimadzu SPD 6AUV detector connected to a Shimadzu C-R6A integrator. The injection volume was 20 μL. Compounds were identified and quantified by comparison of retention times (RT) and peak areas of standards analyzed in the same conditions: hydroxytyrosol (RT = 10 min); tyrosol (RT = 15 min); *p*-coumaric acid (RT = 25 min); caffeic acid (RT = 30 min); oleuropein (RT = 38 min). OMW phenolic extracts were prepared by using the liquid−liquid extraction method.[18] OMW phenolic extraction was carried out in a separatory funnel, where OMW sample (5 mL) was mixed with the same volume of ethyl acetate. The mixture (solvent−OMW) was vigorously shaken for 10 min to achieve equilibrium and then centrifuged for 3 min at 3000 rpm. The organic phase was separated and filtered with a syringe filter (0.45 μM) for HPLC analysis.

LC-MS Analysis. The LC-MS/MS experiments were carried out with an Agilent 1100 LC system consisting of a degasser, a binary pump, an autosampler, and a column heater. The column outlet was coupled with an Agilent MSD ion trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were carried out on a personal computer with Data Analysis software (Chemstation). For the chromatographic separation a Zorbax 300 Å Extend-C-18 column (2.1 × 150 mm) was used. The column was held at 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in ACN) for 1 min, followed by an 11 min step gradient from 5 to 100% B, and then it was kept for 4 min with 100% B. Finally, the elution was achieved with a linear gradient from 100 to 5% B in 2 min. The flow rate was 200 μL min^{-1}, and the injection volume was 5 μL. The following parameters were employed throughout all of the MS experiments: for electrospray ionization with negative ion polarity, the capillary voltage was set to 3.5 kV, the drying temperature to 350 °C, the nebulizer pressure to 40 psi, and the drying gas flow to 10 L min^{-1}. The maximum accumulation time was 50 ms, the scan speed was 26000 m/z s^{-1} (ultra scan mode), and the fragmentation time was 30 ms.

RESULTS AND DISCUSSION

The management, treatment, and safe disposal of OMW impose serious environmental concerns due to the presence of phenolic compounds. Many researchers have evaluated the feasibility and economic processes for recovering olive phenols from OMW or solid wastes.[3,7,19] The main concept of the herein proposed methodology is that the polyphenols contained in OMW could be selectively hydrolyzed by enzymes, affording extracts rich in free compounds useful for the pharmaceutical and cosmetic industries, on the one hand, and producing a wastewater free of polyphenols and significantly reduced COD, on the other hand. Hydroxytyrosol was selected as a pattern for the biotransformation studies because it is the main polyphenol in OMW and furthermore possesses the most potent antioxidant activities.[5,6] In this study, we suggest the biotransformation of OMW polyphenols using an enzyme preparation, obtained from the fermentation of wheat bran by *A. niger* and presenting a high β-glucosidase activity. Different parameters of the proposed enzyme hydrolysis reaction were studied and optimized to maximize hydroxytyrosol release.

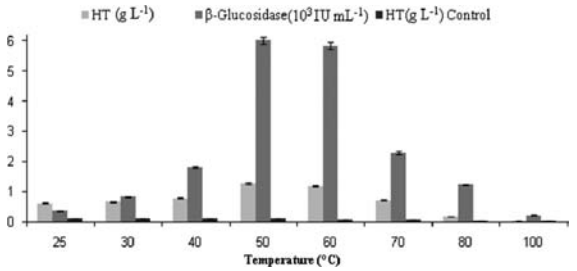

Figure 1. Effect of temperature on β-glucosidase activity and on hydroxytyrosol production. Error bars are standard deviation (n = 2).

Temperature Dependency on β-Glucosidase Activity and Bioconversion Reaction. Different assays of β-glucosidase activity and OMW bioconversion reactions were carried out in separate batches monitored at different temperature conditions. The effect of this parameter on β-glucosidase activity and on hydroxytyrosol production through the enzymatic hydrolysis of OMW is shown in Figure 1. The temperature of reactions was varied from 25 to 100 °C. Results show that the β-glucosidase activity increased progressively with the increase of temperature from 25 to 50 °C. However, the β-glucosidase activity was significantly higher at temperatures ranging between 50 and 70 °C than at temperatures ranging from 25 to 40 °C. As is obvious in Figure 1, crude enzyme preparation showed higher catalytic activity at 50 °C. It was completely inactivated at an incubation temperature of 100 °C (Figure 1). The optimum temperature of our enzyme is similar to the majority of the earlier reported bacterial and fungal β-glucosidases,[20,21] and yet lower than those of some thermostable β-glucosidases.[22] However, the heat tolerance of our β-glucosidase is higher than reported for most β-glucosidase experiments.[23]

