Chapter 14

The Possibility of Recovering of Hydroxytyrosol from Olive Milling Wastewater by Enzymatic Bioconversion

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Additional information is available at the end of the chapter

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Abstract

This chapter discusses an innovative approach to obtain liquid fractions from olive mill wastewater (OMW) rich in hydroxytyrosol. The method is based on bioconversion combined with membrane separation techniques. An enzymatic bioconversion of three types of OMW was tested. TheS total volumes of OMW are 15 and 40 L. The reaction was monitored in mechanically stirred systems for 2 h at 50°C. Maximum hydroxytyrosol concentrations of about 1.53, 0.83 and 0.46 g/L in the presence of 5 IU *Aspergillus niger* β-glucosidase per milliliter from North OMW and South OMW were procured by two different olive millings, which are milling super press (MSP) and milling continuous chain (MCC), respectively. Enzymatic pretreatment was followed by two tangential flow membrane separation stages, microfiltration (MF) and ultrafiltration (UF). The ultrafiltration permeate was concentrated by evaporation at 45°C for 2 h. The latter exhibited a chemical oxygen demand (COD) level of 48.44 g/L. The UF permeate dehydration increased the hydroxytyrosol concentration to 7.2 g/L. A new natural product that contains some minerals beneficial to health and devoid of heavy metals or chemicals was obtained by this innovative work which describes an environmentally friendly process at pilot-scale.

Keywords: olive milling waste water, bioconversion, membrane separation, hydroxytyrosol, antioxydants

1. Introduction

Ancestral food of the Mediterranean, olive oil saw its production increases steadily, due to the recognition of its high dietetic nutritional value. After milling of the olive by pressing, the oil phase and the aqueous phase are separated and the liquid organic residue called vegetable

water is discarded. This by-product poses significant pollution problems. The olive mill wastewater (OMW) treatment is difficult due to the high concentration of pollution load toxicity microflora and low biodegradability. The pollution load of olive industry output is exceptional: it usually exceeds 120 g COD/L and can reach up to 200 g COD/L. The toxicity of this effluent is very high due to its high content of phenolic compounds. Each year, 40 million m^3 of vegetable water is produced around the Mediterranean basin. Most of these liquid wastes are treated by gathering in evaporation ponds built in the open. This produces the accumulation and concentration of the bulk of the organic matter present. Various methods have been tested for the treatment of vegetable waters.

The olive mill wastewater (OMW), with a high concentration of aromatic compounds (2.5–20 g/L), is a potential source of molecules or precursors to valuable molecules, particularly ortho-diphenols. These compounds, of 10–100 times higher concentration than in the vegetation water of olive oil [1], are known for their antioxidant properties and their beneficial role in preventing certain diseases such as cardiovascular disease. Ortho-diphenols are of major industrial interest for the food company, antioxidants as potential natural alternates to synthetic antioxidants. The ortho-diphenol hydroxytyrosol is a very powerful antioxidant. It also has a wide range of biological activities, in particular antibacterial, antiinflammatory and antihypertensive.

The process of producing hydroxytyrosol from the OMW has been developed in recent years [2]. A period of storage of OMW is necessary for the hydroxytyrosol recovery after its spontaneous fermentation. This storage time sometimes exceeds 100 days [3]. However, the stability of hydroxytyrosol and resistance to its oxidation and its fungal and microbial degradation require reflection and technical practices such as the search for the concentration of ethanol to be added to the effluent throughout the enrichment period. Indeed, the search for new, more attractive techniques overcoming these problems will be mandatory.

The theme of our chapter is related to a part of the search for a gentle and inexpensive method for increasing hydroxytyrosol concentration in OMW [4]. We were interested in finding an optimal method of enrichment hydroxytyrosol. A good approach is that the polymerized phenols in the OMW can be modified by bioconversion, thus producing an extract rich in simple phenolic compounds. The latter are of practical interest for the pharmaceutical, food and cosmetics. Besides, the process significantly reduces the chemical oxygen demand (COD) required for the degradation of these compounds.

2. Olive mill wastewater: a source of natural phenolic compounds

Ninety-seven percent of the total olive production of the world was delivered in Mediterranean basin. Nine percent of the world's olive oil was produced in the olive oil industry of Mediterranean basin [5]. The industrial method of olive oil has undertaken many changes. The traditional discontinuous pressing process was initially switched by continuous centrifugation, using a three-phase system and, subsequently, a two-phase system. The different olive oil production methods would certainly yield different waste materials.

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2.1. Composition of olive mill wastewater

Three phases and two wastes materials: olive oil (20%), solid waste (30%) and aqueous liquor (50%) were produced by classic production of olive oil. The olive pulp and stones were mixed in solid waste. The aqueous liquor originates from the vegetation water and from the soft tissues of the olive fruits, with water added during the modification process, the so-called olive mill wastewater (OMW). High chemical oxygen demand (COD) values (up to 220 g/L) and mineral salts are a major problem for the wastewater treatment which is due to the presence of large amounts of organic substances such as oil, olive oil phenol, protein and polysaccharides [6]. A very important category of antioxidant is present in OMW which is phenolic compounds that are useful for the pharmaceutical and cosmetic industries [7]; 50–1000 μg/g of phenol compounds are present in olive oil ranges. This variation depends on the olive variety and the extraction system. The antioxidants quantity in olive oil is only 1–2% of the available pool of antioxidants in the olive fruit [8]. In OMW, more than 40 biophenols have been identified. Potential antioxidant, cardioprotective and cancer preventive actions in humans were manifested in these compounds [9]. Hydroxytyrosol, tyrosol and caffeic acid are among prominent components [9] (**Figure 1**). The recovery process of these compounds from OMW is more economical and more practical than other OMW valorization method [7]. 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 OMW as by-products [6, 7]. Therefore, OMW can be converted into value-added products. Many possible applications were tested in this study: (i) bioconversion into useful products; (ii) recovery of natural components; and (iii) enrichment of OMW in hydroxytyrosol.

