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Use of *Pleurotus sajor-caju* for the Biotreatment of Olive Mill Wastewater

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Abstract

The use of olive mill wastewaters (OMW) and their possible treatment by Pleurotus sajor caju were investigated. From the physical and chemical properties of OMW, the fungus was able to utilize OMW without any addition of nutrients. OMW was a suitable substrate for the induction of fungal ligninolytic enzymes that are involved in the treatment of OMW and reduction of its toxic compounds. The phenolic compounds content was reduced significantly (from 5.7 to 2.1 g/l) along with enzymes secretion and action. By the end of the treatment process, some phenolic and non-phenolic compounds were completely degraded and the color of OMW was reduced by 60-70%. This treatement process contributed to a notable reduction in COD and BOD contents of OMW indicating the possible application of such treatment on a larger scale.

Keywords: *Olive mill wastewater, P. sajor-caju, Enzymes, Phenolic compounds, Treatment*

Introduction

The olive oil industry represents one of the most important economic agro-food sectors in the Mediterranean countries especially in Jordan. The extraction process of olive oil yields several huge amounts of colored often toxic and harmful wastewater. The olive oil wastewaters (OMW) consist of a mixture of soluble and insoluble carbohydrates and, therefore, can be used by a wide range of microorganisms in several applications [1]. OMW contain polyphenols, volatile acids, polyalcohols, and nitrogenous compounds, which contribute to a high toxicity and antimicrobial activity [2]. In Jordan, OMW is frequently dumped, untreated, into wastewater ponds at specific locations around cities which may contribute to many environmental pollution problems such as phytotoxicity, bad odors, proliferation of insects, contamination of underground water, and increasing salinity.

A possible solution would be the treatment of OMW prior to its utilization in any process. In many biotreatment experiments, some fungal species have proved their capability of utilizing complex substrates such as lignocellulosics and a variety of wastewaters as a carbon source [3, 4]. White rot fungi produce various isoforms of extracellular oxidases and peroxidases, which are involved in the degradation of complex substrates in their natural environment such as highly toxic phenolic compounds and azo dyes [5, 6]. These enzymes are synthesized during secondary metabolism in response to nitrogen, carbon or sulphur limitation [7, 8, 9].

In a previous study, *Pleurotus sajor-caju* was employed to treat OMW for the reduction of its toxicity and reuse as a fermentation medium by yeast to produce ethanol [10]. A follow up study was performed to evaluate the effects of simple and complex carbon sources such as OMW on *P. sajor-caju* growth and enzymes production [4]. The results revealed that OMW was a suitable medium for fungal growth and metabolism without the need for any nutrient addition. Nevertheless, based on these previous findings OMW was used as a medium in an anaerobic fermentation process using microflora collected from OMW collecting sites for the production of Acetone- butanol- ethanol (ABE) [11]. The results revealed that OMW is a potent substrate for the production of ABE without any pretreatment. Therefore, this information was important for designing a fungi-based technique for treatment of OMW and thus, the aim of this work was to investigate in laboratory cultures the ability of *P. sajor-caju* to treat OMW with respect to its enzymatic system.

Materials and methods

2.1. Olive mill wastewaters (OMW) OMW were obtained from 3 different olive oil mills (three phase centrifugal) located at Irbid and Mafraq cities of Jordan. After collecting, OMW were maintained at 4°C in order to prevent the undergoing biodegradation of theses wastes due to microbial action. The chemical and physical properties of OMW were studied before the treatment process was started and the data are shown in Table 1.

2.2. Maintainance of cultures Mycelia of *P. sajor-caju* were obtained from the Agriculture Department laboratories (Jordan University of Science and Technology, Jordan). The fungus was maintained in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) and stored at 4 ºC till use.

2.3. Inoculum preparation cultures of *P. sajor-caju* were performed using 250mL Erlenmeyer flasks incubated in an incubator shaker (Human Lab., Korea). Each flask contained 50 mL of Sternberg medium of (g L^{-1}): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4.7H_2O$, 0.0016; $ZnSO_4.7H_2O$, 0.0014; $CoCl_2.7H_2O$, 0.002; protease peptone, 0.75; urea, 0.3; and Tween 80, 1; with a final pH of 5.5 [12]. OMW was used as a carbon source at a final concentration of 5% v/v. The flasks were sterilized by autoclaving and after cooling they were inoculated with 3 agar disks (5 mm in diameter) containing mycelia of *P. sajor-caju* maintained at PDA Petri plates. Thereafter, the flasks were incubated at 27 ºC in continuous agitation at 150 rpm for 4 days.