The hydrolytic effect of the enzyme preparation on OMW at different temperatures was assayed by measuring the amount of hydroxytyrosol released at the end of the reaction (Figure 1). The identification and quantification of this product were achieved by HPLC apparatus and based on the comparison of the chromatographic retention time and UV absorbance spectra with those of an authentic standard and confirmed by using a LC-MS system operating in a negative mode. Simple phenolic compounds recovered from the hydrolyzed OMW, determined by HPLC, were hydroxytyrosol (peak 1, as major compound) and tyrosol (peak 2, as minor compound). The most notable effect was the increase of hydroxytyrosol concentration, coupled with a concomitant disappearance of oleuropein (peak 3) (Figure 2). Chemically, oleuropein is the ester of oleoside 1,1-methyl ester and hydroxytyrosol.

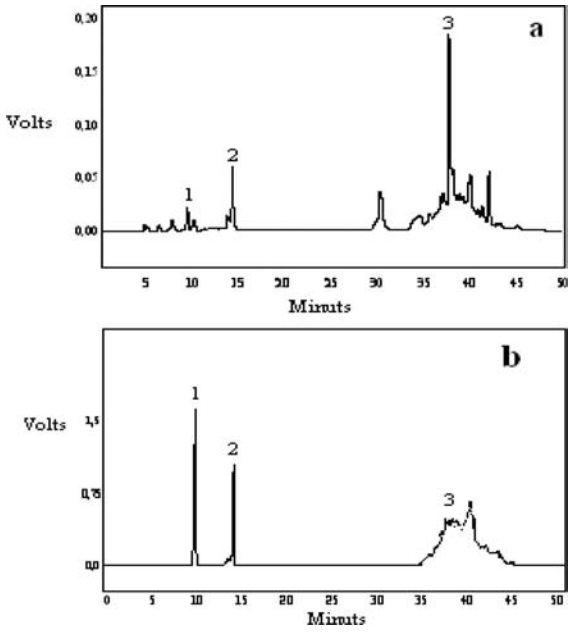

Figure 2. HPLC chromatograms at 280 nm of OMW extract before (a) and after (b) enzymatic hydrolysis: (peak 1) hydroxytyrosol; (peak 2) tyrosol; (peak 3) oleuropein.

The latter is the main product of oleuropein hydrolysis. The β-glucosidase enzymes break the glucoside bond, releasing oleuropein aglycone, which rapidly gives result to hydroxytyrosol and elenolic acid in a chemical and biochemical way.[7] Identification of hydroxytyrosol was also confirmed by LC-MS (Figure 3). The mass spectrum present in Figure 3 showed a peak m/z 153 at 2.2 min that corresponded to hydroxytyrosol. The fragmentation of such a peak led to fragments having molecular masses m/z 109 and 123 resulting from succession to those published by Savarese et al.[24] Also, HPLC profiles (Figure 2) show an increase of tyrosol concentration after hydrolysis treatment of raw OMW. Oleuropein, hydroxytyrosol, and tyrosol are related structurally. Hydroxytyrosol and tyrosol are structurally identical except that hydroxytyrosol possesses an extra hydroxy group in the meta position. In fact, OMW generally contains in addition to oleuropein other glucosylated phenolic molecules, such as ligstroside and demethyloleuropein.[8,24,25] When those conjugates are hydrolyzed, they give rise to hydroxytyrosol, tyrosol, and several forms of elenolic acid. Our results show that the enzymatic pretreatment of OMW induced hydrolysis of oleuropein and other complex phenolic molecules.