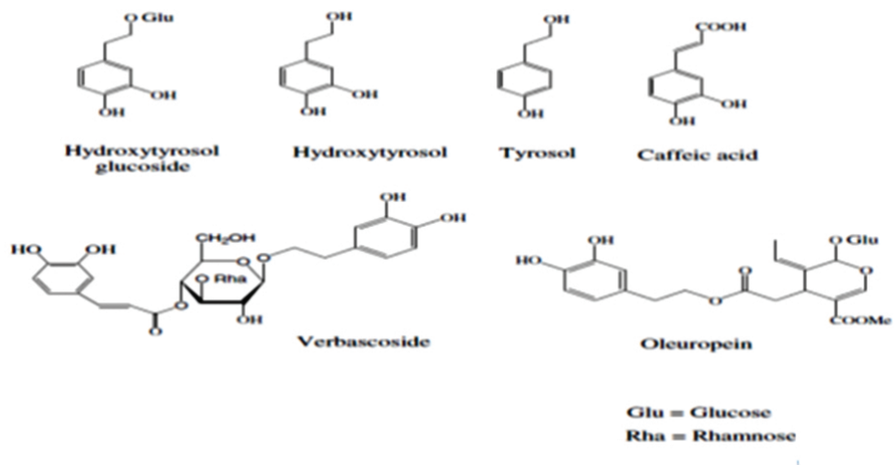
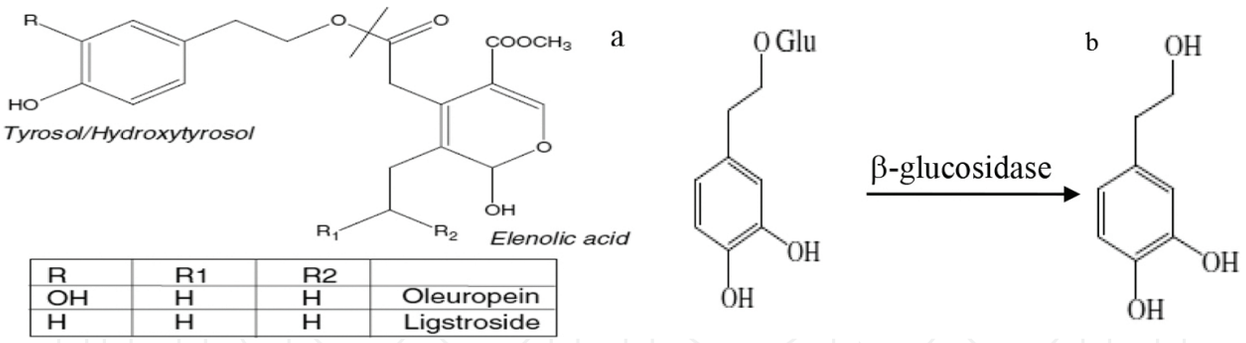


Figure 1. Chemical structures of phenolic compounds of OMW [9].

Hydroxytyrosol is the most valuable because of its amazing pharmacological and antioxidant actions, and it belongs to the major phenolic compounds present in olive fruit [10, 11]. Through

concern to the positive possessions of hydroxytyrosol, numerous approaches have been developed to produce this compound by means of chemical synthesis and enzymatic conversion [12, 13]. Further biological procedures have also been developed to produce hydroxytyrosol [14, 15]. Capasso et al. [12], Briante et al. [13] and Liebgott et al. [14] have tried the bioformation of pure substrate (oleuropein and tyrosol) to hydroxytyrosol. In the other hand, an ethyl acetate fraction and the corresponding aqueous exhausted fraction of dry olive mill residue were used as substrate for the culture of some saprobe fungi that led to the production of hydroxytyrosol [15].

The use of raw OMW as a natural source of hydroxytyrosol is very important because it is widespread in nature to leaf extract or synthetic oleuropein [16–18]. Indeed, hydroxytyrosol is present in OMW in two forms, free and combined. Combined molecules are oleuropein, demethyloleuropein, verbascoside and hydroxytyrosol glucosides [19–21] (**Figures 1** and **2**). Conjugated hydroxytyrosol cannot be recovered by membrane technology or solvent extraction. Furthermore, high amount of chemicals (acids, bases) for acidification to pH2 followed by neutralization was needed in chemical hydrolysis of OMW. Then, the label "BIO" would disappear. Consequently, this study purposes at discovery procedure to hydrolyze olive mill wastewater by a β-glucosidase preparation to acquire maximum hydroxytyrosol regaining. The key functioning variables governing the enzymatic hydrolysis process (temperature, time, pH, agitation) are studied. The amount among the enzyme and the substrate has also been estimated. The proposed enzymatic reaction has been practical to two diverse categories of OMW: the first is produced from the traditional discontinuous pressing process (milling super press, MSP), and the second is made from a continuous centrifugation using a three-phase system (milling continuous chain, MCC).



R	R1	R2	
OH	H	H	Oleuropein
H	H	H	Ligstroside

Figure 2. Release of hydroxytyrosol and tyrosol after degradation of oleuropein and ligstroside, respectively, [22] (a); hydrolyzate of hydroxytyrosol 4-β-D-glucoside (4-beta-D-glucosyl-hydroxyphenylethanol 3) (b).

2.2. Properties of hydroxytyrosol

Hydroxytyrosol is characterized by a strong antioxidant activity, which is similar to that of several synthetic and antioxidants, namely 2,6-di-tert-butyl-p-hydroxytoluene (BHT) and 3-tert-butyl-6-hydroxyanisole (BHA) [23]. Aeschbach et al. [24] showed that the antioxidant activity of this molecule was comparable to that of thymol, carvacrol, 6-gingerol and zingerone. It contributes to the stability of virgin olive oil [25]. It also inhibits low-density lipoprotein

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(LDL) oxidation and also confers good cell protection and the dietary properties of virgin olive oil [25]. In addition to its antioxidant activity, hydroxytyrosol has an important interest in relation to human health. It has been shown that this ortho-diphenol opposes the cytotoxic effect of reactive oxygen metabolites in the cell, which prevents cell damage [26]. Hydroxytyrosol also exerts a marked antiinflammatory action. Petroni and his team showed that hydroxytyrosol inhibits the formation of a pro-inflammatory eicosanoide referred to as "leukotriene B4" [27]. De la Puerta and his team [27] found that hydroxytyrosol, tyrosol, oleuropein and caffeic acid inhibit the formation of leukotriene B4 by reducing the activity of 5-lipoxygenase, the enzyme that catalyzes this synthesis. Furthermore, it was reported that this enzyme is inhibited by olive extract, and the substances responsible for this effect are hydroxytyrosol, oleuropein and caffeic acid [28].

2.3. Synthesis of hydroxytyrosol

The only natural source of hydroxytyrosol remains for the moment the olive and olive oil by-products [29, 30]. Olive oil contains only a small amount of hydroxytyrosol concentrations of 0.01–1 mg/100 g of olive oil. This is due to the high solubility of hydroxytyrosol in water (about 5 g/100 mL); it is present in the OMW, which is in many cases discharged into the environment. A first pathway of hydroxytyrosol production relies on its purification from the olive mill wastewater. This process offers an impure product given that the great diversity and the large amount of polyphenols listed in the OMW (2.5–3% by weight, about a hundred different phenols [31]). Moreover, obtaining a purified fraction containing the hydroxytyrosol from the waste involves several chromatographic steps using large amounts of solvents [29]. Therefore, researchers have attempted to find a less expensive process for the purification of hydroxytyrosol. Other production pathways based on chemical methods include the synthesis of hydroxytyrosol by chemical reduction of the 3,4-dihydroxyphenylacetic acid [32, 33] and by catalytic conversion of tyrosol to hydroxytyrosol with a mixture of methylrhenium trioxide (MTO) and hydrogen peroxide. Two enzymatic production routes for hydroxytyrosol have also been described:

1. A hydroxytyrosol production by hydrolysis of oleuropein in the presence of β-glucosidase [34]. The β-glucosidase used in the process of Briante et al. is produced by a recombinant strain of *Escherichia coli*; the β-glucosidase from *Sulfolobus solfataricus* is a hyperthermophilic bacterium. This method has several disadvantages: the enzyme and oleuropein must first be purified from the bacterial culture and from olive leaves, respectively. The final extract is constituted by a mixture of hydroxytyrosol and two forms of the elenolic acid.

