International Journal of Advances in Engineering and Management (IJAEM)
Volume 4, Issue 12 Dec. 2022, pp: 863-871 www.ijaem.net ISSN: 2395-5252

Biodegradation and treatment of Olive Mill Wastewater with fungi (Laccase)

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Submitted: 18-12-2022 Accepted: 31-12-2022

ABSTRACT
This study was designed to harness the environmentally friendly biological process which is economical in the treatment of industrial wastes. The study employed the effectiveness in the biodegradation and treatment of the olive mill wastewater (OMWW) by fungi, using standard procedures in the enzyme synthesis as well as data analysis. The result of the standard calibration curve showed that the OMWW have an optimum pH value in the range of 3 with phenolic degradation to laccase enzyme at 76.12% in 15minutes, allowing the OMWW to be degraded directly without adjustment in pH. It was also observed that an increased enzyme activity significantly increased the absorbance level with the most suitable temperature range found to be between 40.5°C to 41.5°C. The study has revealed the suitability of using fungi in remediation of industrial wastewater.

Key words: Olive mill wastewater, fungi, phenol, laccase, degradation, enzyme.

I. INTRODUCTION

Olive oil is a fat obtained from olive fruit (Olea europaea), a tree crop of the Mediterranean Basin and commonly used in cooking, cosmetics, pharmaceuticals, and soaps, and as a fuel for traditional oil lamps. Olive oil is used throughout the world and is often associated with Mediterranean countries especially Greece andCyprus. Olive oil consumption has suffered an increase in the last two decades. The highest demand of this natural fat may be related to the use of olive oil as the main fat source in the so-called Mediterranean diet that has been proved to prevent the incidence of cardiovascular and neurodegenerative diseases in humans, and also some types of cancer (Tuck and Hayball 2002; Boudet 2007; Yang et al. 2007).

The olive oil processing industry has two by-products whether the press system or three-phase centrifugation is used. The rest is termed “pomace" or “husk". It is the solids resulting from the squeezed residue. The other is a dark red to black liquid called olive mill wastewater" (OMW) (Adhoum&Monser, 2004; Zenjariet al., 2006).

OMW is a complex mixture of water (83-92%), organic matter (4-16%), and minerals (1-2%). The organic matter includes sugars (1-8%), nitrogenous compounds (0.5-2.4%), fatty acids (0.5-1.5%), polyalcohols, polyphenols and pectins (1.0-1.5%), and fats (0.02-1%). These wastewaters present high organic loads, usually indicated as chemical oxygen demand (COD), up to 100-200 gO_2/L (Asses et al. 2000), and low biodegradability assessed by the ratio between COD and biochemical oxygen demand (BOD) which varies from 2.05 to 2.35 (Vlyssideset al. 2004). Sugars (18%), N-compounds (0.52.4%), organic acids (0.51.5%), fats (0.021%), phenols and pectins (11.5%) are organic fractions of olive mill wastes (Hanafi, Assobhei, &Mountadar, 2010). As they have high phenol, lipid and organic acid concentrations, they are phytotoxic materials and cause serious environmental concerns that are also related to treatment costs. However, these wastes also contain valuable resources such as a large proportion of organic matter and a wide range

International Journal of Advances in Engineering and Management (IJAEM)
Volume 4, Issue 12 Dec. 2022, pp: 863-871 www.ijaem.net ISSN: 2395-5252

ofnutrients that could be recycled (Hachicha et al., 2009c; Azbar et al., 2004).

Thedischargeofolivewasteintothesoilmaycause,inplants,leafandfruitabscission and inhibition of seed germination if it's being disposed uncontrollably. Discharging into water bodies increase the phosphorous content, causing color alteration and bad smell While disposing olive wastes in open space causes microbial fermentation, with the production of methane and a wide array of harmful or simply bad smelling gases.

