Ethanol Production from Olive Mill Wastewater (OMW) Pretreated with *Pleurotus sajor-caju*†

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The olive oil extraction process yields liquid waste byproduct known as olive mill wastewater (OMW), which presents a major environmental problem, if not treated before dissipation. The large amounts of OMW generated, combined with its high polluting power as well as its high chemical oxygen demand (COD) concentrations, represent the main difficulties in finding a solution for the management of this kind of wastewater. However, it could be upgraded by removing or reducing its phenolic compounds and using its carbohydrate fraction to produce biofuels. Phenolic compounds can be degraded by a few microorganizms, such as white-rot fungi, which produce a variety of enzymes that are capable of oxidizing phenols. The capability of *Pleurotus sajor-caju* to degrade phenols of OMW preconditioned by different treatments, namely, thermally processing (at 100 °C) diluted and undiluted OMW and thermally processing pretreated OMW with hydrogen peroxide, was investigated in this work. Results showed that *P. sajor-caju* removed phenolic compounds from OMW cultures, under all different conditions examined. The degradation of phenols reached up to 68% for the thermally processed OMW, 50% for the diluted OMW, 53% for the thermally processed OMW treated with hydrogen peroxide, and 58% for the thermally processed undiluted OMW. The impact of such biological conversion upon lowering the phenols content of OMW was tested by yeast fermentation of the product to produce ethanol because yeast cells are very sensitive to a high phenol concentration. Ethanol production was enhanced by the pretreatment of OMW with *P. sajor-caju*. The maximum ethanol production of 14.2 g/L was obtained after 48 h of yeast fermentation using 50% diluted OMW that was thermally processed and pretreated with *P. sajor-caju*. According to the results obtained, bioconverted OMW by *P. sajor-caju* is a promising substrate for the bioethanol production process, with additional benefits of its use with regard to environmental and economical aspects.

1. Introduction

Biofuels from plant biomass represent one of the most promising alternative sources of energy. These plant-derived fuels are replenishable via photosynthesis in green plants, unlike fossil fuels, such as petroleum, whose reserves are rapidly being exhausted. After the energy crisis in the 1970s, there were numerous research efforts directed toward the development of alternative energy sources, and ethanol production from agricultural products was a major constituent of these projects.[1]

The economic prospects for ethanol production from biomass is tied not only to petroleum prices but is also related to costs of fermentable sugars.[2,3] Plant residues are often considered as wastes and require net energy input for treatment without product gain, or they are decomposed in the environment by aerobic organisms without capturing biochemical products or biofuels. These residues though are much more economical than sugar crops (sugar cane, sugar beet, or sweet sorghum).

The olive oil industry represents one of the most important economic agro-food sectors in the Mediterranean countries. The extraction process of olive oil yields a highly contaminating byproduct, olive mill wastewater (OMW), that causes serious environmental concerns, still unsolved in the olive-oil-producing countries. The overall annual production of OMW in the Mediterranean region is estimated to be over 30×10^6 m^3.[4–7] The olive oil mill effluent consists of a mixture of soluble and insoluble carbohydrates and, therefore, can be used by a wide range of microorganizms for fuel production.[8] OMWs contain polyphenols, volatile acids, polyalcohols, and nitrogenous compounds, which contribute to a high toxicity and antimicrobial activity.[9]

Despite the existing laws, OMW is frequently dumped, untreated, either on the soil or into water sources.[5] This causes problems of phytotoxicity, destruction of cultures, bad odors, proliferation of insects, contamination of underground water, increasing salinity and reducing the permeability of the soil, and decreasing the degree of aeration.[10] However, Fiorelli et

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Table 1. Physical and Chemical Characteristics of OMW[a]

		results	
parameter	unit	minimum reading	maximum reading
TDS	mg/L	16 984	80 355
TSS	mg/L	14 207	46 188
COD	mg/L	78 536	160 096
BOD_5	mg/L	23 248	63 271
T-P	mg/L	158	403
NH_4-N	mg/L	22	68
TKj-N	mg/L	398	1036
Na	mg/L	130	384
Ca	mg/L	276	757
Mg	mg/L	38	63
K	mg/L	2053	5492
Cl	mg/L	486	1111
phenolics	mg/L	7739	10 432

[a] TDS, total dissolved solids; TSS, total suspended solids; COD, chemical oxygen demand; BOD_5, biological oxygen demand (after incubation for 5 days at 30 °C); T-P, total phosphorus; NH_4-N, ammoniacal nitrogen; TKj-N, total nitrogen.