2. An enzymatic synthesis by conversion of tyrosol in the presence of a tyrosinase extracted from a fungus [35]. This second enzymatic method also has its limitations: firstly, the method requires the preliminary purification of tyrosinase with several steps which further increase the cost of production; secondly, the method requires the presence of ascorbic acid for inhibiting cresolase activity of tyrosinase and to avoid the formation of quinones; an additional final purification step must be implemented to eliminate the ascorbic acid from the reaction mixture.

Another type of production was approved by whole cells grown on tyrosol. Producing bacteria *Serratia marscecens* [36], *Pseudomonas aeruginosa* [2, 37], *Pseudomonas putida* F6 [38] and *Halomonas* sp. HTB24 [14], the enzyme system responsible for the bioconversion of tyrosol hydroxytyrosol has never been identified in these different bacterial species.

3. *A. niger* choice: generally recognized as a safe "GRAS" microorganism

One of the most important Ascomycota multi-uses in biotechnological applications is *A. niger*. Its use fascinated production of extracellular enzymes, such as glucoamylase [39], pectinase [40], the acidic lipase [41], esterase feruloyl [42] and xylanase [43]. This species is also known for the production of some organic acids such as gluconic acid [44] and citric acid [45]. Citric acid and several enzymes produced by *A. niger* are considered as generally recognized as a safe (GRAS) by the "Food and Drug Administration" of the United States [46]. In addition, various biotransformation of ferulic acid in vanilla [47], progesterone in polyethylene [48], isosteviol in diperpenoide and isosteviole [49], terpenes in "2-alpha, 3-beta, 13-trihydroxystemodane" [50], linalool oxide mixture of cis-and trans-linalool furanoid and cis-and trans-pyranoid oxide linalool are formed through *A. niger* [51]. During the past two decades, *A. niger* was the most broadly used food enzyme [46].

Besides its many applications in industry, *A. niger* also has an important role as environmental microorganism. It is involved in the biodegradation of the toxic chemicals, e.g., dioxins [52], in the treatment of waste molasses from beet and OMW [53, 54] and the bioconversion of sewage sludge [55]. *A. niger* biomass is also used in the biosorption toxic heavy metals such as cadmium, chromium and copper [56, 57].

The natural by-product was acknowledged as a solid support and a source of energy and carbon. Agricultural wastes were used for mushroom cultivation; this offers the advantage of combining the use of an inexpensive substrate and an interesting road to recover these by-products. Many by-products generated by the food industry were used as substrate for fungi such as sugar cane residue, wheat bran, rice and barley straw, beet pulp and pulp coffee [44, 58–61].

4. Bioconversion

4.1. Microbiological synthesis

This production method, also called "fermentation," uses bacteria or fungi cultured in the presence of the reagent selected as a precursor to synthesize the molecule. This type of biological reaction is relatively easy to implement. The proposed culture can be improved by optimizing the composition of the culture media of strains and experimental conditions [62]. Bioproduction processes involve various reactions such as hydroxylation, oxidation, reduction, hydrolysis, esterification, decarboxylation, methylation, condensation and isomerization.

Bioproduction reactions have been applied to the production of several types of molecules, in particular flavorings and aromatic antioxidants [63]. A typical example for the production of aroma is the synthesis of vanilla. Several studies have shown that some bacteria (*Pseudomonas putida*, *Streptomyces setonii*, *Amycolatopsis*, etc.) and some fungi (*Pycnoporus cinnabarinus*, *A. niger*, etc.) are able to convert ferulic acid to vanillin [64]. Vanillin was also produced by the bioconversion of isoeugenol by means of a *Bacillus* sp. type of bacteria [65].

4.2. Enzymatic synthesis

This method involves enzymes purified and immobilized on a suitable support. The choice of the nature of the enzyme depends on the chemical structures of the precursor and the product to be synthesized. Thus, to produce an ortho-diphenol, it is necessary to use a monooxygenase. Starting from a precursor free of hydroxyl group, it is necessary to employ a dioxygenase. This type of bioconversion reaction requires the use of cofactors such as NADH, NADPH and ATP [37]. In practical terms, the bioconversion reactions using cells (stationary growth) seem to be more interesting than those involving purified enzymes. Furthermore, regeneration of cofactors generally constitutes a handicap in the use of many enzymes. Besides, enzymatic hydrolysis of oleuropein by β-glycosidase has been studied [66]. It generates oleuropein aglycone. Immobilization by inclusion of a recombinant β-glucosidase and thermophilic *Sulfolobus solfataricus* of chitosan on a support so as to hydrolyze oleuropein enzymatically at 60 and 70°C was contemplated by Briante et al. [67]. The enzymatic hydrolysis attacks only the glycosidic linkage; the biotransformation generates unstable aglycone species by cleavage at a temperature above 60°C and releases hydroxytyrosol. An immobilized thermophilic enzyme in a bioreactor can solve the technical problems of hydrolysis of different substrates. Indeed, the elevated temperatures used limit microbial growth and assist in the solubilization of the substrate [68].

5. Analytical methods

5.1. High-performance liquid chromatography (HPLC) analysis

The identification and quantification of phenolic monomers were carried out by HPLC. The assays were achieved on a Shimadzu apparatus composed of an LC-10ATvp pump and an SPD-10Avp UV/visible detector. The column was a C-18 (4.6 × 250 mm; Shim-pack VP-ODS), and its temperature was kept at 40°C. The flow rate was 0.5 mL/min. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 50 min, and the next amounts of solvent B were used for the elution: 0–30 min, 20–50%; 30–35 min, 50%; and 35–50 min, 50–20%. The flow rate was 0.6 mL/min, and the injection volume was 20 μL. Identification and quantification of HT were based on its spectrum and on its retention time in comparison with standard analyzed under the same conditions.

5.2. Enzyme activities assays

The β-glucosidase activity was determined using 1 mM p-nitrophenyl-β-glucoside (pNPG) as substrate (in 100 mM citrate buffer pH 4.8). An aliquot of 0.2 mL of 1 mM pNPG in 50 mM sodium citrate buffer (pH 4.8) was incubated with 0.280 mL of citrate buffer and 20 μL of an appropriate diluted enzymatic preparation at 50°C for 15 min. The reaction was stopped by adding 0.5 mL Na_2Co_3 1 M; the liberated p-nitrophenol (pNP) was measured at 400 nm [68]. In the blank, 20 μL of water was used in place of the enzyme sample. The activity was defined as μM of p-nitrophenol produced per minute below the analyzed conditions (IU). Activities were measured in triplicate and were expressed in International Unit per milliliter (IU/mL) with 1 IU being defined as the amount of enzyme that catalyzes the release of 1.0 μM of p-nitrophenol per minute.