For these reasons, the development of suitable OMW treatments has been a challenge for the researchers. However, until now none of the proposed treatments can be pointed out, both as economically feasible and effective, for treating this type of wastewaters. Proposed treatments include physical, chemical and biological operations, isolated or combined in several ways (Azbaret al. 2004; Niaounakis and Halvadakis 2006; Arvanitoyannis et al.2007). Chemical and physical processes to treat OMW fail to be widespread because they are costly, with low efficiencies, and present serious sludge disposal problems (Mantzavinos and Kalogerakis 2005). Biological processes gained popularity due to their environmental compatibility, lower management costs and equivalent efficiencies compared to physical-chemical ones. Usually, biological methods for OMW treatment include aerobic-activated sludge, co- digestion and anaerobic digestion (Tziotzioset al. 2007; McNamara et al.2008).

II. TREATMENT METHODS OF OLIVE OILWASTEWATER

2.1 PhysicalMethods

In practical terms, the most common treatment method is through evaporation in storage ponds in the open because of the low investment required and the favorable climatic conditions in Mediterranean countries. The large area needed, bad odor, infiltration and insect proliferation are negative properties of evaporation (Roiget al., 2006).

Another alternative OMW treatment is vacuum evaporation producing distillate and concentrate. Distillate is produced continuously, and a discontinuous concentrate automatically discharged. Sodium hydroxide was used to neutralize wastewater during distillation in a study that was carried out by Azbaret al. (2004). As a result, the distilled water volume reached 90% of the wastewater input. Although the distil-late is a color-less liquid, its COD is 3000-4000 mg/L showing that further treatment is still required. Biological oxidation of the distillate pre-ceded by pH adjustment and C: N: P correction gave a treated water complying with the waste water discharge regulations. The concentrate and the olive mill solid residues can be mixed. This mixture can either be de-oiled and burned or used for various purposes such as animal fodder or fertilizer, as it contains 14% protein and 5% potassium (Azbaret al.,2004).

2.2 ChemicalMethods

Sterilization of OMW prior to the incubation with the isolates to be tested is performed and has been reported by several authors, justifying this previous step as a mea- sure to ensure that the alterations suffered by the sample are only due to the inoculums, and not to other microorganisms present in the OMW. (Sayadi and Ellouz 1995; Vinciguerraet al. 1995; D'Annibale et al. 1998; Ben Hamman et al. 1999; Asses et al. 2000; García-García et al. 2000; Tsioul- pas et al. 2002; Aggeliset al. 2003; D'Annibale et al. 2004; Kachouriet al. 2006; Aissamet al. 2007; Martinez-García et al. 2009; Asses et al. 2000). Nevertheless, it has been pointed out that the results obtained with sterilized samples should be cautious, since the heating process may lead to physicochemical alterations on some compounds. COD values may de- crease, and oxidation of phenolics and quinoids may cause precipitation and darkening of the water (Fontoulakiset al. 2002; Aggeliset al. 2003; Kachouriet al.2005).

In reports using sterilized samples OMW were tested atseveral dilutions and with different supplements. The addition of supplements is performed to prevent lack of essential nutrients as nitrogen and phosphorus, which should bein agreement with high C/N ratios for an efficient microbialgrowth (Fadilet al. 2003).

2.3 PhysicochemicalMethods

The most important inorganic occupants that have been used for OMW treatment are ferric and ferrous chloride, ferric sulphate, and aluminum sulphate. In addition, aluminum chloride, calcium hydroxide, and their combinations added as anionic polyelectrolytes, also sulphuric acid, were tested by several researchers. None of these reagents should be used if the precipitated material is to be used as animal feed (Azbaret al., 2004; Tsagarakiet al., 2007). In one study, calcium hydroxide and aluminium sulphate were used to reduce COD to 20 30% of initial values (Roiget al., 2006).

One of the major disadvantages is that they are only a partial solution and must be

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followed by a secondary treatment to comply with legal requirements because the treated liquid still has a high polluting load. Thus, they are more suitable as pre-treatment methods. The other one is the large quantities of sludge that cause other environmental problems (Panizza &Cerisola, 2006; Tsagaraki, Lazarides, &Petrotos, 2007).

2.4 BiologicalMethods

Biological treatments are also generally used for the treatment of OMW.