al.[11] found that OMW led to enrichment of soils in nitrogen-fixing bacteria, an important factor for soil fertility. Hamdi[12] describes the application of OMW as a fertilizer, but this was hampered by phytotoxicity (because of polyphenols) and transport costs. Biological methods, such as bioremediation, using lignin-degrading fungi might be an efficient alternative.[13,14] The lignin-degrading ability of white-rot fungi seems to be associated with the release of extracellular enzymes, capable of destroying the benzene rings that are common in both phenolics and lignin compounds present.[15,16]

The purpose of the present work was to investigate the ability of a "white rot" basidiomycete fungus, *Pleurotus sajor-caju*, to use OMW as a substrate, reducing its phenolic compound content, and to examine the effect of this pretreatment on its subsequent anaerobic fermentation of OMW by *Saccharomyces cerevisiae* L-6 to produce ethanol as a fuel.

2. Experimental Section

2.1. OMWs. OMW samples were obtained from three different olive oil mills (three-phase centrifugal olive mills) located at Irbid and Mafraq cities of Jordan. After OMW was collected, it was maintained at 4 °C to prevent it from undergoing biodegradation by its naturally occurring microbial action. The physical and chemical properties of OMW were determined as proposed by standard methods,[17] and the data are shown in Table 1.

2.2. Maintenance of the *P. sajor-caju* Culture. A subculture of *P. sajor-caju* was obtained from the Plant Pathology and Mycology Research Laboratory at the Faculty of Agriculture/Jordan University of Science and Technology, Jordan. The fungus was maintained on potato dextrose agar (PDA) Petri plates (9 cm in diameter) and stored at 4 °C.

2.3. Growing *P. sajor-caju* on Agar Culture Media of OMW. Solid culture experiments on OMW were conducted using Petri dishes to examine the capability of *P. sajor-caju* to grow on a substrate, which consisted only of thermally processed OMW (at 100 °C for 1 h) and agar (15 g/L). The pH of the OMW was set to pH 6.00 with the addition of NaOH (1 N). OMW was used at dilutions of 25, 50, 75, and 100% OMW. Initially, all solid cultures were inoculated with mycelia (radius of 10 mm and 2 mm in thickness) previously grown on PDA. After inoculation of the solid cultures, the Petri dishes were kept in an incubator at 27 °C. The mycelium of *P. sajor-caju* exhibited a radial growth; therefore, its growth rate could be estimated by measuring the radius (mean colony diameter) of the mycelium throughout the growth period.

2.4. OMW Liquid Cultures Media of *P. sajor-caju*. Liquid cultures were conducted to examine more parameters, such as the phenol concentration, color, and biological oxygen demand (BOD), during the growth of *P. sajor-caju*. A total of 100 mL of OMW was placed in 250 mL Erlenmeyer flasks and then inoculated by a mycelium plug (radius of 10 mm and 2 mm in thickness) cut at the advancing edge of *P. sajor-caju* grown on the solid cultures media. Each flask was then plugged with hydrophobic cotton plugs and placed in an incubator at 27 °C. Three kinds of pretreated OMW were used, namely, OMW treated with 10% H_2O_2 (overnight and then sterilized at 121 °C for 15 min), thermally processed 50% diluted OMW, and thermally processed (100 °C) undiluted OMW. All cultures were set at pH 6.00, as described before. Every experiment lasted about 20 days, and a measurement was taken every 2 days. Two flasks without and with *P. sajor-caju* were used for each measurement. After analysis, these flasks were waste.

2.5. Yeast Fermentation of *P. sajor-caju*-Treated OMW. Three batch reactors (bottles with a round nick, 500 mL working volume) were placed in a shaking water bath at 35 °C for 48 h. Three different types of OMW were fed to these reactors at 50% of the working volume, and the rest was filled with yeast biomass obtained from a log phase of yeast inoculum (*S. cerevisiae* L-6). The first type was biotreated OMW with *P. sajor-caju* that was treated with H_2O_2. The second was thermally processed OMW biotreated with *P. sajor-caju*, and the third was thermally processed 50% diluted OMW and biotreated with *P. sajor-caju*. Anaerobic conditions were maintained during the filling phase of the reactors, by flushing with nitrogen gas. The nick of each flask was closed properly with a rubber stopper that was connected to a Z-shaped glass tube filled with 4 mL of glycerol to ensure anaerobic conditions and prevent evaporation of the bottle contents. The pH value in all three reactors was initially set to pH 5 using 1 N NaOH/1 N HCl.