6. Scale-up of bioconversion of OMW phenolic compounds

The basic assumption of the method proposed by Hamza et al. [4, 16, 17] is that the polyphenols contained in OMW could be selectively hydrolyzed by enzymes to generate extracts or free compounds useful for the pharmaceutical and cosmetic industry and produce a wastewater that is free from polyphenols and with significantly reduced COD.

6.1. Enzymatic bioconversion of OMW

Figure 3a, **c**, and **e** shows the HPLC chromatogram of the ethyl acetate extract of raw MCC, MSP and North OMW. This profile shows that fresh OMW is rich in oleuropein (peak 4) and luteolin (peak 3). Simple phenolic bioactive compounds, such as HT (peak 1) and tyrosol (peak 2), were too present at moderate concentrations. The HT concentrations recorded in raw MCC, MSP and North OMW were 0.17, 0.23 and 0.86 g/L, respectively. This content corresponded to the free fraction of HT in raw OMW [4].

Further enzymatic analysis revealed that the β-glucosidase activities in the raw OMW of MCC, MSP and North OMW were 0.7, 0.7 and 1.03 IU/mL, respectively (**Figure 3**). This enzyme could be derived from olive fruit [20] and from the activity of endogenous microorganisms [69]. The enzyme concentration described above (as well as of other hydrolytic enzymes) is not plenty for the hydrolysis of molecules present in fresh OMW. With the time of storage of OMW, the HT concentration was previously shown to increase as a result of natural fermentation and cleavage of HT conjugates [3]. The increase in bioactive compound concentrations was tested through the enzymatic hydrolysis of OMW during 2.0 h at 50°C using 5 IU of *A. niger* β-glucosidase per milliliter of OMW [16]. As shown in **Figure 3**, important quantities of HT were free after the enzymatic pretreatments of OMW. A progressive rise was detected for the HT concentration in OMW over the response time of the β-glucosidase action in subsequent OMW enhancement by this enzyme. Blank controls deprived of enzyme were also run, and no significant HT production rates were detected (**Figure 2**). The HT quantities in the blank controls of MCC, MSP and North OMW were 0.2, 0.23 and 0.8 g/L, respectively. Several factors are known to affect the quantitative phenolic profiles of olive fruits. Among those factors, the

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geographical origin, cultivar type, irrigation treatment and ripeness degree have the most pronounced impacts on phenolic composition [20]. The initial hydroxytyrosol concentrations recorded in this study corresponded to an average of about 41.14% of their final value in the hydrolyzed OMW. In the absence of broth culture, the substrate was not converted into HT even at high temperature (50°C). HPLC chromatograms indicated an increase in the HT peak (1) and tyrosol peak (2) with a simultaneous reduction in the oleuropein peak (4) and luteolin peak (3), respectively, subsequently the enzymatic pretreatment of OMW (**Figure 3**). These results established that β-glucosidase played a significant role in cleavage of the glycosidic bonds in the molecules present in OMW. Hence, HT could have created from oleuropein, via its aglycone, by the opening of the elenolic acid loop with a final reorganization into the secoiridoid compound and numerous procedures of elenolic acid [70]. This result is in agreement with the findings previously reported in the studies of Capasso et al. [29], Briante et al. [71], Hamza et al. [4, 16, 17] and Khoufi et al. [18].

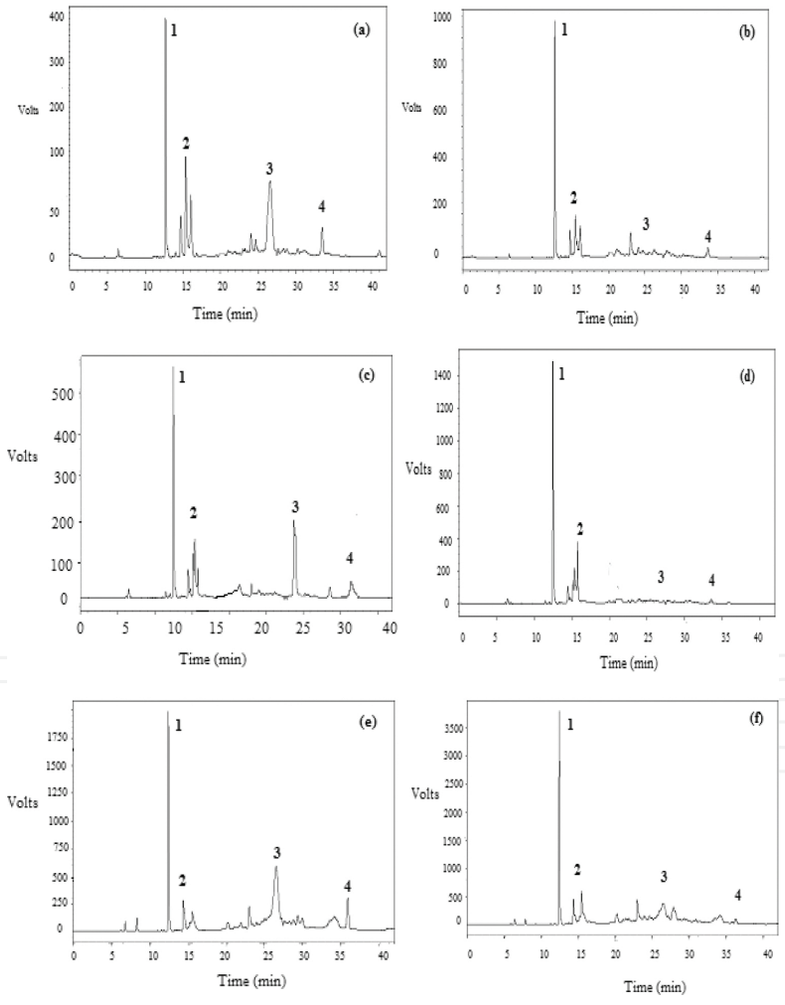

Figure 3. HPLC chromatogram of phenolic compounds [4] (detection at $\lambda = 280$ nm) extracted from raw MCC (a) and hydrolyzed MCC by *A. niger* β-glucosidase (b), raw MSP (c), hydrolyzed MSP by *A. niger* β-glucosidase (d), raw OMW North Tunisia (e) and hydrolyzed OMW North Tunisia by *A. niger* b-glucosidase (f). 1, hydroxytyrosol; 2, tyrosol; 3, luteolin; 4, oleuropein.