Aerobic treatments:

Another way to manage OMW is by aerobic treatment with micro-organisms, which has also been used to remove the pollution effect of OMW (Anastasiou, Christou, Michael, Nicolaides, &Lambrou, 2011). Aerobic bacteria have been tested primarily to remove phytotoxic com-pounds (i.e., monoaromatic or simple phenolics) from OMW, although some studies have also focused on reduction of COD. The bacteria ap-pear to have a minimal effect on more complex polyphenolics responsible for the dark coloration of OMW. Furthermore, they appear to be very effective against some phenolic compounds and relatively ineffective against others. For example, Bacillus pumilus was able to completely degrade protocatechuic acid and caeic acid but had much less effect on tyrosol. On the other hand, Arthrobacter transformed tyrosolto 4-hydroxyphenyl acetic acid.

The production of energy (biogas) and the potential re-use of the effluent in irrigation are the main reasons to use anaerobic digestion for OMW treatment. The major limitation of this treatment is the inhibition of methanogenic bacteria by the phenolic compounds and the organic acids present in the OMW. Anaerobic digesters or anaerobic sludge bed reactors are suitable for treating OMW but a pre-treatment stage is necessary to remove undesirable compounds. It was proposed that sedimentation infiltration pre-treatment prior to anaerobic digestion is a useful way of OMW disposal (Roiget al.,2006).

III. MATERIALS AND METHODS

3.1 Materials

1. Petridish 2. Potatoinfusion3. Dextrose4. Agar5. TartaricacidAntibiotics6. Distilledwater 7. Erlenmeyerflask Spatula8. Weightbalancemachine9. Hot platestirrer10. Autoclave11. Biosafetycabinet12. Incubator13. pH meter 14. Whiterot fungi 15. Centrifugemachine 16. 180gWheatbran 17. 20g Soybean 18. 140ml $NaPO_4$ Buffer (0.1M,5.5pH) 19. $NaPO_4$ Buffer (0.1M, 5.0pH) 20.Cruteenzyme.

3.2. Methods

3.2.1 Potato Dextrose Agar (PDA)Preparation

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. PDA is a general-purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth.

Table 3.1: Grams of potato infusion, dextrose and agar in 1litre.

Item	Quantity (g)
Potato infusion from200g	4
Dextrose	20
Agar	15

a. Calculate and measure the grams of potato infusion, dextrose and agar to200mlofdistilledwater.(39gPDAin1000ml ⟶7.89gPDAin200ml)
b. After mixing them, add sterile tartaric acid or antibiotics. (Final pH: 3.5)
c. Dissolve 7.89g PDA in 200ml of distilledwater.
d. Mix and heat at 100°C for 10minutes.
e. Autoclavetoliquidcycleat121°Cfor15minutes.(Usedcotton, do notevaporation.)
f. Sterilize the cabinet and pour the liquid mixture onto petri dish before it solidifies and put it at37°C.

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Figure 3.1: Potato Dextrose Agar (PDA)

3.2.2. Enzyme production from solid state fermentation

Procedure:

a. Cut a piece of $1m^2$ from the white rot fungi and inoculated onto thePDA.
b. Mixed180gwheatbranand20gsoybean tomakeasolid-statefermentationinto the erlenmeyerflask. Prepared 5.5 pH 0.1M $NaPO_4$ buffer, added to the mixture and autoclaved it at 121°C, 1L Mpa for 15minutes.
c. Cut the fungi in 8 pieces and add onto theSSF.
d. Put it at 28°C for 14 days and dry at 60°C for 1day.
e. After all, grind the sample to get the enzyme as apowder.
f. Weigh 1 g enzyme powder and add to 10 ml buffer for 15 minutes at 6000rpm.
g. At the end, obtain the supernatant as a crute enzyme.

Figure 3.2: 5th day of the TrametesVesicolor. **Figure 3.3:** Fungi in the SSF asan enzyme.

3.2.3 Laccase activity onthe oil mill wastewater

Laccase enzymes catalyze phenolic compounds, changing the color of OMWW. In these experiments, we looked the enzyme activity on the OMWW for the best degradation.