Figure 1 shows a clear positive correlation between the concentration of hydroxytyrosol released and the β-glucosidase activity. OMW hydrolysis using β-glucosidase-enriched enzyme preparation carried out at 50 °C and without ajustement of OMW pH showed high hydroxytyrosol concentration versus low oleuropein concentration after 2 h of incubation time (Figures 1 and 2). The concentration of hydroxytyrosol reached a value of 1.26 g L^{-1} at the end of the reaction. However, the control (OMW treated in the same condition but without enzyme addition) did not show any increase of hydroxytyrosol concentration (0.015 g L^{-1}). The enhancement of substrate bioconversion to hydroxytyrosol and tyrosol is probably due to the action of β-glucosidase, esterase, and other enzymes present in the *A. niger* enzyme preparation. These preliminary results confirm that β-glucosidase is the key factor of hydroxytyrosol release from oleuropein, whereas a previous study has demonstrated the enzymatic bioconversion of this compound by using homogeneous recombinant β-glucosidase from hyperthermophilic archaeon *Sulfolobus solfactaricus* expressed in *Escherichia coli*.[8]

From these results, we can conclude that the highest hydroxytyrosol concentration recorded in these enzymatic experiments, which corresponded to the highest β-glucosidase activity, was >1 g L^{-1}. Consequently, it is possible to state that 50 °C is the optimum temperature condition for this enzymatic bioconversion.

pH Dependency on β-Glucosidase Activity and Bioconversion Reaction. The optimum pH for β-glucosidase activity was determined in the assay mixture over a pH range of 2.0−8.0. The same study was operated for OMW bioconversion in the presence of β-glucosidase-enriched enzyme preparation. The effects of pH on β-glucosidase activity and hydroxytyrosol yield are presented in Figure 4. Controls were also carried out by adding water instead of enzyme preparation. Figure 4 shows that the pH has no effect on hydroxytyrosol release. However, the addition of enzyme preparation shows an increase of hydroxytyrosol concentration at all pH values. The results indicated that β-glucosidase exhibited a maximum activity at pH 4.8. It was completely inactive at pH values above 6 and under 3. The β-glucosidase activity was comparatively higher in the acidic range than in the alkaline range. These data show a relatively higher pH optimum for *A. niger* β-glucosidase than for most other fungal and bacterial β-glucosidases.[20,21,26] This corresponds very well with the natural pH of OMW, which ranges between 4.6 and 5.

The effect of pH also demonstrates a clear positive correlation between the release of hydroxytyrosol and the β-glucosidase activity. The bioconversion reaction at pH 4.8 gives the highest concentration of hydroxytyrosol (1.54 g L^{-1}), which is the result of the highest β-glucosidase activity recorded in these experiments.

Effect of OMW Type on the Hydroxytyrosol Release. The manufacturing process of olive oil has undergone evolutionary changes. The traditional intermittent pressing process was replaced by the continuous centrifugation process using a three-phase system. In this study, OMW generated by the first and second processes is called "Margine Super Press" (MSP) and "Margine Chaine Continue" (MCC), respectively. MSP and MCC samples are originated from 'Chemlali' olive variety and processed at the same time. A comparative study of enzymatic hydrolysis of these two kinds of OMW by the same enzyme preparation was operated in batch condition. Table shows the concentration of total simple phenolic compounds and the amount of hydroxytyrosol before and after OMW enzymatic hydrolysis. The initial hydroxytyrosol concentration was very low in MCC (0.015 g L^{-1}) and in MSP (0.018 g L^{-1}) (Table 1). After biotransformation, total simple phenolic compounds concentration sharply increased, as a result of the cleavage of polyphenolic−saccharide links and other bound (cell wall-associated) forms of polyphenols.[6] For the two kinds of OMW, quantitative analyses show that the main simple phenolic compound released was hydroxytyrosol. The concentration of this antioxidant reached values of 0.89 and 1.52 g L^{-1}, respectively, in MSP and MCC. This shows that the release of hydroxytyrosol depends on the type of OMW. Of course, this could be attributed to the differences in the processing technique, although we think that it could also be due to the amount of added water during the oil extraction process. This comparative study of bioconversion demonstrated that MCC has more potential for biotransformation than