2.6. Analytical Procedures. Total phenolic compounds were determined spectrophotometrically according to the Folin–Ciocalteu method.[18] BOD_5 was measured according to the 5210D respirometric method at the Ecological Studies Center (The Hashemite University), where 5 mL of treated OMW were added to 155 mL of distilled water in a brown bottle and placed in a BOD apparatus (respirometer system).[19] The apparatus was set to run at 350 mg/L for 5 days, and the BOD_5 was recorded. For the measurement of the mycelium dry weight in the liquid cultures, the mycelium was collected from each flask by filtration using filter paper Whatman number 1. After filtration, the mycelium was placed in a preweighted glass plate and then dried at 80 °C for 24 h. The difference between the two weight measurements of the glass plate, before and after the sample, was taken as the "dry weight" of the sample. Laccase activity was determined at pH 5 by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm for 2 min.[20] A 100 μL sample was added to 890 μL of a solution containing 50 mM sodium malonate and 1 mM 2,6-DMP (pH 4.5) in a 1 mL cuvette. The enzyme activity was expressed as units/mL, where 1 unit was defined as 1 mmol of substrate oxidized per minute. The ethanol concentration was measured on the basis of stoichemistry and by a gas chromatograph (Shimadzu GC-2010) equipped with a thermal conductivity detector (TCD) and a Carbosieve column.

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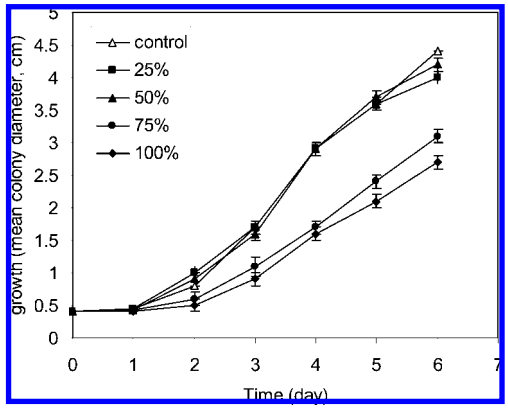

Figure 1. Growth of *P. sajor-caju* on solid cultures of different concentrations of OMW. Error bars indicate standard deviations from mean values.

Helium was used as a carrier gas, at a flow rate of 30 mL/min. The column temperature program was 150 °C for 8 min with a 20 °C/min ramp to 200 °C.

3. Results

3.1. Growth of *P. sajor-caju*. The growth of *P. sajor-caju* mycelium on solid media of OMW is shown in Figure 1. The growth of the fungus on PDA cultures was used as a control for comparison purposes. The starting radius of the mycelium on PDA was 0.33 cm. At the sixth day of growth, the mycelium radius was 4.2 cm. The growth of the fungus on solid cultures with thermally processed OMW was conducted using four different dilutions containing 25, 50, 75, and 100% OMW (Figure 1). During incubation, *P. sajor-caju* grew at all dilutions with a short lag period. The grown mycelium had high-density hyphae, with minor differences for the various dilutions. The fungal growth exhibited a faster growth rate, employing 25 and 50% OMW than other dilutions. The radius of the mycelium at the 50% OMW was slightly larger on the sixth day of incubation, having a value of 4.2 cm, compared to all other dilutions, which were 3.97, 3.53, and 3.2 cm, for the 25, 75, and 100% OMW, respectively.

Liquid cultures were conducted using OMW pretreated with hydrogen peroxide, thermally processed (100 °C) and diluted 50% with water, and thermally processed (100 °C) undiluted OMW. It was observed that, although the pH of the control cultures (with no mycelium) containing thermally processed OMW did not change during the 20 days of growth, the fungi flasks caused a reduction in the pH from 6 to 5.3, after 20 days. The growth of the fungus as presented by dry weight is shown in Figure 2. For the H_2O_2-pretreated OMW, the initial weight of biomass was 0.008 g and, after 20 days of growth, reached the value of 0.2 g. Although the initial weight of the thermally processed OMW (diluted 50%) was the same, it reached 0.5 g after only 18 days of incubation. Finally, the thermally processed undiluted OMW had an initial weight of 0.009 g and reached up to 0.42 g after 21 days. It is clear that the thermally processed 50% diluted OMW is most suitable for fungus growth and metabolism.

3.2. Phenolic Compound Removal and Laccase Enzyme Production from OMW. The fungus cultivated in thermally processed diluted OMW (50%) showed a remarkable capability to excrete laccase into the growth medium and eliminate phenolic compounds. In representative examples, given in Figure 3, it is shown that laccase activity was rapidly increased at the 6–10th days after inoculation, in parallel to the phenolic compound removal. The maximum laccase activity was measured at the 10–14th days after inoculation, and at this time, the maximum phenolic removal was often achieved. However, laccase activity slightly decreased after the 12–14th days.