Procedure:

a. Change the concentration of the wastewater to observe the enzyme activity on the wastewater.
b. Take 0.1ml from each solution and add 1.9ml ABTS into the enzyme solution, then wait for5minutes.
c. Read them at425nm.

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Solution concentration	**Enzyme**	**Wastewater**	**Buffer**	**Total Amount**
0%	2ml	0ml	2.00ml	= 4ml
1%	2ml	0.04ml	1.96ml	= 4ml
2%	2ml	0.08ml	1.92ml	= 4ml
5%	2ml	0.20ml	1.80ml	= 4ml

Table 3.2: Enzyme inhibition activity on different wastewater concentrations.

IV. RESULT AND DISCUSSION ON RESULT

Enzyme InhibitionActivity

The result of the laccase inhibition on the oil mill wastewater is presented in table 4.1 below. The result indicates the highest laccase inhibition of the oil mill wastewater at 3.52U of the laccase activity.

Therewasadecreasingontheabsorbanceandthelaccas eactivityfrom0%to5%.So, the enzyme inhibition is increased with the concentration of wastewater. Basically, the graph showed the laccase activity that was decreasing due to the OMWconcentrations.

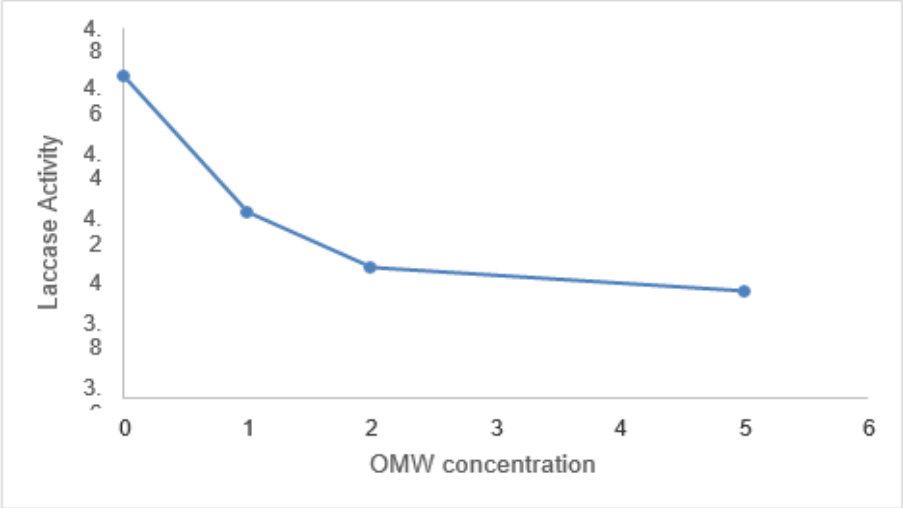

Figure 4.1: Laccase activity versus OMW concentration.

Table 4.1: Percentages of laccase inhibition with different concentrations of wastewater.

Amount of wastewater (%)	**Abs (425nm)**	**Laccase Activity (U)**	**Inhibition (%)**
0	0.84	4.57	0
1	0.72	3.91	14.50
2	0.67	3.64	20.34

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5	0.65	3.52	22.85

Standard Curve PhenolAnalysis

Table 4.2: Standard phenol concentrations analysis.

Phenol Concentration (mg/L)	Abs (500nm)
0	0
1	0,226
2	0,408
3	0,599
4	0,816
5	1,013

The standard curve is obtained from this table and the curve gave an equation that would provide to calculate the concentrations of all phenol analysis with using the absorbance values.

Figure 4.2: Calibration curve (standard curve).

