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Improvement of antioxidant activity of olive mill wastewater phenolic compounds by *Lactobacillus plantarum* fermentation

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ABSTRACT

Olive mill wastewater (OMW) contains a substantial amount of valuable antioxidant phenols that can be recovered for industrial application as food additives. In this study, we investigated the use of *Lactobacillus plantarum* strain to obtain high-added-value compounds, such as antioxidants, from the degradation of phenolic compounds present in OMW. *L. plantarum* growth on fresh OMW decreased pH and reduced phenols content and colour by 63 and 69%, respectively. Gel filtration chromatography showed an important reduction in molecular mass distribution of polyphenols. HPLC analysis showed a significant decrease in the concentration of total simple phenol, especially for tyrosol and hydroxytyrosol. The antioxidant and radical-scavenging activity of treated OMW increased by 50% after 5 d. The increase in antioxidant activity may be due to reductive conversion of phenolic compounds. Fourier transform infrared results showed that the biodegradation favours the production of new aromatic structures, such as vinyl hydrocarbon compounds. The production of lignin peroxidase (LiP) and tannase by *L. plantarum* were performed and results showed that a maximum activity of tannase (3.576 UI/mL) was obtained at beginning and LiP showed a highest activity (31 U/L) in 5 d.

Keywords: *Lactobacillus plantarum*; Olive mill wastewater; Polyphenols; Antioxidant activity; Lignin peroxidase; Tannase

1. Introduction

The extraction of the olive oil usually yields olive oil (20%), a semi-solid waste (30%) and an aqueous liquor (50%) called olive mill wastewater (OMW). The OMW comes from the vegetation water and the soft tissues of the olive fruits in addition to the water used in different stages of oil production. The olive fruit is very rich in phenolic compounds, but only 2% of the total phenolic content of the olive fruit pass into the oil phase, while the remaining amount is lost in the OMW (approximately 53%) and in the pomace (approximately 45%) [1]. The OMW waste is claimed to be one of the most polluting effluents produced by the agro-food industries because of its high polluting

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load. It also exhibits high toxicity to plants, bacteria and aquatic organisms, due to its composition of organic substances (14–15%) and phenols (up to 10 g/L) [2]. These latter compounds, characterized by high specific chemical oxygen demand (COD) and resistance to biodegradation, are responsible for its black colour, depending on their state of degradation and the olives they come from, and its phytotoxic and antibacterial properties [3]. Several methods have been proposed for OMW treatment and mainly include physicochemical processes such as decantation, chemical oxidation, concentration, ultrafiltration and reverse osmosis [4].

Biological methods used for OMW treatment are aerobic-activated sludge and anaerobic digestion. Several fungi such as *Aspergillus niger* [5,6,7], *Phanerochaete chrysosporium* [8], *Lentinusedodes*, *Pleorotusostreatus*, *Funaliatrogii*, *Geotrichum* spp. and *Geotrichum candidum* [9,10] or yeasts such as *Candida Oleophila* and *Candida tropicalis* [11,12] or lactic acid bacteria such as *Lactobacillus plantarum* [13] and *Pediococcus pentosaceus* [14] have been proposed to detoxify and/or decolourize OMW.

OMW represents a possible resource for simple and complex sugars that might be a basis for fermentation processes [15]. In addition, OMW contains an important amount of residual oil, depending on the extraction process efficiency. For these reasons, several authors used OMW as a growth substrate for suitable micro-organisms in order to reduce COD, BOD and dark colour as well as media to produce biomass and enzymes [16]. *G. candidum* and other which utilize a wide range of simple aromatic compounds have the ability to express peroxidase enzymes [9–17]. OMW remediation by means of ligninolytic fungi has been mostly addressed with an emphasis on the identification of ligninolytic enzymes responsible for polyphenols conversion and on the effects of operating conditions on enzyme secretion [18].

For a long time, OMW has been regarded as a hazardous waste with negative impacts on the environment and an economic burden on the olive oil industry. However, this view is changing. OMW has the potential to become a low-cost starting material rich in bioactive compounds, particularly phenolics, which can be extracted and applied as natural antioxidants for the food and pharmaceutical industries. The production of functional foods from OMW extracts constitutes a viable alternative for transforming this agro-industrial waste stream into a useful and relevant ingredient [19].

The aims of the study were to use the *L. plantarum* to stabilise OMW during storage and to produce functional foods from OMW with high-added-value compounds, such as antioxidants, from the degradation of phenolic compounds by reductive conversion enzymes such as Tannase and Lignin peroxidase (LiP).

2. Materials and methods

2.1. Olive mill wastewater (OMW)

OMW used in this study was obtained from local olive oil manufacturer (Tunisia). It was centrifuged at 4,000 g for 15 min to eliminate solids and materials and stored at 20˚C. Diluted fresh OMW was characterized by a pH of 5.16 ± 0.01, phenols concentration of 4.48 ± 0.05 g gallic acid/L, phenols at 280 nm of 211 ± 0.03, colour at 390 nm of 34.25 ± 0.02 and intensity colour (C^*) of 5.55 ± 0.01.

2.2. Micro-organism and culture procedures

L. plantarum was isolated from fermented olives [20,21] and maintained on MRS (Man Rogosa and Sharpman) agar at 4˚C. This strain was selected with an antioxidant activity (TAA = 43.47% ± 0.663, AAC = 172.65% ± 5.57 and DPPH PI = 57.07% ± 0.57 at a dose of 8.2×10^9 CFU/mL) [22] and was identified by API 50CHL kit (biomérieux Inc., Marcy l'Etoile, France) and 16s rDNA sequencing analysis.

Cultures of *L. plantarum* were conducted in 500-mL Erlenmeyer flasks containing 100 mL of diluted and sterilized (heat treated for 5 min at 100˚C) fresh OMW. The centrifuged and sterilized OMW was diluted with distilled water. The medium was supplemented with glucose (10 g/L), yeast extract (1 g/L) and ammonium sulphate (2 g/L) as nitrogen source (OMW sample at the beginning). After inoculation with *L. plantarum* cells at an initial concentration of 5.1×10^7 CFU/mL, culture was incubated at 37˚C for 5 d (treated OMW). The initial pH was adjusted to 6.0. Culture was grown in triplicate.

2.3. Analytical methods

Bacterial growth was monitored by measurement of final pH determination and by direct counting of colony forming units (CFU/mL) determined by plating 0.1 mL of serial dilutions on MRS agar, pH 6.0 and incubating at 37˚C for 24 h.