2.4. OMW Liquid cultures of *P. sajor-caju* A total of 2 L of diluted 50% OMW were placed in a vessel of stirred bioreactor (Winpact, Taiwan). The vessel with its components was sterilized by autoclaving at 121 ºC for 20 min. After cooling, the vessel was connected to the control unit and inoculated with 5 % v/v of freshly prepared inoculum. The initial pH of OMW was set to 6 with the addition of sterile NaOH (1 N). The culture was maintained at 27 ºC for incubation temperature, 50 rpm for agitation speed, and 1 vvm of air flow rate under batch culture operation for 20 days.

2.5. Analytical Procedures The physical and chemical properties of OMW were analyzed at the Royal Scientific Society laboratories (Amman-Jordan) according to standard methods of analysis [13]. Sample withdrawn from the culture vessel were centrifuged at 10000 × g for 15 min using Refrigerated Centrifuge (Nuve, Turkey). The supernatant was

assayed for enzymes activity, phenolic compounds, culture decolorization, and dissolved proteins content. Total phenolic compounds were determined spectrophotometrically according to the Folin–Ciocalteu method [14].

Laccase enzyme activity was determined at pH 5 by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm for 2 min. [15]. The enzyme activity was expressed as units mL^{-1}, where 1 unit was defined as 1 mmol of substrate oxidized per min. Lignin peroxidase (Lip) activity was assayed using a method described by Tien et al. [16]. The assay mixture contained 2 mM veratryl alcohol, 0.4 Mm H_2O_2 in 50 mM sodium tartrate buffer (pH 6.8) and 0.2 ml of filtered supernatant. Veratryl alcohol oxidation was followed at 310 nm. Manganese peroxidase activity (MnP) was estimated according to Giardinat et al. [17]. All enzyme activities were expressed in unit L^{-1}. The dissolved protein content was estimated by using Bradford method [18].

Culture decolorization was determined by measuring the optical density (OD_{600}) of the culture supernatant before and after treatment. The phenolic and non-phenolic compounds of OMW were determined by Gas Chromatography device (GC-MS, Shimadzu, Japan) equipped with a capillary column Agilent and a flame ionization detector. The temperature of injection was 220°C and peak identities of the products were confirmed by both retention time and spectra matching of standard compound delivered by Sigma Aldrich.

Results and discussion

3.1. OMW analysis The collected fresh OMW samples were thick, oily, dark brown in color. The physical and chemical properties of OMW are shown in Table 1. It is noted that OMW contain significant amounts of COD and BOD indicating high contents of organic matter. The solid content mainly comes from olive fruit residue such as olive pulp, stone, and lignocellulosic derivatives that are hardly degradable. On elemental basis, OMW contain relatively high amounts of minerals that would support the growth of a wide range of microorganisms. On the other hand, OMW contain frequently high amounts of phenolic compounds which contribute to its dark color, high toxicity and antimicrobial activity [2].

3.2 Ligninolytic enzymes production throughout OMW treatment The enzymes Lip, MnP, and laccase production were studied throughout the growth of *P. sajor-caju* on OMW (Figure. 1). As shown in figure, LiP was produced significantly during the first stages of fungal growth on OMW. LiP activity was detected initially on the second day after inoculation and increased rapidly to reach the maximum activity after 4 days of incubation indicating the early need for this enzyme. MnP activity was detected from the first day of incubation and increased slowly, achieving maximum activity after 7 days of incubation. At this stage, the production of LiP was declining. On the other hand, laccase enzyme production started a bit later after 7 days of incubation. The maximum laccase activity (128 U L^{-1}) was observed in all cultures after 12 days of incubation. This result is in agreement with Tsioulpas et al. [2] and Massadeh and Modallal [10] where they reported late production of laccase enzyme by *Pleurotus* sp. These results suggest that the production of these enzymes was necessary for fungal growth and metabolism along its growth phases.

Table 1 Physical and chemical characteristics of OMW.