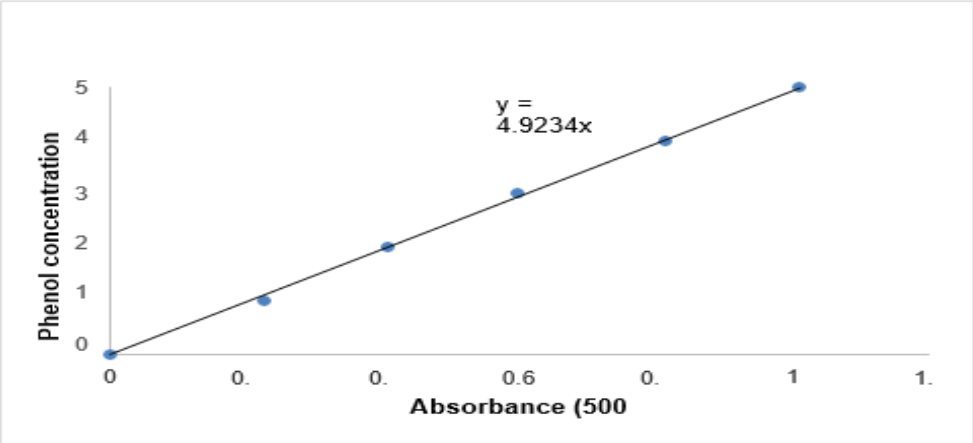

Table 4.3: Different pH conditions for the best phenol removal.

pH	Phenol Removal (%)
3	76.12
4	47.01
5	45.52
6	38.80
7	1.46

These results showed the best pH and time to enzyme activity for understand the best phenolicdegradationconditionsoflaccaseenzyme.Aswecansee,thebestpHwas3and the best time for the activity at pH3 was 15minutes.

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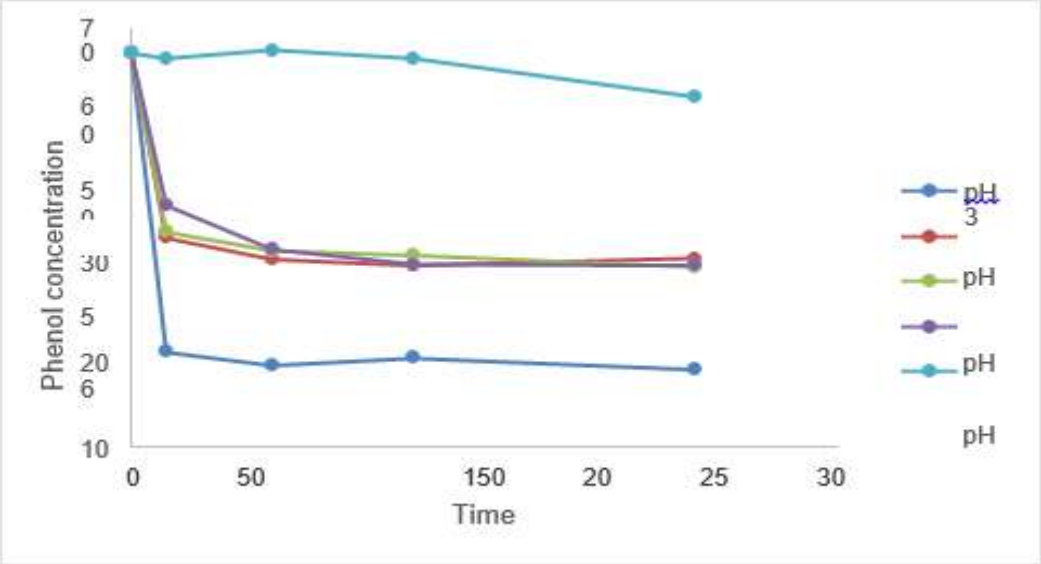
Figure 4.3: Phenol concentration versus time.



V. CONCLUSIONS

The by-products obtained during olive oil production exhibits high levels of phenolic compounds such as hydroxytyrosol and oleuropein that are hazardous to the environment due to the non-degradable nature of phenols and can be considered highly toxic hence; the methods for phenol removal cannot be over emphasized.

Nevertheless, the use of safer and eco-friendly methods such as the use of laccase producing fungi to degrade OMW must be considered in a pilot scale due to their great potential for olive mill waste degradation. Additionally, this method is time and cost effective as the raw materials used are agricultural wastes, not expensive and easy toobtain.

The mesophilic white-rot fungi (Trametesvesicolor) secreted an extracellular ligninolytic enzyme called laccase known for its degradation of phenols and decolorization of OMW than any other biological method.

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