Decolourization and phenols were assayed by the measurement of absorbance, respectively, at 390 and 280 nm (Jenway UV–visible spectrophotometer).

The colour intensity was measured with a hand-held Tristimulus reflectance colorimeter (Spectrocolorimetre mobile color-test/ Erichsen SARL). Colour was recorded using the CIE- $L^*a^*b^*$ uniform colour space (CIE-Lab), where L^* indicates lightness, a^* indicates chromaticity on

a green to red axis, and b^* chromaticity on a blue to yellow axis. These recorded colour values (a^* and b^*) or some of their combinations should be considered as the physical parameters to describe the visual colour degradation. Ahmed et al. [23] founded out that a representation of visual quality in terms of total colour may be more relevant. This is why they founded that $L^*(a^*/b^*)$ or ($L^*a^*b^*$) are the best combination. The intensity or the saturation colour was expressed by Chroma (C^*):

$$C^* = [a^{*2} + b^{*2}]^{1/2} \quad (1)$$

2.4. Phenolic analysis

Phenol (with respect to gallic acid) concentrations were determined spectrophotometrically according to the Folin–Ciocalteu method [10] using a Jenway UV–visible spectrophotometer. Phenolic compounds were prepared as follows. Samples were acidified with HCl (1 N) to pH 2 and extracted with ethyl acetate (10/30) at ambient temperature. The organic layer was combined with anhydrous Na_2SO_4 for 30 min. The extract was concentrated to dryness in a rotary evaporator (at 45˚C) and dissolved with a mixture of methanol/water (60:40). Total phenol content was measured using the Folin–Ciocalteu's phenol reagent (Merck), involving the successive additions of 5 mL of sodium carbonate (200 g/L) and 2.5 mL of Folin–Ciocalteu's phenol reagent to 50 mL of properly diluted sample. After 60 min at 20˚C, the absorbance was measured at 725 nm against distilled water.

2.5. Molecular mass distribution of polyphenolics

Gel filtration on Sephadex G-50 was used to analyse the polymeric aromatic fraction present in different samples of OMW. Two millilitres of sample were filtered and placed on a Sephadex coarse G-50 column (2.5 × 60 cm) previously equilibrated with $NaNO_3$ 0.05 M containing 0.02% sodium azide at a flow rate of 0.6 mL/min. The effluent was collected on the basis of 3 mL per tube. The optical density of these fractions was measured spectrometrically at 280 nm. The column was calibrated with syringic acid (MM = 198 Da), lysozym (MM = 15 kDa) and blue dextran (MM = 200 kDa).

2.6. HPLC analysis

A reversed-phase high-performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds contained in the ethyl acetate extracts of fresh and fermented OMW. The HPLC chromatograph was performed on a Shimadzu apparatus composed of a LC-10 ATVP pump and a SPD-10 AVP detector. Elutes were detected at 280 nm. The column was (4.6 × 250 mm) model Shimpach VP-ODS and its temperature was maintained at 40˚C. The following rate was 0.5 mL/min. The mobile phase used was 0.1% phosphoric acid in water (A) vs. 70% acetonitrile in water (B) for a total running time for 40 min. The gradient was charged as follow: solvent B started at 20% and increased immediately to 50% in 30 min. After that, elution was conducted in the isocratic mode with 50% solvent B within 5 min. Finally, solvent B decreased to 20% until the end of running.

2.7. Fourier transform infrared (FTIR) spectroscopy

A quantity of 1.5 mg of sample was compressed under vacuum with 250 mg of KBr. The pellets obtained were analysed with a Perkin–Elmer series 783 Fourier transform infrared (FTIR) spectrophotometer (Nicolet Analytical Instruments, Madison, WI) covering a frequency range of 4,000–400 cm^{-1}.

2.8. Antioxidant activity of phenolic extracts

2.8.1. OMW extracts

The phenolic extract was obtained by a liquid–liquid extraction of the OMW. First, the pH of OMW samples (5 mL) was adjusted to pH 2 using HCl (2 M). After defatting with n-hexane, extractions with ethyl acetate were performed thrice, and the three extracts were brought to dryness by vacuum evaporation at 40˚C, and then recuperated in 5 mL of methanol. The resulting extract is called "phenolic extract".

2.8.2. β-carotene bleaching test

The antioxidant activity of phenolic extract was evaluated by β-carotene–linoleate model system [24]. β-carotene (0.2 mg), 20 mg of linoleic acid and 200 mg of Tween 40 (polyoxy-ethylene sorbitanmonopalmitate) were mixed with 0.5 mL of chloroform. Chloroform was removed at 45˚C, under vacuum, using a rotary evaporator. The resulting mixture was diluted with 50 mL of oxygenated distilled water. Aliquots (4 mL) of this emulsion were transferred into different test tubes containing 0.2 mL of test samples. A control containing 0.2 mL of corresponding solvent and 4 mL of the above emulsion was prepared. The tubes were

placed, at 50 °C, in a water bath. Absorbances of all the samples at 470 nm were taken at zero time ($t = 0$), measurement of absorbance was continued, until the colour of the β-carotene disappeared in the control reaction ($t = 120$ min), at 15 min intervals. A mixture prepared as above, without β-carotene, served as blank. All determinations were performed in triplicate. The antioxidant activity coefficient (AAC) was evaluated in terms of bleaching of the β-carotene using the following formula:

$$\mathrm{AAC} = [(A_{s(120)} - A_{c(120)})/(A_{c(0)} - A_{c(120)})] \times 1000 \quad (2)$$

where $A_{c(0)}$ is the absorbance value measured at zero time of the incubation for test control, $A_{s(120)}$ and $A_{c(120)}$ are the absorbances measured in the test sample and control, respectively, after incubation for 120 min.