During enzymatic experiments, maximum HT concentrations of about 1.53, 0.83 and 0.46 g/L were obtained in the presence of 5 IU *A. niger* β-glucosidase per milliliter of OMW in North OMW, MSP and MCC, respectively. The results of this relatively large-scale study were inferior than those described in a preceding small-scale study by the authors [17]. So the initial HT concentration in large volumes of fresh OMW is much higher than in small volumes of fresh OMW acquired from separators in oil mills. The findings from this phase of the study suggested that OMW was a source of hydrolysable phenols, including oleuropein, ligstroside and verbascoside, which possess glycosidic and ester links between the main phenol components, polysaccharides and/or lignin (**Figure 1**) [70, 72]. Consequently, a higher quantity of HT was released when enzymatic hydrolysis was carried out on OMW using specific enzyme preparations. The proposed enzymatic pretreatment proved useful not only on a laboratory scale but also on large-scale applications involving OMW recycling.

6.2. Membrane separation technologies

Several studies have recently shown that membrane filtration is preferably applied in a cross flow mode. In cross flow or tangential flow filtration, the feed is pumped into the membrane module where it is separated into two streams, namely the filtrate (or permeate) and the retentate, in which the retained species has been concentrated. The tangential movement of the fluid targets removes most of the rejected materials from the membrane surface and, consequently, minimizes their accumulation at the membrane surface. Membrane technologies are known to employ special filters (membrane) that operate in a special fluido-dynamic condition (tangential flow), which reduces the filter fouling and, consequently, assures a high permeate flux as a function of time [73]. These technologies are commonly applied worldwide not only for the treatment of wastewaters but also for the recovery of dispersed solutes, often pollutants and generation of purified water.

The present study opted for the application of membrane technology for the treatment of OMW to enhance the extraction and recovery of its valuable phenol content and to facilitate its proper disposal in the environment. According to the proposed process, the OMW was submitted to enzymatic hydrolysis by a β-glucosidase enzyme to remove oleuropein and, thus, enhance the free HT in OMW (**Figure 3**). The liquid fraction separated from the degradation products was then used in a membrane system including tangential microfiltration (MF), tangential ultrafiltration (UF) and evaporation units in sequence. Both membrane retentate fractions constituted the new refined products with the addition of purified water obtained after the evaporation steps and the last concentrate recuperated which is phenol enriched, respectively. The ultimate concentrate of the system after evaporation was rich in hydroxytyrosol.

The initial fluxes of OMW processing by MF and UF were 42.85 and 103.89 L/m^2h in transmembrane pressures of 1.8 and 2.2 bar, separately. The determination of chemical oxygen demand (COD), pH, conductivity, hydroxytyrosol, total solids (TS) and total phenolic content were evaluated at different operating conditions. The COD load was due to high organic contents, which are nitrogen compounds, sugars, organic acids, oils, cellulose and phenolic compounds, with a concentration of 165 g/L in the raw OMW. A substantial decrease in COD and total solids (TS) was detected. The membrane technology under study generated a slightly

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colored permeate, with less COD for its oxidation (3.42% of the initial COD) (**Table 1**). Microfiltration was prominent to eliminate 72.12% of COD. In fact, all the organic matter of the OMW in the form of an insoluble material suspension was retendered by microfiltration. However, the MF permeate contains the hydrophilic compounds, which are reducing sugars, hydroxytyrosol and minerals. Only 24% of hydroxytyrosol was recovered in the ultimate concentrate. This proportion was considerably enhanced by diluting the MF and UF retentates with pure water. Accordingly, diafiltration was required to recuperate more hydroxytyrosol. The utilization of moderate temperatures in evaporation step led to a hydroxytyrosol-rich concentrate. The results provided evidence that the process of this study can be used as an efficient treatment of OMW at mild conditions. Besides, 41% of the enhancement in total phenols was due to the discharge of hydroxytyrosol (**Table 1**). A ecological production of great quantities of hydroxytyrosol can, consequently, be realized. A promising alternative method was funded by the use of a pilot-scale method for the recovery of natural hydroxytyrosol. An additive in the food industry or an anaerobic substrate for methane production can be recycled by the retentate of MF and UF units which are rich in polyphenolic compounds. The total recovery of the chemical components in OMW using membrane technologies was previously reported on a similar process by Pizzichini and Russo [74].

Raw OMW		**Hydrolyzed OMW**		**MF**	**UF**	**Cc**
P	**R**	**P**	**R**			
pH						
5.03 ± 0.2	4.93 ± 0.2	4.78 ± 0.2	4.73 ± 0.2	4.81 ± 0.2	4.79 ± 0.2	4.84 ± 0.2
Total solids (g 100/g)						
9.33 ± 0.5	9.23 ± 0.5	6.28 ± 0.5	11.12 ± 0.5	4.71 ± 0.5	5.72 ± 0.5	41.95 ± 0.5
Conductivity (ms/cm)						
11.26 ± 0.5	11.25 ± 0.5	11.17 ± 0.5	10.62 ± 0.5	10.29 ± 0.5	10.7 ± 0.5	15.11 ± 0.5
COD (g/L)						
165.76 ± 1.5	165.83 ± 1.5	57.76 ± 1.5	119.25 ± 1.5	48.44 ± 1.5	59.62 ± 1.5	nd
Total phenols (g/L)						
4.5 ± 1.2	5.5 ± 1.2	3.31 ± 0.5	7.27 ± 1.2	1.96 ± 0.2	2.7 ± 0.5	17.6 ± 2.5
Reducing sugar (g/L)						
26 ± 3.2	60 ± 4.2	nd	nd	nd	nd	158.21 ± 8.5
Hydroxytyrosol (g/L)						
0.23 ± 0.2	0.93 ± 0.2	0.85 ± 0.1	0.72 ± 0.1	0.79 ± 0.1	0.807 ± 0.1	7.2 ± 1
Mineral composition of ultimate concentrate (mg/kg)						
Zn (240 ± 0.05); Cd (0.0264 ± 0.01); Ca (1156 ± 0.2); Fe (46 ± 0.01); K (61.36 ± 5.1); Mg (617.2 ± 6.3); Na (46.4 ± 0.5); Cr; (0); Pb (0); Ni (0); Cu (0)						

P, permeate; COD, chemical oxygen demand; R, retentate; nd, not determined; MF, microfiltration; UF, ultrafiltration; Cc, concentration with evaporation.

Table 1. pH, total solids, conductivity, total simple phenol, chemical oxygen demand (COD) content and hydroxytyrosol concentration [4] before and after the enzymatic hydrolysis of olive mill wastewater and after microfiltration, ultrafiltration and concentration; the mineral composition of ultimate concentrate from purified OMW.

6.3. Characterization of final product

Table 1 shows the characteristics of the OMW fractions at the different steps. The final product had a pH value of 4.84 which remains constant during the process. Conductivity was reserved constant, except after concentration step was increased. The ultimate concentrate contains a high concentration of reducing sugar (about 158.21 g/L) (**Table 1**). The inorganic content of this new product was found to consist mainly of metals. The metal content of the ultimate concentrate is shown in **Table 1**. This product was exempt of heavy metals but rich in calcium, iron, potassium, manganese and sodium (**Table 1**).