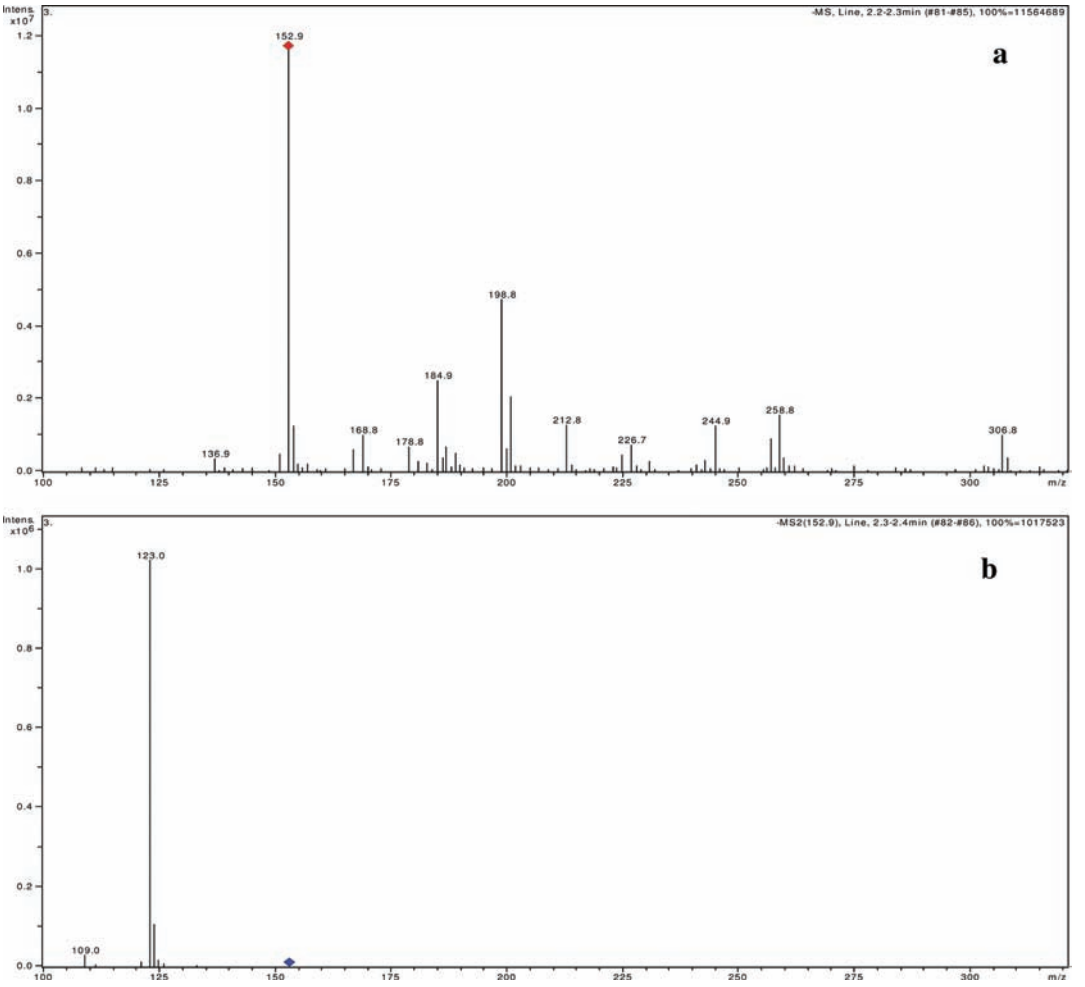

Figure 3. Mass spectra of 2.2 min total ion chromatographic peak in hydrolyzed OMW: (a) MS; (b) MS^2.

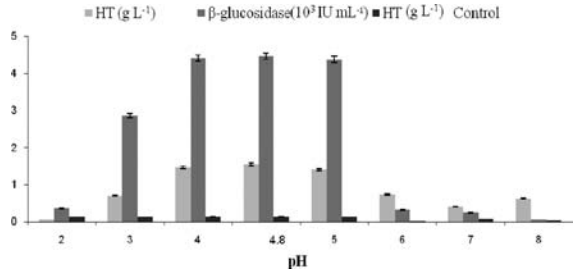

Figure 4. Effect of pH on β-glucosidase activity of *A. niger* culture broth and on hydroxytyrosol production. Error bars are standard deviation (n = 2).

MSP (Table 1). The presence of more hydrolyzable polyphenols such as oleuropein, ligstroside, or verbascoside could explain the high release of hydroxytyrosol in MCC.[27] For this reason, we have suggested the use of MCC for the subsequent experiments.