2.8.3. DPPH· free radical-scavenging activity

Free radical-scavenging ability of different phenolic extracts was determined using a stable 2,2-diphenyl-2-picrylhydrazyl radical (DPPH·). The free radical working solution was prepared by dissolving 4 mg of DPPH in 100 mL of ethanol. A 100-μL aliquot of the sample (500–5,000 mg/L) adequately diluted with ethanol was placed in a cuvette and reacted with 3 mL of DPPH· working solution. The mixture was shaken vigorously and left to stand for 60 min at room temperature in the dark. The decrease in absorbance was measured at 517 nm after 60 min, against ethanol as a blank. Low absorbance of the reaction mixture indicates high free radical-scavenging activity. All determinations were performed in duplicate. The affinity of the test material to quench DPPH radicals (% inhibition of DPPH·) was calculated according to the following equation:

$$\%\ \mathrm{Inhibition} = [1 - (A_{\mathrm{sample}}/A_{\mathrm{control}})] \times 100 \quad (3)$$

where A_{control} was measured as the absorbance of DPPH· in ethanol (3 mL) plus ethanol (100 μL) instead of samples. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

2.8.4. ABTS radical cation decolourization assay

ABTS also forms a relatively stable free radical, which decolourizes in its non-radical form. The spectrophotometric analysis of $ABTS^{\cdot+}$-scavenging activity was determined according to the method of Re et al. [25]. In this method, an antioxidant is added to a preformed ABTS radical solution, and after a fixed time period the remaining $ABTS^{\cdot+}$ is quantified spectrophotometrically at 734 nm. $ABTS^{\cdot+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 4 h. The $ABTS^{\cdot+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). After the stock solutions were diluted, 10-μL aliquots of each sample (500–5,000 mg/L) were added to 1.0 mL of diluted $ABTS^{\cdot+}$ solution into the assay. The absorbance was recorded 30 min after mixing and the percentage of radical scavenging was calculated for each concentration relative to a blank containing no scavenger. The extent of decolourization is calculated as a percentage reduction of absorbance.

The scavenging capability of test compounds was calculated using the following equation:

$$ABTS^{\cdot+}\ \text{scavenging}\ (\%) = [1 - (A_{\mathrm{sample}}/A_{\mathrm{control}})] \times 100 \quad (4)$$

where A_{control} is absorbance of a control (blank) lacking any radical scavenger and A_{sample} is absorbance of the remaining $ABTS^{\cdot+}$ in the presence of scavenger. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

The Trolox Equivalent Antioxidant Capacity (TEAC assay) [25] represents the ratio between the slope of this linear plot for scavenging of $ABTS^{+}$ radical cation by the sample compared to the slope of this plot for $ABTS^{+}$ radical cation scavenging by Trolox (mM Trolox), used as an antioxidant standard.

2.9. Enzyme assays

2.9.1. Lignin peroxidase activity

LiP activity was determined using veratryl alcohol as substrate [26]. The assay mixture contained 2 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5. The method is based on determination at 310 nm of the initial rate of oxidation of veratryl alcohol to veratraldehyde. Enzyme activity was expressed in international units (IU).

2.9.2. Assay of tannase activity

Tannase activity was assayed by measuring the amount of hydrolysed substrate. The method reported

by Iibushi et al. [27] was based on estimation of decrease absorbancy at 310 nm. Four mL 0.35% (w/v) tannic acid in 0.05 M citrate buffer, pH 5.5, was preincubated at 37˚C, to which 1 mL of the supernatant of the culture was added. The reaction mixture was maintained at 37˚C. After t_1 and t_2 min, 40 µL was withdrawn and diluted 100 times with 90% (v/v) ethanol to stop the enzyme reaction. The absorbance at 310 nm was read and the difference between absorbencies of t_1 and t_2 was calculated. One IU of tannase activity is defined as the amount of enzyme which hydrolyses 1 µmol ester bond in 1 min.

2.10. Statistical analysis

Statistical analyses were performed using the ANOVA test: DATASET1.ISD by GraphPad in stats demo version 3.0 Software to determine differences between means. Statistic significance was determined at 5% probability level.

3. Results and discussion

3.1. Antioxidant activity of OMW sample

The antioxidant and radical-scavenging activity of OMW were evaluated using three tests, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH·) scavenging, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical-scavenging activity and β-carotene blanching test. According to the results presented in Fig. 1(a), we can note that DPPH free radical-scavenging activity of the phenolic extract of OMW sample was increased with increasing concentrations (R^2: 0.9545). IC_{50} for phenolic extract of OMW sample was 5.33 mg/mL. Moreover, the phenolic extract exhibited effective radical cation-scavenging activity. As seen in Fig. 1(b), phenolic extract of OMW sample is an effective $ABTS^{\cdot+}$ radical scavenger in a concentration-dependent manner (0.5–5 mg/mL, R^2: 0.9832). IC_{50} for phenolic extract in this assay was 4.89 mg/mL. The antioxidant activity coefficient assessed with β-carotene bleaching test was 420. The obtained results reveal the considerable antioxidant capacity of the OMW that can be considered as an inexpensive potential source of high added value with powerful natural antioxidants compared to some synthetic antioxidants commonly used in the food industry. The antioxidant capacity of the OMW may be due to the antioxidant activity of phenolic compounds. Olives and derivative products are recognized as a valuable source of so-called "functional food" because of their natural phenolic antioxidant content. The phenolic content of OMW is high up to 11.5 g/L with numerous

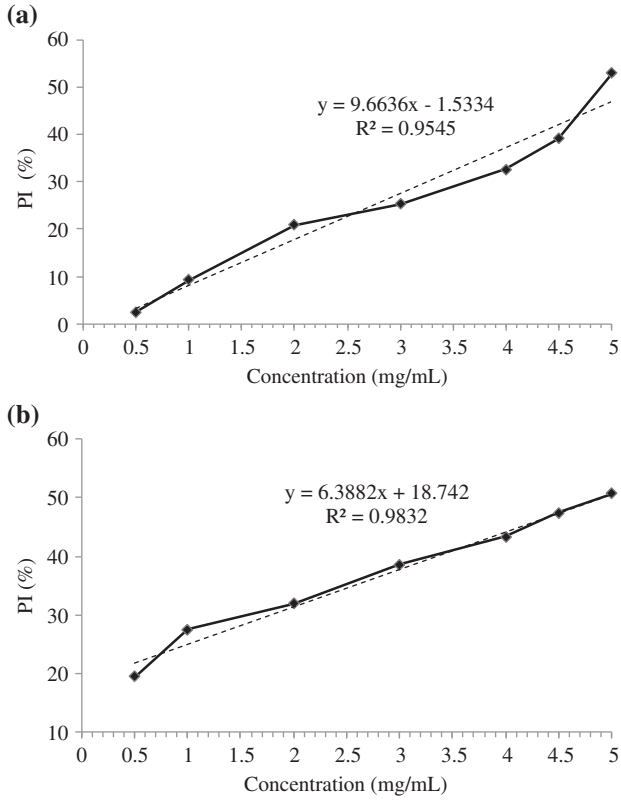

Fig. 1. Antioxidant activity using DPPH (a) and ABTS (b) tests at different concentrations of OMW sample.