Metals remain the key from nutritional and toxicological perspectives. Certain metals, principally iron, copper and zinc, are indispensable constituents for the human body, and their deficiency can have chronic and severe effects [75]. OMW comprises certain added significant metal ions, such as magnesium and calcium, which have often been reported to diminish the danger of heart sicknesses [76]. Trace elements, such as copper and iron, should not be eliminated when they are in short dietary supply. Elements, such as cadmium and lead, which can accumulate in the body, should be minimized. Consequently, the characteristics of the raw material and extraction techniques have a significant effect on the composition of the final extracts, and, in industrial applications, the composition of olive phenol extracts must be standardized. In fact, an 'aqueous extract' of OMW suitable for foods and beverages can be easily obtained by simple ultrafiltration [77]. The commercialization scenarios will, therefore, depend on the intended use, whether an individual compound, a multicomponent mixture or crude aqueous extract of OMW are to be recovered.

7. Conclusion

This chapter discusses the potential of a scale-up enzymatic treatment for OMW with the aim of increasing HT concentration. Hydrolytic treatments were investigated using a culture broth of *A. niger* on wheat bran in a pilot-scale 100-L fermentor for 7 days. The use of the filtrate from the *A. niger* culture broth as a biocatalyst released 1.53, 0.83 and 0.46 g of HT per liter of North OMW, MSP and MCC, respectively. This process produced a natural and a bioactive product from a vegetal source as opposed to the molecule obtainable through chemical synthesis. Taken together, the findings suggested that the proposed enzymatic pretreatment may be considered useful not only at laboratory-scale applications but also at pilot-scale applications involving OMW recycling. The application of membrane filtration processes allowed for the recovery of four main liquid fractions in different volumetric percentages, all of which may have a potential for commercial use in the food, nutraceutical and cosmetic industries. In the ultimate concentrate obtained, only 24% of HT was recovered. The new product obtained met principally the dietary and other requirements: rich in HT, slightly acidic, with a reduced sugar content, devoid of heavy metals and chemicals and rich in minerals.

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References

[1] Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J. C., Lorquin, J., Delattre, M. (2001). Simple phenolic content in olive oil residues as a function of extraction systems. Food Chem. 75, 501–507.

[2] Allouche, N., Damak, A., Ellouz, R., Sayadi, S. (2004). Use of whole cells of Pseudomonas aeruginosa for synthesis of the antioxidant hydroxytyrosol via conversion of tyrosol. Appl. Environ. Microb. 70, 2105–2109.

[3] Feki, M., Allouche, N., Bouaziz, M., Gargoubi, A., Sayadi, S. (2006). Effect of storage of olive mill wastewaters on hydroxytyrosol concentration. Eur. J. Lipid Sci. Technol. 108, 1021–1027.

[4] Hamza, M., Sayadi, S. (2015). Valorisation of olive mill wastewater by enhancement of natural hydroxytyrosol recovery. Inter. J. Food Sci. Technol., 50, 826–833.

[5] Fiorentino, A., Gentili, A., Isidori, M., Monaco, P., Nardelli, A., Parrella, A., Temussi, F. (2003). Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components. J. Agric. Food Chem. 51, 1005–1009.

[6] Khoufi, S., Aloui, F., Sayadi, S. (2008). Extraction of antioxidants from olive mill wastewater and electro-coagulation of exhausted fraction to reduce its toxicity on anaerobic digestion. J. Hazard. Mater. 151, 531–539.

[7] Vlyssides, A. G., Loizides, M., Karlis, P. K. (2004). Integrated strategic approach for reusing olive oil extraction. J. Clean Prod. 12, 603–611.

[8] Rodis, P. S., Karathanos, V. T., Mantzavinou, A. (2002). Partitioning of olive oil antioxidants between oil and water phases. J. Agric. Food Chem. 50, 596–601.

[9] Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., Robards, K., Stockmann, R. (2005). Bioactivity and analysis of biophenols recovered from olive mill waste. J. Agric. Food Chem. 53, 823–837.

[10] Obied, H. K., Bedgood, Jr., D. R., Prenzler, P. D., Robards, K. (2007). Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities. Food Chem. Toxicol. 45, 1238–1248.

[11] Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gomez-Caravaca, A. M., Segura-Carretero, A., Fernandez-Gutierrez, A., Lercker, G. (2007). Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules. 12, 1679–1719.

[12] Capasso, R., Evidente, A., Visca, C., Gianfreda, L., Marmonti, M., Greco, G. J. R. (1996). Production of glucose and bioactive aglycone by chemical and enzymatic hydrolysis of purified oleuropein from Olea europaea. Appl. Biochem. Biotechnol. 61, 365–377.

[13] Briante, R., Patumi, M., Febbraio, F., Nucci, R. (2004). Production of highly purified hydroxytyrosol from Olea europaea leaf extract biotransformed by hyperthermophilic β-glycosidase. J. Biotechnol. 111, 67–77.

[14] Liebgott, P.-P., Amouric, A., Comte, A., Tholozan, J.-L., Lorquin, J. (2009). Hydroxytyrosol from tyrosol using hydroxyphenylacetic acid-induced bacterial cultures and evidence of the role of 4-HPA 3-hydroxylase. Res. Microbiol. 160, 757–766.

[15] Aranda, E., Garcia-Romera, I., Ocampo, J. A., Carbone, V., Malorni, A., Sannino, F., De Martino, A., Capasso, R. (2007). Reusing ethyl acetate and aqueous exhausted fractions of dry olive mill residue by saprobe fungi. Chemosphere. 66, 67–74.

[16] Hamza, M., Khoufi, S., Sayadi, S. (2012). Changes in the content of bioactive polyphenolic compounds of olive mill wastewater by the action of exogenous enzymes. J. Agric. Food Chem. 60(1), 66–73.

[17] Hamza, M., Khoufi, S., Sayadi, S. (2012). Fungal enzymes as a powerful tool to release antioxidants from olive mill wastewater. Food Chem. 131, 1430–1436.

[18] Khoufi, S., Hamza, M., Sayadi, S. (2011). Enzymatic hydrolysis of olive wastewater for hydroxytyrosol enrichment. Bioresource Technol. 102(19), 9050–9058.

[19] Romero, C., Brenes, M., Garcïa, P., Garrido, A. (2002). Hydroxytyrosol-4-β-d-glucoside, an important phenolic compound in olive fruits and derived products. J. Agric. Food Chem. 50, 3835–3839.

[20] Jemai, H., Bouaziz, M., Sayadi, S. (2009). Phenolic composition, sugar contents and antioxidant activity of Tunisian sweet olive cultivar with regard to fruit ripening. J. Agric. Food Chem. 57, 2961–2968.