Parameter	Amount (g L^{-1})	
	Minimum reading	Maximum reading
Total Dissolved Solids	16.984	80.355
Total Suspended Solids	14.207	46.188
COD	78.536	160.096
BOD_5	23.248	63.271
Total Phosphorus	0.158	0.403
Ammoniacal nitrogen	0.022	0.068
Total nitrogen	0.398	1.036
Sodium	0.130	0.384
Calcium	0.276	0.757
Magnesium	0.038	0.063
Potassium	2.053	5.492
Chloride	0.486	1.111
Phenolics	7.739	10.432

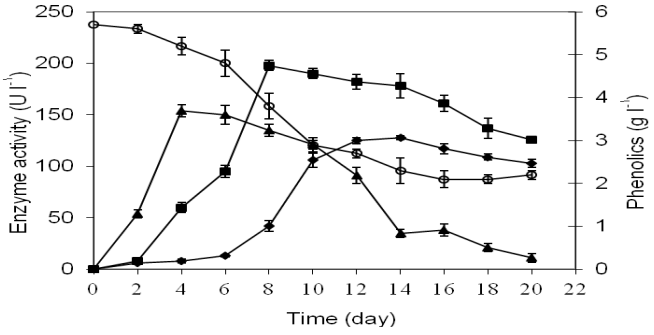

Fig. 1 Time profile for *P. sajor-caju* activity on OMW as represented by enzymes activity and phenolic compounds removal. (▲)LiP activity; (■) MnP activity; (♦) Laccase activity; (○) Phenolic compounds.

3.3. Degradation of Phenolic compounds of OMW As previously mentioned, OMW contain all essential elements that make them suitable substrates for microbial growth, but they also contain several growth inhibitors such as organic acids and phenolic compounds. Dhouib et al. [19] claimed that white-rot fungi are known for their capability to degrade lignin and their enzymatic system takes part in the transformation of aromatic compounds. Therefore, the fungus *P. sajor-caju* cultivated in OMW medium showed a remarkable capability to excrete ligninolytic enzymes to the medium, to significantly reduce OMW's brown color, and to eliminate their phenolic compounds (Figure 2). In representative examples, given in Figure 2, it is shown that MnP activity was rapidly increased after 4 days of inoculation, in parallel to the phenolic compounds removal. The maximum laccase activity was detected after 12 days of fermentation and it lasted stable until the end of the experiment and at this time the maximum phenolic removal was often achieved. Nevertheless, LiP activity was not necessary for phenolic compounds removal but for fungal accessibility of OMW during the early stages of growth.

On the other hand, the fungal growth as monitored indirectly by measuring dissolved proteins content exhibited a typical growth curve showing all stages of growth (Figure 2). The first 2 days of growth represents a period of lag phase that was necessary for fungal adequacy to

medium components. Thereafter, any increase in the fungal growth was correlated to phenolic compounds reduction and treatment of OMW in general. The maximum phenolic removal ranged between 60% and 70% of the initial phenolic content. In all cultures, decolorization of OMW was observed as the absorbance of those cultures was reduced significantly at 600 nm (Figure 2). The absorbance reduction started significantly after the 6th day of the experiment and this reduction in color is correlated directly to the phenolic compounds removal.