interesting compounds. At present, more than 50 different phenolic compounds have been identified in OMW [28]. However, the major phenolic compounds in OMW are oleuropein, hydroxytyrosol and tyrosol. These phenolic compounds possess a high spectrum of biological activities, including antioxidant, anti-inflammatory, antibacterial and antiviral functions. El-Abbassiet al. [29] showed that all the antioxidant activity of the OMW cannot be ascribed exclusively to the phenolic content. Most likely, some non-phenolic compounds contribute to the overall antioxidant activity of the OMW or at least enhance the antioxidant activity of the phenolic compounds especially the total antioxidant capacity. Furthermore, the antioxidant activity of various OMW extracts was reported to be directly correlated with percentage of free hydroxytyrosol and their antioxidant properties was found to be the result of their phenol composition rather than their phenol content [30].

HPLC analysis obtained from the OMW sample at the beginning of storage revealed the presence of several phenolic compounds as shown in Table 1. Hydroxytyrosol was the major compounds detected. The concentration in OMW sample was 2,200 mg/L. Hydroxytyrosol has been an important focus of

Table 1
Main phenolic compounds present in OMW sample at beginning and treated OMW by *L. plantarum* after 5 d of storage (mg/L)

Compounds	OMW sample	Treated OMW
Gallic acid	150 ± 0.017	–
Hydroxytyrosol	2,200 ± 0.243	810 ± 0.041
Tyrosol	647 ± 0.04	328 ± 0.038
p-coumaric acid	2.6 ± 0.011	0.63 ± 0.007
Caffeic acid	0.8 ± 0.013	0.3 ± 0.007
Oleuropeinaglycone	2,180 ± 0.17	2,176 ± 0.14
Total	5,180.4	3,314.93

research since its discovery. Hydroxytyrosol-4-β-glucoside, hydroxytyrosol and caffeic acid are the hydrolysis products of verbascoside and the hydroxytyrosol-secoiridoid, and they show a powerful antioxidant activities [31]. The caffeic acid also was found in OMW sample, but at very low concentration (0.8 mg/L). Obied et al. [32] reported that caffeic acid shows a higher antioxidant activity (DPPH test) than hydroxytyrosol and oleuropein with an IC_{50} of 1.7, 2.3 and 7.6 μg/mL, respectively. Gallic acid which accounts in the OMW sample for 150 mg/L was also found to be a strong antioxidant in lipid systems and exhibits the antihyperglycaemic and antioxidant properties [33]. Gallic acid is used in processed food, cosmetics and food packing materials to prevent rancidity induced by lipid peroxidation and spoilage [34]. Tyrosol, which showed higher concentration (647 mg/L) in OMW sample, is reported to be effective in preserving cellular antioxidant defences [35]. p-coumaric acid, which is known to be a weak antioxidant compound [36], showed low concentration (2.6 mg/L). Finally, oleuropeinaglycone, which is a hydrolysis product of oleuropein, was found to be useful in the treatment of various inflammatory diseases [37]. This compound was identified at a high concentration (2,180 mg/L).

Nowadays, interest in novel sources of natural antioxidants is steadily growing. Since OMW is available in huge quantities and exhibits high concentrations of phenolic compounds, they may turn into a natural source of valuable and powerful antioxidants within the few coming years. Studies on the safety and efficacy of olive polyphenols [38] show that polyphenols from olive fruit and its by-product can be considered as safe and non-toxic for human consummation.

Moreover, the production of functional foods from OMW extracts constitutes a viable alternative for transforming this agro-industrial waste stream into a useful and relevant ingredient. But, the quality attributes of a functional food may change during storage. Poor storage conditions of functional foods and beverages may lead to loss of bioactive ingredients and undesirable colour and odour change [39]. Therefore, it is of great importance to measure the rate of change of a given quality parameter with storage.

Several studies have examined the stability of phytochemical constituents and colour in food products during storage. Alighourchi and Barzegar [40] reported that a storage temperature of 4˚C, maintained the anthocyanin content and chromatic parameters of pomegranate juice during long-term storage, while Gössinger et al. [41] found that a storage temperature of 4˚C was suitable to stabilize the colour of strawberry nectars for more than one year.

The acidic pH of OMW may play an important role in its stability during storage since it was found that mildly acidic infusions (pH 4.6) retain the colour and the total phenolic content (90%) for approximately 4 months. In OMW, after 5 months of storage, significant accumulation of hydroxytyrosol was observed [42]. The corresponding concentration increased from 0.77–0.98 to 3.1–3.5 g/L. However, the concentrations of the other phenolic compounds were markedly decreased. On the other hand, it was possible to maintain the concentration of hydroxytyrosol constant during storage by adding 10% ethanol [42]. Obied et al. [32] compared different conditions of OMW storage, in an attempt to identify optimal conditions which preserve the phenolic compounds and their antioxidant properties as it is just after olive oil extraction. The phenol content and antioxidant capacity of OMW stored at 4˚C and of OMW preserved by 40% (w/w) ethanol and 1% (w/w) acetic acid and stored at 4˚C were monitored for 30 d and compared with those of OMW stored at room temperature. None of these storage conditions could prevent the rapid decrease in phenolic concentrations and antioxidant capacity, which happened within the first 24 h [32].

Different reactions may take place during storage, some antioxidants may disappear and/or new molecules can be produced affecting the antioxidant activity. Moreover, the variation of the phenolic content and its antioxidant activity seems to be also affected by many factors such the initial physicochemical parameters of OMW samples, temperature of storage and, finally, the bacterial and fungal flora existing in OMW.