Dependence of Hydroxytyrosol Production on β-Glucosidase Concentration. The effect of enzyme concentration on OMW polyphenol bioconversion was also investigated. For this study, the volume of OMW in medium reaction was fixed and the enzyme content varied. Here, the enzyme preparation has a β-glucosidase activity of 4600 IU mL^{-1}. The bioconversion

Table 1. Total Simple Phenol Content and Hydroxytyrosol Concentration before and after Enzymatic Hydrolysis of MCC and MSP[a]

OMW	$[Ph]_i$ (g L^{-1})	$[Ph]_f$ (g L^{-1})	$[HT]_i$ (g L^{-1})	$[HT]_f$ (g L^{-1})
MCC	7.420	12.900	0.018	1.520
MSP	4.340	9.420	0.015	0.890

[a] i, initial concentration; f, final concentration; HT, hydroxytyrosol; Ph, phenols.

reactions were carried out at pH 4.8 and heated at 50 °C, which were determined as optimal conditions for the *A. niger* β-glucosidase activity (Figures 1 and 4). Enzyme concentration was varied from 0 to 1125 IU mL^{-1} reaction medium. The relationship between hydroxytyrosol production and enzyme concentration was also examined. Results presented in Figure 5 show a significant increase of hydroxytyrosol concentration in the presence of different concentrations of enzyme. Nevertheless, the control (without addition of enzyme) did not show any improvement of hydroxytyrosol concentration over incubation time. This demonstrates that *A. niger* culture broth exhibited high hydrolytic activity on the polymeric compounds present in OMW. The increase of hydroxytyrosol production was proportional to the enzyme activity present in the reaction medium. Results show that as the enzyme

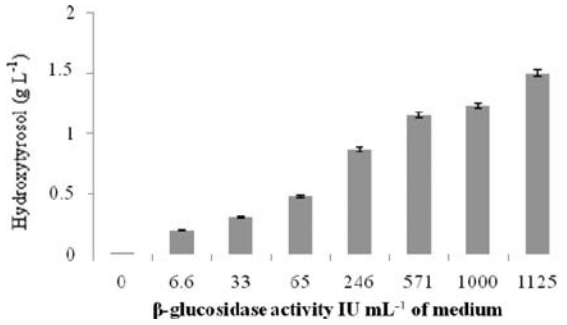

Figure 5. Effect of β-glucosidase concentration on hydroxytyrosol production. Error bars are standard deviation (n = 2).

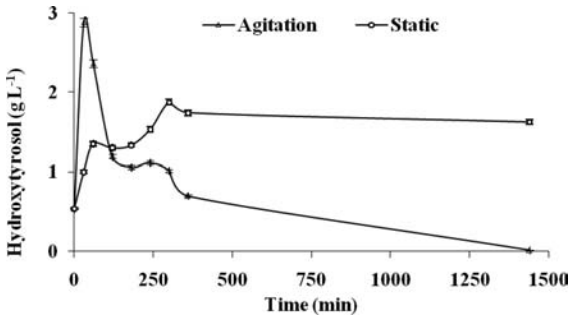

Figure 6. Evolution of hydroxytyrosol production during the enzymatic hydrolysis of MCC in static and agitated condition. Error bars are standard deviation (n = 2).

concentration increased in the reaction medium, the hydroxytyrosol concentration increased as well. These findings indicate that β-glucosidase is the key enzyme in this process; also, the β-glucosidase concentration is essential for OMW polymers bioconversion to release hydroxytyrosol. This was demonstrated by Capasso et al.,[7] who have studied the enzymatic hydrolysis of oleuropein, extracted from olive plant, by free β-glucosidase from almond. It should also be taken into account that the amount of hydroxytyrosol in OMW comes not only from the hydrolysis of oleuropein and verbascoside but also from the hydrolysis of hydroxytyrosol 4-β-D-glucoside, where the β-glucosidase is the key enzyme for hydroxytyrosol release.[11,28,29]

The highest hydroxytyrosol yield (1.5 g L^{-1}) was observed at an enzyme concentration of 1125 IU mL^{-1} reaction medium (Figure 5). Here, it could be noted that there is an increase of hydroxytyrosol concentration to about 0.4 g L^{-1} by doubling the enzyme concentration from 571 to 1125 IU mL^{-1}. The obtained yield could be considered as not satisfactory by comparison to the reaction cost. The ideal system would involve the quickest possible reaction rate by equilibrating the amount of β-glucosidase used. To economize the enzyme quantity, we suggest the use of 500 IU mL^{-1} as optimum concentration that generates sufficient release of hydroxytyrosol.