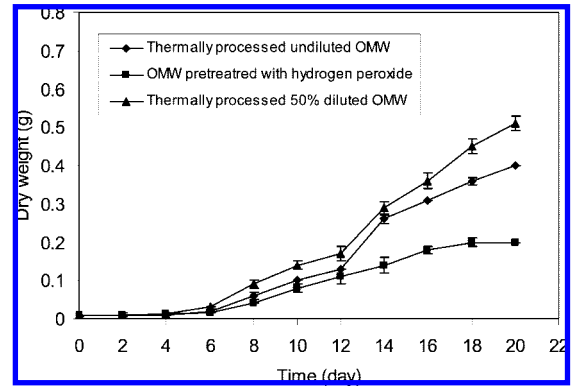

Figure 2. Growth of *P. sajor-caju* in liquid cultures of OMW expressed as dry weight. Error bars indicate standard deviations from mean values.

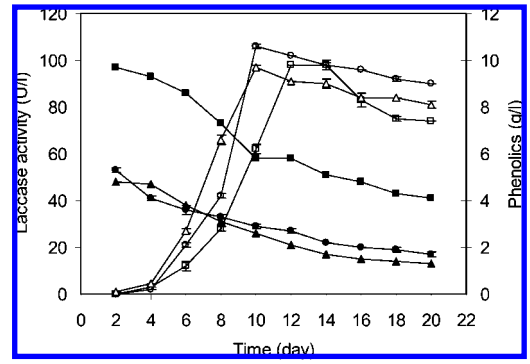

Figure 3. Phenolic compound removal and laccase enzyme production by *P. sajor-caju* in liquid cultures of thermally processed 50% diluted OMW (●) phenols and (○) laccase, thermally processed undiluted OMW (■) phenols and (□) laccase, and OMW pretreated with H_2O_2 (▲) phenols and (△) laccase. Error bars indicate standard deviations from mean values.

Maximum phenolic removal ranged between 60 and 68% of the initial phenolic content (Figure 3). However, the phenolic removal capability of the fungus was not closely associated to the laccase activity in the growth medium. For example, when the fungus was grown on thermally processed undiluted OMW, it was capable of removing large amounts of phenolic compounds (58%), although lower laccase activities were measured in their growth media. In all cultures, decolorization of the OMW was observed but was highly observed in cultures using 50% diluted and thermally processed OMW. The absorbance of those cultures was reduced significantly at 600 nm after the 6th day of the experiment (Figure 4). There was a notable correlation between the removal of phenolic compounds and the decolorization of the culture (i.e., as the concentration of phenolics in the culture decreases, the decolorization of the culture increases).

3.3. Ethanol Production. The results for ethanol production by yeast fermentation using the three types of *P. sajor-caju* cultures are shown in Table 2. The concentration of ethanol in the reactor that contained pretreated (thermally processed 50% diluted OMW and biotreated for 20 days with *P. sajor-caju*) OMW was much higher than in the other two reactors. The amount of ethanol detected in those cultures reached up to 14.2 g/L after 2 days of yeast fermentation. The reactor containing the pretreated OMW (thermally processed undiluted OMW and

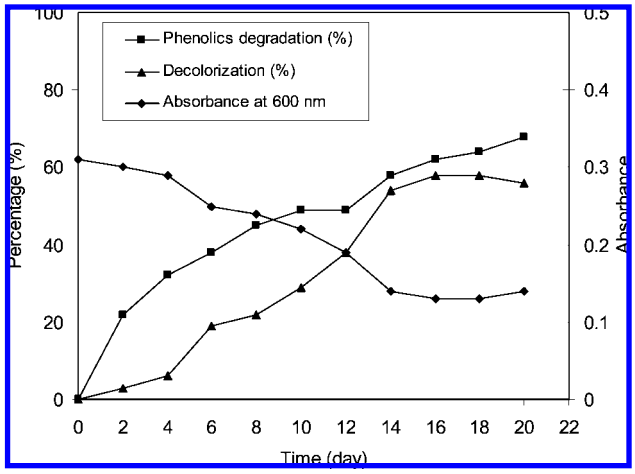

Figure 4. Effect of *P. sajor-caju* growth on the decolorization of OMW (thermally processed 50% diluted OMW) as represented by absorbance (at 600 nm) in relation to phenolic compound degradation and decolorization for the whole period of cultivation.