3.2. *Degradation of OMW during storage by* L. plantarum *fermentation*

The application of *L. plantarum* on fresh OMW during storage favours an important decrease in pH from 6 to 4 (Fig. 2), due to the consumption of sugar

content. Nevertheless, pH value remained favourable to enzymatic decolourization. In fact, the decolourization and the intensity of colour decreased after 5 d of storage, respectively, by 69 and 88% (Fig. 3(a) and (b)). In addition, the phenols content decreases by 88% (Fig. 3(d)), which may be attributed to the depolymerization of phenolic compounds with high molecular masses that are responsible for its black colour. In fact, Ayed and Hamdi [13], showed that *L. plantarum* have the capacity to reduce the redox potential and to realize the inverse reaction of auto-oxidation of phenolic compounds to tannins present in OMW by reductive depolymerization. Previous work carried out in our laboratory [20] studied the transformation of phenolic compounds contained in OMW into valuable products using *L. plantarum* in order to increase their transportation from OMW to olive oil. Incubation of olive oil samples with fermented OMW by *L. plantarum* caused polyphenols to decrease in OMW and increase in oil. Fermentation with *L. plantarum* induced reductive depolymerization of OMW which is more soluble in olive oil. The analysis of the phenolic compounds found in olive oil after storage showed that the application of *L. plantarum* favours the increase of all phenolic compounds in olive oil, especially by depolymerization and by reductive conversion of phenolic compounds of olive and oxygen fixation.

Gel-filtration chromatography was performed on the liquid phase sampled at the beginning and the end of incubation. The elution of phenolic compounds in the OMW sample on Sephadex G-50 (Fig. 4) shows that they are constituted of two fractions. The first includes compounds of high molecular weight that are responsible for the black colour (from the 42th to the 80th fractions). The second fraction, however, includes compounds of low molecular weight (from the 81th to the 120th fractions). The treated OMW with the *L. plantarum* shows a depolymerization of

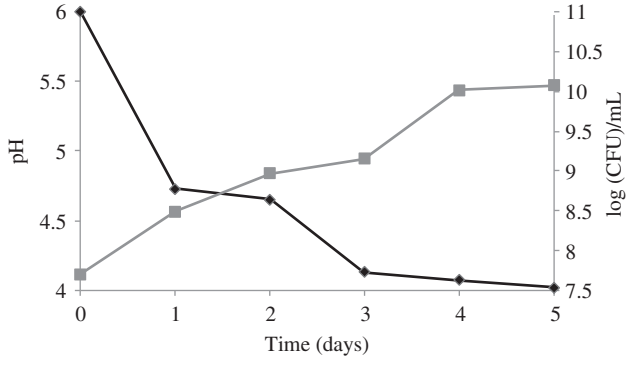

Fig. 2. pH (-♦-) and cell concentration of *L. plantarum* (-■-) of treated OMW during 5 d of storage.

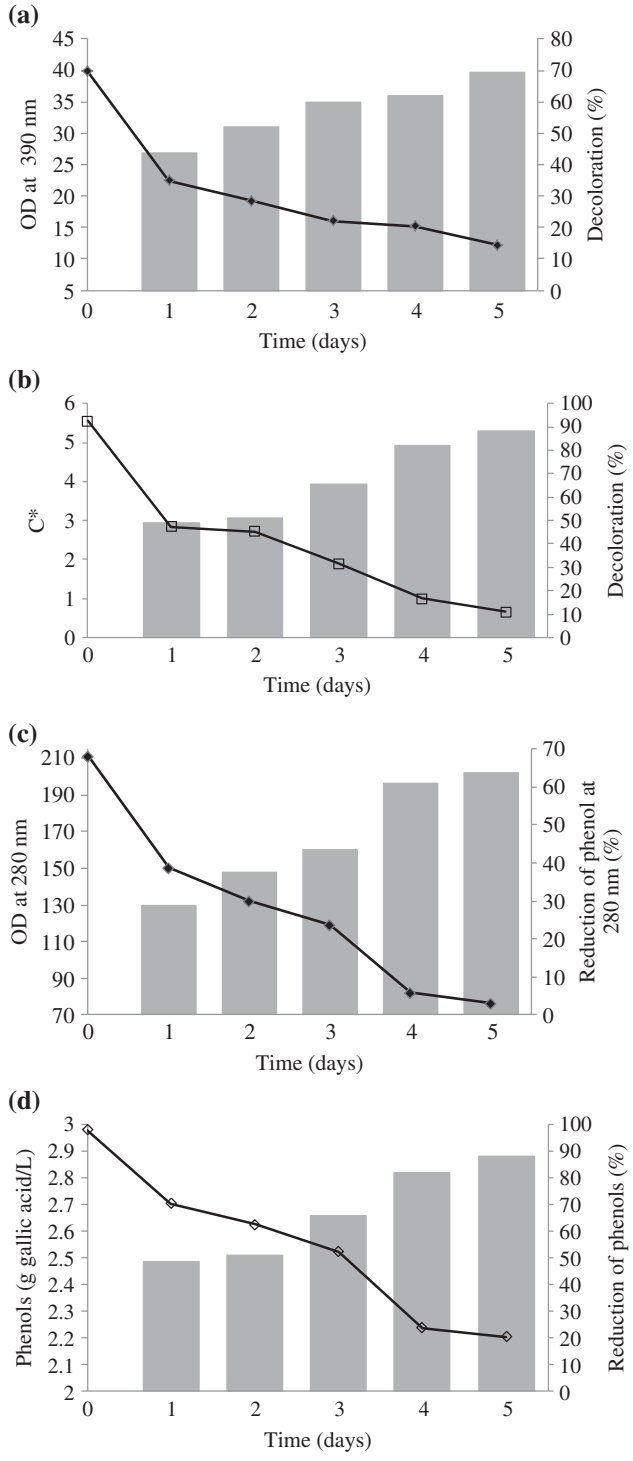

Fig. 3. Decolourization at 390 nm (-■-) and percentage of decolourization (-■-) (a), intensity colour (-□-) and percentage of decolourization (-■-) (b), phenols at 280 nm (-♦-) and percentage of reduction of phenols at 280 nm (-■-) (c) and phenols content (-◇-) and reduction of phenols content (d) of treated OMW by *L. plantarum* during 5 d of storage.

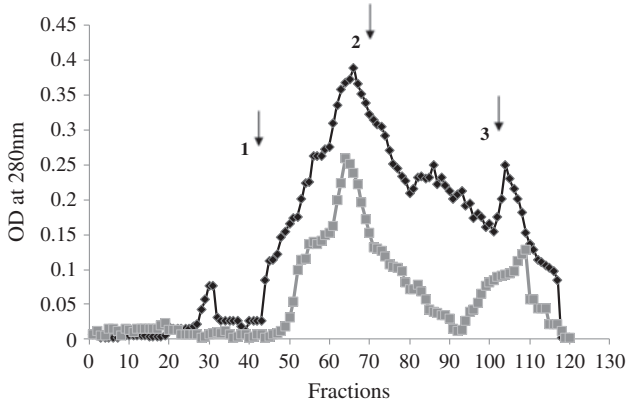

Fig. 4. Changes in molecular mass distribution of OMW sample at beginning (-♦-) and treated OMW by *L. plantarum* after 5 d (-■-). 1: Blue dextran (MM = 200 kDa), 2: Lysosym (MM = 15 kDa), 3: Syringic acid (MM = 198 Da).

high molecular weight of polymers and a reduction and/or a conversion of low molecular weight monomers.