Effect of Agitation on the Bioconversion Reaction. The effect of agitation on the rate of hydroxytyrosol release was studied. The time course of OMW hydrolysis by β-glucosidase-enriched enzyme preparation was also evaluated by the determination of hydroxytyrosol concentration. The evolution of hydroxytyrosol accumulation during the bioconversion reaction of

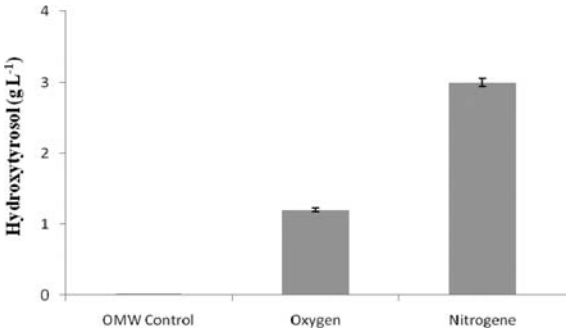

Figure 7. Hydroxytyrosol concentration after hydrolysis treatment of OMW during 1 h of agitation in the presence of atmospheric oxygen and nitrogen.

MCC is shown in Figure 6. The bioconversion reactions were carried out at optimum conditions determined previously.

The optimum β-glucosidase concentration was assessed to be 500 IU mL^{-1} reaction medium. Results show that the production of hydroxytyrosol was rapid and excellent in agitation condition. Maximum hydroxytyrosol concentrations of 2.9 and 1.5 g L^{-1} were registred, respectively, after 30 min of agitation and after 5 h in static condition (Figure 6). Here, we note that the agitation acceleration improves the enzymatic hydrolysis of OMW. This result could be explained by the saturation of active catalytic sites of *A. niger* β-glucosidase in static condition. On the basis of these results, the agitation is an important parameter for optimum OMW enzymatic hydrolysis. However, our findings show that the static condition resulted in the greatest stability of hydroxytyrosol in the reaction medium. In fact, the molecule of hydroxytyrosol is very sensitive to oxidation essentially in agitated condition. Indeed, in previous studies, stabilization of olive oil residues with ethanol was found to be an essential step to prevent enzymatic and nonenzymatic oxidative reactions responsible for phenolic compound degradation and/or polymerization.[28,30] Consequently, from this work, the destruction of hydroxytyrosol occurs after a few hours of reaction due to its oxidation by oxygen. This was demonstrated by two experiments of enzymatic hydrolysis monitored at optimum conditions during 1 h of agitation. The first was carried out in the presence of atmospheric oxygen, and the second was monitored in the presence of nitrogen. The second experiment showed maximum hydroxytyrosol production and higher stability in comparison to the first one (Figure 7). Besides, the enzymatic hydrolysis of OMW in agitated condition only for the first 30 min showed the stability of hydroxytyrosol concentration during the following 6 h of reaction, and then it decreased progressively with time. For this reason, 30 min of enzymatic reaction is sufficient to release a high amount of hydroxytyrosol from MCC (2.9 g L^{-1}).

In comparison with a long-term storage method published earlier, the suggested enzymatic method is very promising.[28] After 5 months of storage, significant accumulation of hydroxytyrosol was observed. The corresponding concentration increased in OMW by a factor of 3.5.[28] Indeed, the long-term storage method is very simple but has a high management cost. It requires a huge space for several months and produces sludge and a bad smell.

In conclusion, a raw enzyme preparation rich in β-glucosidase has been successfully used for hydroxytyrosol release from OMW. A high concentration of hydroxytyrosol (2.9 g L^{-1}) was obtained after 30 min of the proposed enzymatic reaction. This concentration has not been previously reported, and it is one of the highest

concentrations of hydroxytyrosol reported for OMW after chemical or biological conversion.

The proposed enzymatic pretreatment of OMW allowed the recovery of natural hydroxytyrosol with chemical-free methods, food-grade solvents, which made this process economically feasible. Preliminary calculation of process cost gave a very interesting value of U.S. $695/m^3. This bioprocess will certainly open new horizons in marketing of hydroxytyrosol as a powerful natural antioxidant. Hydroxytyrosol from hydrolyzed OMW can be recovered by ultrafiltration or simple liquid−liquid extraction. A direct ultrafiltration process would lead to a concentrate containing the target phenolic compounds. Alternatively, the solvent extraction process of the hydrolyzed OMW resulted in an extract containing the target compounds and an exhausted fraction representing the aqueous phase of OMW. This fraction remains at its initial pH, is free of polyphenols, and has a significantly reduced COD in comparison to raw OMW. These characteristics make the hydrolyzed wastewater very easy to treat by biological process.

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