[21] Ziogas, V., Tanou, G., Molassiotis, A., Diamantidis, G., Vasilakakis, M. (2010). Antioxidant and free radical-scavenging activities of phenolic extracts of olive fruits. Food Chem 120, 1097–1103.

[22] Visioli, F., Galli, C. (2003). Olives and their production waste products as sources of bioactive compounds. Curr. Top. Nutr. Res. 1, 85–88.

[23] Chimi, H., Sadik, A., Le Tutour, B., Rahmani, M. (1988). Contribution to the comparative study of antioxidant power in the olive oil tyrosol, hydroxytyrosol, caffeic acid, oleuropein and B.H.T. Rev. FR. Transfus Immu. 35, 339–344.

[24] Aeschbach, R., Loeliger, J., Scott, B., Murcia, A., Butler, J., Halliwell, B., Aruoma, O. L. (1994). Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem. Toxicol. 32, 31–36.

[25] Attya, M., Benabdelkamel, H., Perri, E., Russo, A., Sindona, G. (2010). Effects of conventional heating on the stability of major olive oil phenolic compounds by tandem mass spectrometry and isotope dilution assay. Molecules. 15, 8734–8746. doi:10.3390/molecules15128734

[26] Manna, C, Galletti, P, Cucciolla, V, Moltedo, O, Leone, A, Zappia, V. (1997). The protective effect of the olive oil polyphenols (3-4-dyhydroxyphenyl)-ethanol counteracts reactive oxygen metabolite induced cytotoxicity in caco-2-cells. J. Nutr. 12, 286–292.

[27] Petroni, A., Blasevich, M., Salami, M., Papini, N., Montedero, G. F., Galli, C. (1995). Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. Thromb. Res. 78, 151–160.

[28] Kohyama, N., Nagata, T., Fujimoto, S., Sekiya, K. (1997). Inhibition of arachidonate lipoxygenase activities by 2-(3,4-dihydroxyphenyl) ethanol, a phenolic compound from olives. Biosci. Biotechnol. Biochem. 61, 347–350.

[29] Capasso, R., Evidente, A., Avolio, S., Solla, F. (1999). A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. J. Agric. Food Chem. 47, 1745–1748.

[30] Bezançon, P., Debosque, S., Delpeuch, F., Descomps, B., Gerber, M., Léger, C. L., Padilla, M., Puygrenier, M. (2000). Alimentation Mediterranean diet and health: present and future. Edited by John Libbey Eurotext, Montrouge, France. Martine Padilla (Ciheam-IAM Montpellier) 170 pp.

[31] Labat, M., Augur, C., Rio, B., Perraud-Gainé, I., Sayadi, S. (2000). Biotechnological potentialities of polyphenolic compounds of coffee and comparison with olive. In Coffee Biotechnology and Quality. Sera T, Soccol CR, Pandey A and Roussos S (Eds), Kluwer Acad. Publ., Dordrecht, Chap. 46, 517–531.

[32] Tuck, K. L., Tan, H. W., Hayball, P. J., (2000). Synthesis of tritium-labeled hydroxytyrosol, a phenolic compound found in olive oil. J. Agric. Food Chem. 48(9), 4087–4090.

[33] Verhe, R., Papadopoulos, G., Boskou, D. (1992). Preparation of hydroxytyrosol. Bull. Liason Groupe Polyphenols. 15, 237–244.

[34] Briante, R., La Cara, F., Tonziello, M. P., Febbraio, F., Nucci, R. (2001). Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by hyperthermophilic beta-glycosidase. J. Agric. Food. Chem. 49, 3198–3203.

[35] Espin, J. C., Soler-Rivas, C., Cantos, E., Tomàs-Barberàn, F. A., Wichers, H. J. (2001). Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. J. Agric. Food Chem. 49, 1187–1193.

[36] Allouche, N., Sayadi, S. (2005). Synthesis of hydroxytyrosol, 2-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid by differential conversion of tyrosol isomers using Serratia marcescens Strain. J. Agric. Food Chem. 53, 625–6530.

[37] Bouallagui, Z., Sayadi, S. (2006). Production of high hydroxytyrosol yields via tyrosol conversion by Pseudomonas aeruginosa immobilized resting cells. J. Agric. Food Chem. 54, 9906–9911.

[38] Brooks, S. J., Doyle, E. M., O'Connor, K. E. (2006). Tyrosol to hydroxytyrosol biotransformation by immobilized cell extracts of Pseudomonas putida F6. Enzyme Microb. Techol. 39, 191–196.

[39] Selvakumar, P., Ashakumary, L., Pandey, A. (1998). Biosynthesis of glucoamylase from Aspergillus niger by solid-state fermentation using tea waste as the basis of a solid substrate. Bioresource Technol. 65, 83–85.

[40] Castilhoa, L. R., Medronhob, R. A., Alvesa, T. L. M. (2000). Production and extraction of pectinases obtained by solid-state fermentation of agro-industrial residues with Aspergillus niger. Bioresource Technol. 71, 45–50.

[41] Mahadik, N. D., Puntambekar, U. S., Bastawde, K. B., Khire, J. M., Gokhale, D. V. (2002). Production of acidic lipase by Aspergillus niger in solid-state fermentation. rocess Biochem. 38, 715–721.

[42] Asther, M., Haon, M., Roussos, S., Record, E., Michel Delattre, M., Lesage-Meessen, L., Labat, M., Asther, M. (2002). Feruloyl esterase from Aspergillus niger a comparison of the production in solid state and submerged fermentation. Process Biochem. 38, 685–691.

[43] Duarte, J. C., Costa-Ferreira, M. (1994). Aspergilli and lignocellulosics: Enzymology and biotechnological applications. FEMS Microbiol. Rev. 13, 377–386.

[44] Pandey, A., Soccol, C. R., Nigam, P., Soccol, V. T. (2000). Biotechnologica potential of agro-industrial residues. I: sugarcane bagasse. Bioresource Technol. 4, 69–80.

[45] Kumar, D., Jain, V. K., Shanker, G., Srivastava, A. (2003). Citric acid production by solid-state fermentation using sugarcane bagasse. Process Biochem. 38(12), 1731–1738.

[46] Schuster, E., Dunn-Coleman, N., Frisvad, J. C., Van Dijck, P. W. M. (2002). On the safety of spergillus niger. A review. Appl. Microbiol. Biotechnol. 59, 426–435.

[47] Bonnin, E., Brunel, M., Gouy, Y., Lesage-Meessen, L., Asther, M., Thibault, J.-F. (2001). Aspergillus niger I–1472 and Pycnoporus cinnabarinus MUCL39533, selected for the biotransformation of ferulic acid to vanillin, are also able to produce cell wall polysac-

charide-degrading enzymes and feruloyl esterases. Enzyme Microb. Technol. 28, 70–80.