HPLC analysis showed a significant decrease in the concentration of total monomers of phenols after 5 d of storage by 64% (Table 1). From this result, we suggest that the reduction and/or the conversion of phenolic compounds of OMW by *L. plantarum* fermentation could probably induce a positive effect in the increase in antioxidant activity. OMW shows a toxic action to some plants and micro-organisms since they exhibit a substantial concentration of phenolic compounds. These latter are the largest family of naturally occurring antioxidants in plants that include a wide variety of structures with a common motif, the phenol molecule. They expand from the simplest structures, such as phenolic acids and alcohols, to the most complex oligomeric ones such as proanthocyanidins.

3.3. *Improvement of antioxidant activity of OMW by* L. plantarum *fermentation*

Treated OMW by *L. plantarum* showed a significant increase in antioxidant and radical-scavenging activity during storage. According to the results presented in Fig. 5(a), we can note that DPPH free radical-scavenging activity of the phenolic extract of treated OMW was increased by 53% after 5 d of storage. IC_{50} was decreased from 5.33 mg/mL (8.56 Trolox Equivalent mM/mg) to 2.5 mg/mL (18.25 Trolox Equivalent mM/mg). The lower IC_{50} value indicates a higher DPPH free radical-scavenging activity. The antioxidant analysis by the $ABTS^{\cdot+}$-scavenging capacity confirmed our previous results. In fact, the effective radical

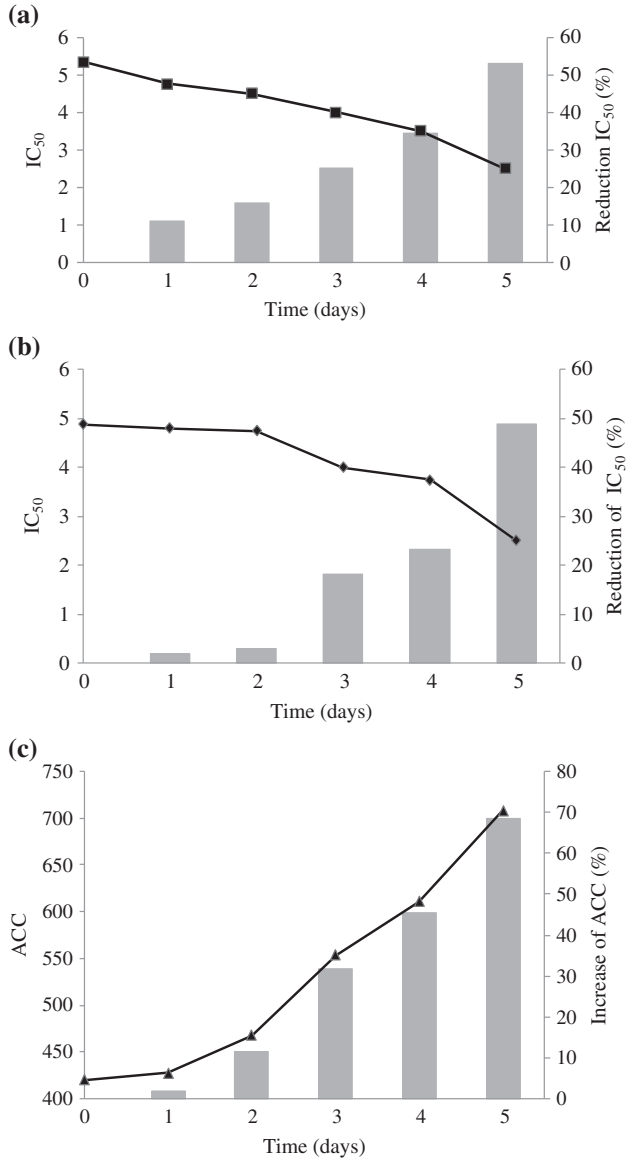

Fig. 5. Antioxidant activity of treated OMW by *L. plantarum* during 5 d of storage: DPPH IC_{50} (-■-) and percentage of reduction of IC_{50} (-■-) (a), ABTS IC_{50} (-♦-) and percentage of reduction of IC_{50} (-■-) (b) and β-carotene AAC (-▲-) and percentage of increase of AAC (-■-) (c).

cation-scavenging activity of phenolic extract of treated OMW increased by 48.8% after 5 d of storage (Fig. 5(b)). IC_{50} in this assay was decreased from 4.89 mg/mL (45.19 Trolox Equivalent mM/mg) to 2.5 mg/mL (88.40 Trolox Equivalent mM/mg). In addition, the antioxidant activity coefficient of phenolic extract of treated OMW increased by 68.5% after 5 d of storage (Fig. 5(c)). The enhancement of antioxidant and radical-scavenging activity may be due to the ability of *L. plantarum* to use the oxygen present in

the solution that was responsible for the auto-oxidation of phenolic compounds and/or to the capacity of the strain to convert the phenolic compounds, forming volatile phenols that participate positively in the increase of antioxidant activity. Previous work carried out in our laboratory showed that the application of *L. plantarum* during the olive oil process preserves the phenolic compounds, particularly ortho-diphenols [21]. On the other hand, Kachouri et al. [22] showed that the use of *L. plantarum* during the storage of olives favours the increase in the antioxidant activity correlated with an increase in ortho-diphenols.

Results obtained by Rodriguez et al. [43] showed that the *L. plantarum* was able to degrade some food phenolic compounds giving compounds influencing food aroma as well as compounds presenting increased antioxidant activity. The antioxidant activity of phenolic compounds depends on their chemical structures, number and arrangement of the hydroxyl groups [44]. Cavin et al. [45] showed that the phenolic acids and in particular the cinnamic acids (mainly p-coumaric and ferulic acids) are metabolized by various micro-organisms like *L. plantarum* into derived 4-vinyl, then reduced into derived 4-ethyl. The vinyl derivatives are required in the industry of fine chemistry and the industry of the biopolymers because of their faculty of regeneration of the free radicals. These derivatives are also required in cosmetic and in food industry. Indeed, the derivatives vinyls and ethyls are volatile phenols which contribute potentially to the flavours of the fermented food.