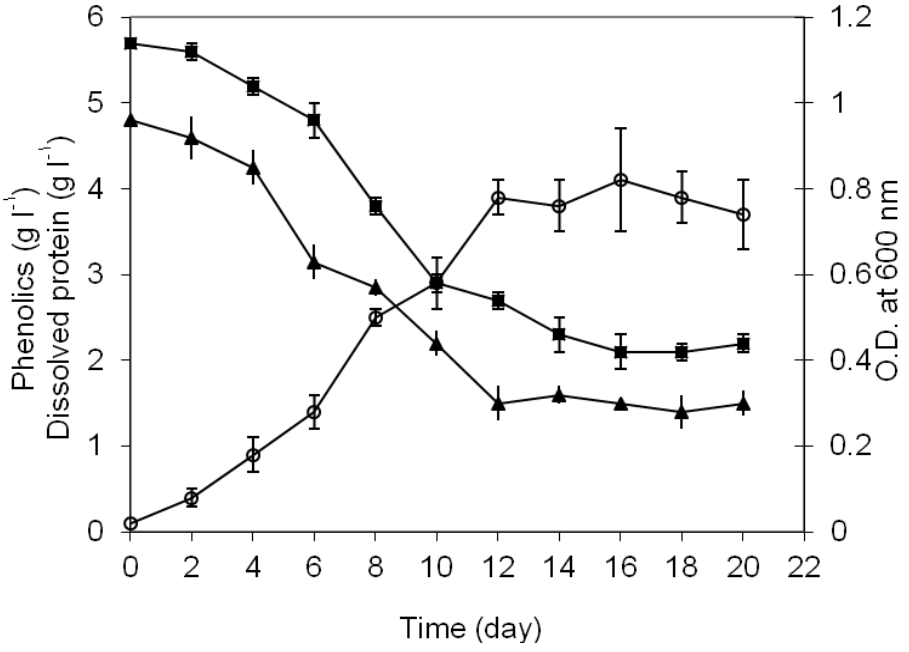

Fig. 2 Efficiency of OMW treatment with *P. sajor-caju* as represented by (▲) Optical density of culture medium; (■) phenolic compounds concentration; (○) Dissolved proteins.

The biodegrdation of phenolic and non-phenolic compounds of OMW by *P. sajor-caju* was evaluated using Gas Chromatography analysis. By the end of the treatment process, it was observed that there were some compounds that were completely degraded i.e. 3-[p-Nitrophenoxy] propionic acid and 2-(1-methylpropyl)-4, 6-dinitro phenol (Table 2). Both P-Nitrophenol and phenol showed significant biodegradation to reach 96.3% and 76.2% respectively. Slight biodegradation was observed for 4-Nitrophenol which was about 59%. On the other hand, non-phenolic compounds were degraded by the enzymatic system of the fungus at different percentages varying from 52-98% (Table 2). This result is in agreement with Dhouib et al. [19] who claimed that the decolorization of OMW corresponds to the depolymerization of high molecular mass aromatics combined with the mineralization of a wide range of monoaromatics that are due to the contribution of fungal enzymes.

This high capability of *P. sajor-caju* to treat OMW was further supported by the COD and BOD values (Table 3). Remarkably, the decolorization of OMW and reduction of their phenolic compounds were accompanied with the reduction in their organic matter contents as represented by the COD and BOD values i.e. the COD and BOD_5 of OMW before treatment were 70600 and 35720 mg L^{-1} respectively and reduced to 31550 and 16160 mgL^{-1} respectively by the end of the treatment process.

Table 2 Removal efficiency of Phenolic and non-phenolic compounds of OMW after the biotreatment with *P. sajor-caju*.

Compound Name	Percentage degradation (%)
Phenolic compounds	
3-[p-Nitrophenoxy] propionic acid	100
2-(1-methylpropyl)-4,6-dinitro phenol	100
P-Nitrophenol	96
Phenol	76
4-Nitrophenol monotms	60
3-Acetyl- 2,4-dihydroxy-6-methyl-benzoic acid	95
Non-Phenolic compounds	
6-Octadecenoic acid, (Z)	98
n-Hexadecanoic acid	95
8,11-Octadecadienoic acid, methyl ester	93
9-Octadecenoic acid, methyl ester (E)	89
Hexadecanoic acid Methyl ester,	88
3-OH-Isovalericacid-monotms	78
Hexahydro-1-oxa-cyclopropa [d] inden-2-one	76
Borazaine , 2-methyl-	76
1-Oxetan-2-one, 4,4-diethyl-3-methylene	75
Cyclohexanone, 2-(1-methylethylidene)	75
1H-Pyrazole ,4-(2-bromoethyl)-3,5-dimethyl-	74
Ethyl Oleate	73
2,5-Dimethoxy-4-ethoxyamphetamine	71
Mandelic acid ,3,4-dimethoxy-methyl ester	66
2,5-dimethoxy-4-propoxybenzaldehyd	63
Lenoleic acid ethyl ester	61
Dibebzothiophene, 1,2,3,4,6,7,8,9 –octah	59
5,5-Dimethyl-1,3- Hexadiene	52

Table 3 Evaluation of OMW treatment with *P. sajor-caju*

Time (day)	COD (mg L^{-1})	BOD_5 (mg L^{-1})	% decolorization
0	70600	35720	0
4	67330	31220	8
8	58110	27750	22
12	44650	24440	48
16	33150	17200	66
20	31550	16160	69

Conclusion

This study showed that *P. sajor-caju* has a great potential for the biotreatmentof OMW. The growth of *P. sajor-caju* on OMW caused important changes in the physical and chemical properties of these wastewaters. The fungus was able to degrade the phenolic as well as non

phenolic compounds. This treatment process has its own advantages: high COD and BOD removal, simple process operation, and high capability of treating a series of toxic compounds.

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