Table 2. Ethanol Production by Yeast Fermentation of Three Types of OMW Treated with *P. sajor-caju*

day[a]	ethanol concentration (g/L)	initial BOD_5[b]	final BOD_5[b]
	Thermally Processed 50% Diluted OMW		
10	10.3	47 500	32 500
20	14.2		23 250
	Thermally Processed Undiluted OMW		
10	7.6	51 000	38 230
20	8.1		33 340
	OMW Pretreated with H_2O_2		
10	6.5	44 100	36 420
20	7.3		31 260

[a] The culture sample was taken after 10 and 20 days of treatment with the fungus for yeast fermentation. [b] BOD values for the three types of OMW before and after treatment with *P. sajor-caju*.

pretreated for 20 days with *P. sajor-caju*) had the lowest production of ethanol (6.8 g/L), among the three reactors.

4. Discussion

The results obtained in this study proved that OMW is a suitable media for cultivating white-rot fungi, which makes this type of wastewater a good candidate for further investigations for growing other types of fungi that are able to biodegrade or bioconvert this type of wastewater and, in the same time, produce fungal metabolites. The growth of *P. sajor-caju* on OMW caused drastic changes in the physical and chemical properties of this wastewater, which may have improved its chemical value. At the beginning of our experimentation, solid cultures were conducted to investigate the capability of *P. sajor-caju* to grow on OMW without any addition of nutrients or other substances. The fungus not only grew using OMW as the sole substrate, but its growth was satisfactory even on 100% OMW. For the liquid cultures, the dilution of OMW with 50% water was used to assist fungus growth in OMW. The idea of raising the OMW temperature from 40 °C to a higher (nonsterilizing) temperature was to prevent bacterial contamination and improve nutrient uptake by the fungus. As can be easily seen from the time course of the fungal growth in liquid cultures as represented by dry weights, all three types of cultures exhibited a lag phase of about 6 days (Figure 2). In 50% diluted and thermally processed OMW, the lag phase period was followed by a faster growth rate because the log phase appeared different from cultures employing OMW pretreated with hydrogen peroxide. It is remarkable to note that, when thermally processed OMW was used, the mycelium yield was significantly higher than in the case of the OMW pretreated with hydrogen peroxide. Hydrogen peroxide probably causes a serious change in the chemical composition of the liquid medium, compared to thermal processing, reducing its nutrient value for fungal growth. The phenol concentration was the most important parameter affected by fungal growth. As shown in Figure 3, a substantial reduction of phenols was observed along with laccase enzyme production, especially in the case of thermally processed 50% diluted OMW cultures. Laccase synthesis was induced in *P. sajor-caju* by OMW. In fact, as reported by Piperidou et al.,[21] this effluent contains some phenolic compounds, which are able to induce laccase synthesis.[22,23] Laccases are remarkably nonspecific, extracellular multicopper enzymes that use molecular oxygen as an electron acceptor, and they are able to oxidize polyphenols, diamines, and a considerable range of other compounds.[24–26] In fungi, laccases have been implicated in sporulation, the formation of rhizomorphs and fruiting bodies, pathogenesis, and the lignin degradation process.[27] In the present work, an early increase of laccase activity was detected in the growth environment, suggesting that this enzyme was produced during primary metabolic growth. These results are in agreement with Tomati et al.,[22] suggesting that white-rot fungi benefit from the laccase reaction, which is involved in a detoxification procedure, preparing the environment for microbial growth.

OMW decolorization was noticed during mycelium growth in the three types of cultures. In Figure 4, it can be observed that during the first 10 days there was no significant decolorization, although most of the phenolics were degraded at that time. Color reduction started on the 10th day. It is possible that the phenolic compounds that were degraded in that time were responsible for the dark color of the waste. In the case of the thermally processed undiluted OMW, no decolorization was observed. This is further supported by the BOD values for the three types of OMW employed (Table 2). There was a reduction by 51% in the BOD values for the culture of *P. sajor-caju* growing on thermally processed 50% diluted OMW compared to 34% reduction in cultures of *P. sajor-caju* growing on thermally processed undiluted OMW and 29% reduction in cultures of *P. sajor-caju* growing on OMW pretreated with hydrogen peroxide. The anaerobic fermentation of pretreated OMW generated very interesting results. The ethanol produced by the thermally processed 50% diluted OMW that was previously treated with *P. sajor-caju* was much higher than that produced by the other two anaerobic reactors.

In conclusion, *P. sajor-caju* appears to be able to grow in OMW without any addition of nutrients and to remove a

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significant part of phenolic compounds. The experimental results showed that *P. sajor-caju* removed phenols from the culture medium, under all of the different conditions examined. The anaerobic fermentation of OMW was enhanced by the pretreatment with *P. sajor-caju*. Such a pretreatment could secure a satisfactory performance of this important treatment approach.

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