3.4. Identification of compounds responsible for the increase in antioxidant activity

The infrared spectra of OMW samples (Fig. 6) and the assignment of infrared absorption bands (Table 2) showed any significant differences between the two samples at the beginning and the end of storage.

The spectrum of the OMW sample at the beginning shows a high intensity for four bands: 3,251, 1,589, 1,399 and 1,072 cm^{-1}. These characteristic bands confirm the presence of high content of phenols, alcohol and organic acids. The absorption occurred at 3,420 cm^{-1} is due to C–H bonds and OH groups of the type alcohol, phenol or carboxyl OH and the hydrogen vibration of amide N–H functions, three bands in the region 2,946, 2,880 and 2,855 cm^{-1} due to aliphatic C–H stretching. The bands located at about 1,589 are attributed to aromatic C=C vibrations, in addition to quinines, conjugated carboxyls and ketones. Signals around 1,399 cm^{-1} are generated by –CH–, $–CH_2–$ and CH_3 radicals. The large peak around 1,025–1,072 cm^{-1} generally attributed to carbohydrates, aromatic ethers and polysaccharides. The principal absorption bands in the FTIR spectra and their corresponding assignments are based on the literature [46].

With treatment of OMW by *L. plantarum*, the first significant change observed was the increase in the intensity of peak 3,251 cm^{-1} due to –OH groups and another increase (1,589 and 1,072 cm^{-1}) due to C–C in aromatic groups, aromatic ethers and polysaccharides. Moreover, the peaks at 2,946, 2,880 and 2,855 cm^{-1} disappeared. It could be attributed to significant biodegradation of aliphatic structures.

The second change was an apparition of new peaks at 1,712, 1,411, 1,257 and 860 cm^{-1}. The absorption occurred at 1,712 cm^{-1} is due to COOH groups, the peak at 1,411 cm^{-1} due to vinyl hydrocarbon compounds C–H vibration, a band at 1,257 cm^{-1} generally linked to the absorbance of ethers and aromatic esters and to the N–H of amides. The peak at 860 cm^{-1} due to aromatic C–H out-of-plane bends 1,3-Di-substitution (meta). The infrared results showed that different reactions that may take place during storage, some antioxidants may disappear and/or new molecules can be produced affecting the antioxidant activity. It could be attributed to the ability of *L. plantarum* to degrade polymers and to convert some monomers of phenolic compounds present in OMW forming volatile phenols that participate positively in the increase in antioxidant activity. Kachouri et al. [22] showed that the application of *L. plantarum* on minimum media growth with 500 mg/L of olive phenolic compounds (p-coumaric acid and tyrosol) favours the production of high-added-value compounds, with higher antioxidant activity. Results showed that the p-coumaric acid has been metabolized by *L. plantarum* into derived 4-vinyl phenol. The tyrosol has been transformed into hydroxytyrosol and 3,4-dihydroxyphenylacetic acid in the presence of *L. plantarum*.

Up to now, metabolisms of phenolic compounds have been described on LAB. Therefore, there is a potential in further research in this field. The elucidation of these metabolic pathways will lead to obtaining biotechnologically useful strains and proteins. These strains or bacterial proteins will be adequate in the elaborate procedures to obtain food with improved sensorial or nutritional characteristics. In addition, it might be possible to use these strains or enzymes to obtain high-added-value compounds, such as antioxidants, from the degradation of phenolic compounds present in food wastes. The capacity of this strain or enzymes to produce high-added-value compounds may be due to the antioxidant defence capacity of this strain to oxygen radical formation by the involvement