[48] Kulkarni, A. G., Lele, S. S., Kulkarni, P. R. (1998). Improved adsorption of Aspergillus niger 589 spores on high-density polyethylene for progesterone biotransformation. J Ferment. Bioeng 86, 510–512.

[49] De Oliveira, B. H., Dos Santos, M. C., Leal, P. C. (1999). Biotransformation of the diperpenoid, isosteviol, by Aspergillus niger, Penicillium chrysogenum and Rhizopus arrhizus. Phytochemistry. 51, 737–741.

[50] Chen, A. R. M., Reese, P. B. (2002). Biotransformation of terpenes from Stemodia maritima by Aspergillus niger ATCC 9142. Phytochemistry. 59, 57–62.

[51] Demyttenaere, J. C. R., Willemen, H. M. (1998). Biotransformation of linalool to furanoid and pyranoid linalool oxides by Aspergillus niger. Phytochemistry. 47, 1029–1036.

[52] Volke-Sepulveda, T. L., Gutierrez-Rojas, M., Favela-Torres, E. (2003). Biodegradation of hexadecane in liquid and solid-state fermentations by Aspergillus niger. Bioresource Technol. 87, 81–86.

[53] Jiménez, A. M., Borja, R., Martin, A. (2003). Aerobic-anaerobic biodegradation of beet molasses alcoholic fermentation wastewater. Process Biochem 38, 1275–1284.

[54] Vassilev, N., Fenice, M., Federeci, F., Azcon, R. (1997). Olive mill wastewater treatment by immobilized cells of Aspergillus niger and its enrichment with soluble phosphate. Process Biochem. 32, 617–620.

[55] Molla, A. H., Fakhru'l-Razi, A., Abd-Aziz, S., Hanafi, M. M., Roychoudhury, P. K., Alam, M. Z. (2002). A potential resource for bioconversion of domestic wastewater sludge. Bioresource Technol. 85, 263–272.

[56] Dursun, A. Y., Uslu, G., Cuci, Y., Aksu, Z. (2003). Bioaccumulation of copper(II), lead(II) and chromium(VI) by growing Aspergillus niger. Process Biochem. 38, 1647–1651.

[57] Kapoor, A., Viraraghavan, T. (1997). Heavy metal biosorption sites in Aspergillus niger. Bioresource Technol. 61, 221–227.

[58] Cordova, J., Nemmaoui, M., Ismaïli-Alaoui, M., Morin, A., Roussos, S., Raimbault, M., Benjilali, B. (1998). Lipase production by solid state fermentation of olive cake and sugar cane bagasse. J. Mol. Catal. 5, 75–78.

[59] Mandviwala, T. N., Khire, J. M. (2000). Production of high activity thermostable phytase from thermotolerant Aspergillus niger in solid state fermentation. J Ind. Microbiol. Biotechnol. 24, 237–243.

[60] Brand, D., Pandey, A., Roussos, S., Soccol, C. R. (2000). Biological detoxification of coffee husk by filamentous fungi using a solid state fermentation system. Enzyme Microb. Technol. 27, 127–33.

[61] Roussos, S., Lonsane, B. K., Raimbault, M., Viniegra-Gonzalez, G. (1995). Advances in solid state fermentation. In Proceedings of the 2nd International Symposium on Solid State Fermentation, FMS-95, Kluwer Academic Publishers. Montpellier, France

[62] Barhini, P., Montebove, F., Ruzzi, M., Scheisser, A. (1998). Optimal conditions for bioconversion of ferulic acid into vanillic acid by Pseudomonas fluorescens BF13 cells. Appl. Microbiol. Biotechnol. 49, 309–314.

[63] Edlin, D. A. N., Narbad; A., Dickinson, J. R., Lloyds, D. (1995). The biotransformation of simple phenolic compounds by Brettanomyces anomalus. FEMS Microbiol. Lett. 125, 311–316.

[64] Muheim, A., Lerch, K. (1999). Towards a high yield bioconversion of ferulic acid to vanillin. Appl. Microbiol. Biotechnol. 51, 456–461.

[65] Eyal, S., Uzi, R., Yuval, S. (2000). Isolation of Bacillus sp. Capable of transforming isoeugenol to vanillin. J. Biotechnol. 78, 1–9.

[66] Limiroli, R., Consonni, R., Ottolina, G., Marsilio, V., Bianchi, G., Zetta, L. (1995). ^{1}H and ^{13}C NMR characterisation of new oleuropein aglycones. J. Chem. Soc. Perkin Trans. 1, 1519–1523.

[67] Briante, R., La Cara, F., Febbraio, F., Barone, R., Piccialli, G., Carolla, R., Mainolfi, P., De Napoli, L., Patumi, M., Fontanazza, G., Nucci, R. (2000). Hydrolysis of oleuropein by recombinant β-glycosidase from hyperthermophilic archaeon Sulfolobus solfataricus immobilised on chitosan matrix. J. Biotechnol. 77, 275–286.

[68] Brini, F., Saibi, W., Amara, I., Gargouri, A., Masmoudi, K., Hanin, M. (2010). The wheat dehydrin DHN-5 exerts a heat-protective effect on b-glucosidase and glucoseoxidase activities. Biosci. Biotechnol. Biochem. 74, 1050–1054.

[69] Ciafardini, G., Zullo, B. A. (2002). Microbiological activity in stored olive oil. Inter. . Food Microbiol. 75, 111–118.

[70] Walter, W. M. Jr., Fleming, H. P., Etchells, J. L. (1973). Preparation of Antimicrobial Compounds by Hydrolysis of Oleuropein from Green Olives. Appl. Microbiol. 26(5), 773–776.

[71] Briante, R., Patumi, M., Limongelli, S., Febbraio, F., Vaccaro, C., Di Salle, A., La Cara, F., Nucci, R. (2002). Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of Olea europaea L. Plant Sci. 162, 791–798.

[72] Bianco, A. D., Uccella, N. (2000). Biophenolic components of olives. Food Res. Int. 33, 475–485.

[73] Cheryan, M. (1986). Ultrafiltration Handbook. Technomic Publishing Co., Lancaster, PA. 369.

[74] Pizzichini, M., Russo, C. (2005). Process for recovering the components of olive mill wastewater with membrane technologies. International. Patent WO2005123603.

[75] Leung, S. W., Siddhanti, S., Williams, B., Chan, A. W. K., Minski, M. J., Daniels, C. K., Lai, J. C. K. (2010). Effects of diet intakes on metal and electrolyte distributions in vital organs. Procedia Environ. Sci. 2, 92–97.

[76] Anne, K. (2011). Magnesium and calcium in drinking water and heart diseases. In J. Nriagu (Ed.). Encyclopedia of Environmental Health . 20 Avenue Appia, 1211 Geneva 27, Switzerland, USA: Elsevier. 535–544.

[77] Galanakis, C. M., Tornberg, E., Gekas, V. (2010). Clarification of high-added value products from olive mill wastewater. J. Food Eng. 99, 190–197.

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