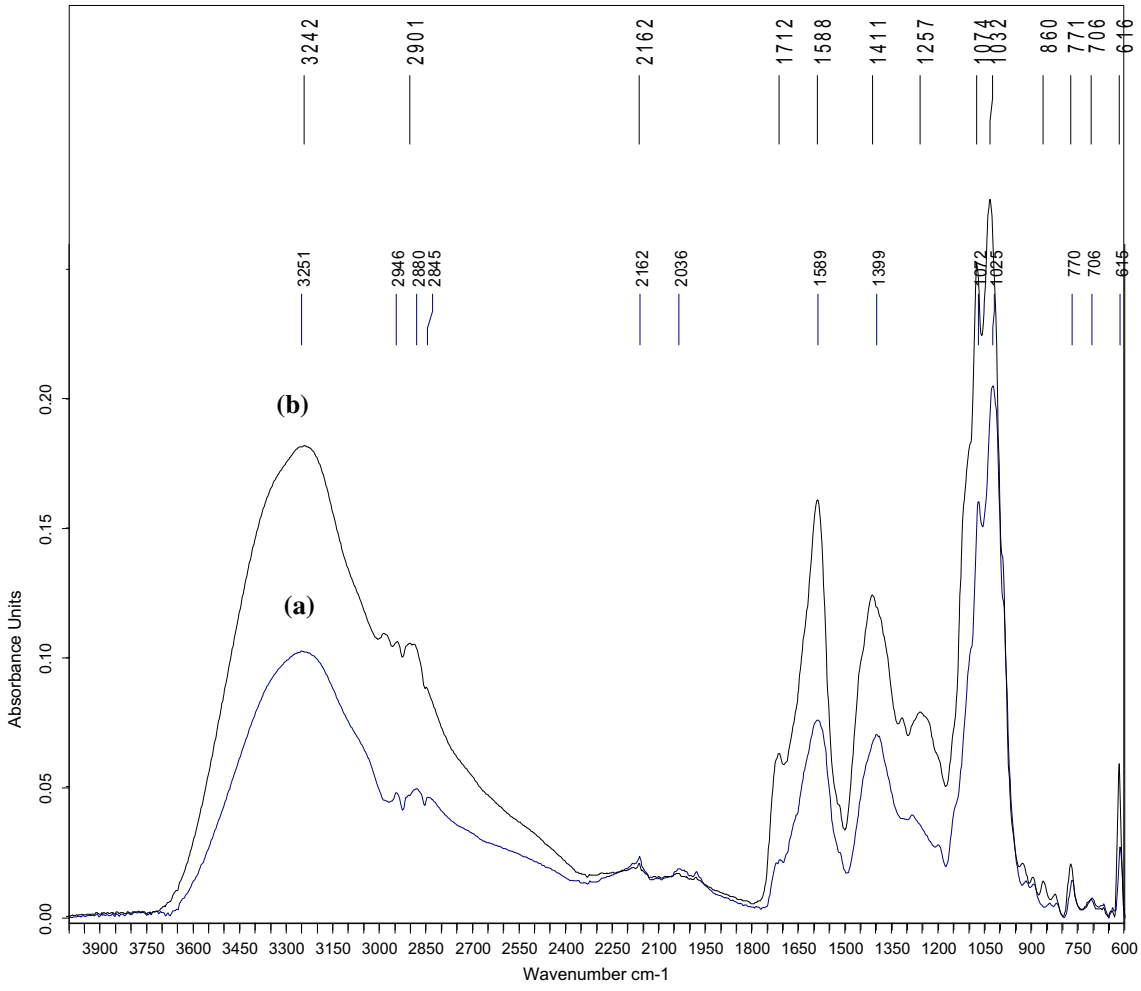

Fig. 6. FTIR spectra of OMW sample at beginning (a) and treated OMW by *L. plantarum* after 5 d of storage (b).

of oxygen in the enzymatic of reductive conversion of phenolic compounds.

In fact, many micro-organisms possess enzymatic and non-enzymatic antioxidative mechanisms and minimize the generation of reactive oxygen species (ROS) to levels that are not harmful to the cells. Lactic acid bacteria lack many of the components of the respiratory chain, which facilitate the utilization of O_2 as a terminal electron acceptor. However, many LAB synthesize the coupled NADH oxidase/NADH peroxidase system which balances the NAD^+/NADH ratio, catalyses the reduction of O_2 to H_2O_2 and decomposes H_2O_2 to H_2O for the purpose of protection. Jänsch et al. [47] showed that the defence of *L. sanfranciscensis* to oxygen toxicity is the involvement of oxygen in the metabolism by NADH-oxidase activity. The current research in antioxidant ability of LAB has shown that some LAB strains are not only able to decrease the risk of ROS accumulation through food ingestion but can also degrade the superoxide anion and hydrogen peroxide [48].

3.5. Study of enzymes responsible for OMW degradation

The enzymatic degradation was further explored in order to test the enzyme produced by *L. plantarum*. The production of LiP and tannase were performed (Fig. 7) and results showed that a maximum activity of tannase (3.576 UI/mL) was obtained at the beginning. A significant decrease in tannase activity has been shown after 5 d of storage. It could be due to the accumulation of the final product which hampers tannase production, or may be due to the accumulation of toxic metabolites secreted during fermentation. The LiP was also secreted. The time course of LiP production by *L. plantarum* growth on OMW showed a high-

Table 2
Interpretation of the main FTIR absorption bands of OMW sample at beginning and treated OMW by *L. plantarum* after 5 d of storage

Wave number (cm^{-1})		Assignment
OMW sample	Treated OMW	
3,251	3,242	O–H stretching, N–H stretching (minor), hydrogen-bonded OH
2,946, 2,880 and 2,845	2,901	Asymmetric and symmetric C–H stretching of CH_2 group
–	1,712	C=O stretching of COOH
1,589	1,588	Aromatic C=C skeletal vibrations, C=O stretching of amide groups (amide I band), C=O of quinone and/or H–bonded conjugated ketones
–	1,411	Vinyl hydrocarbon Compounds C–H vibration/CH_2 in plane deformation vibration scissoring vibration
1,399	–	COO–antisymmetric stretching, C–H bending of CH_2 and CH_3 groups
–	1,257	C–O stretching of aryl esters
1,072 and 1,025	1,074 and 1,032	C–O stretching of polysaccharides or polysaccharide-like substances
–	860	Aromatics C–H out-of-plane bend 1,3-Di-substitution (meta)
770 and 706	771 and 706	Aromatics C–H out-of-plane bend Mono-substitution (phenyl)
615	616	Alkynes C–H bend

est activity (31 U/L) after 5 d. From this result, we suggest that the degradation of phenolic compounds of OMW using *L. plantarum* was due to the possible action of various enzymes, including those of tannase and the ligninolytic oxidative enzymes. In addition, the production of high-added-value compounds, with antioxidant activity, from the degradation of phenolic compounds present in OMW, may be due to the antioxidant defence capacity of this strain to oxygen radical formation by the involvement of oxygen in the enzymatic of reductive conversion of phenolic compounds. Peroxidase catalyses the transfer of oxygen from the hydrogen peroxide to an appropriate substrate and consequently brings about oxidation of the substrate. Peroxidase uses H_2O_2 as electron acceptors for catalysing various oxidative reactions. Many reports have been shown the importance of microbial peroxidases in the degradation of various compounds including dyes, olive mill wastewaters, nitro aromatics, dioxins, chlorinated and many other compounds. Importantly, peroxidases are widely present in both Gram positive and Gram negative bacteria. In bacteria, peroxidases are found as intracellular enzymes and seemed to play important roles in protecting against H_2O_2 induced cell damage [49].

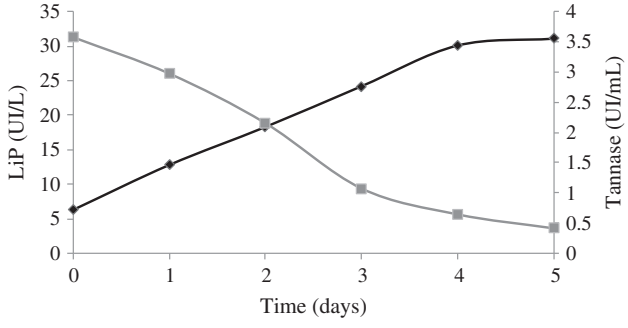

Fig. 7. Lignin peroxidase (-♦-) and tannase (-■-) of treated OMW by *L. plantarum* during 5 d of storage.

4. Conclusion

The application of *L. plantarum* during storage of OMW favours the increase in the antioxidant activity by 50%. This increase is being due to the antioxidant defence capacity of this strain to oxygen radical formation by the involvement of oxygen in the enzymatic of reductive conversion of phenolic compounds such as tannase and lignin peroxidase. This will give the capacity of this strain or enzymes to produce high-added-value compounds, such as vinyl compounds with higher antioxidant activity, from the conversion of phenolic compounds present in the OMW. The production of functional foods from OMW extracts constitutes a viable alternative for transforming this agro-industrial waste stream into a useful and relevant ingredient.

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