A remarkable step in the aerobic biological treatment of Olive Mill Wastewater (OMW): A combination of selected microbial strains that enhance their decolorization and depollution

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Abstract. The olive industry extracts oil from olives but also generates solid co-products called pomace and liquid wastes known as Olive Mill Wastewater (OMW). With global annual production exceeding 30 million tons and approximately 685,000 tons in Morocco alone, these wastes pose environmental challenges due to their high acidity, organic load, and phenolic compounds. Our research aims to depollute and recycle OMW using aerobic biological treatment methods. Samples were collected from various ecological sites across four Moroccan regions. We isolated and purified several strains of molds, yeasts, and bacteria capable of decolorizing OMW. Decolorization experiments revealed promising results, with a combination of seven selected molds showing significant reductions in chemical oxygen demand (COD) by 71.44%, biochemical oxygen demand (BOD5) by 69.91%, and polyphenols content by 84.22%. Encouraged by these findings, we propose further treatment using sourdoughs composed of combinations of different pure strains, including yeasts and selected bacteria such as Bacillus subtilis and Pseudomonas aeruginosa. This approach demonstrates a practical and cost-effective method for depolluting and recycling OMW, contributing to environmental protection and human health preservation. By mitigating the risks associated with untreated OMW discharge, this study offers a viable solution to the environmental challenges posed by olive processing industries globally, particularly in regions like Morocco where olive cultivation is significant. ***Keywords*:** Olive Mill Wastewater, aerobic biological treatment, polyphenols, decolorization, depollution.

1 Introduction

The olive tree is a fruit tree that produces fruits called olives. Its history is deeply rooted in human culture and agriculture for thousands of years. Globally, approximately 10.8 million hectares are dedicated to olive cultivation, with 97% focused on Mediterranean countries [1]. With an estimated total annual production of 1,790,309 tons of olive oil, Spain leads globally, followed by Greece with 327,718 tons. Next, Tunisia ranks the third with 278,300 tons annually, followed by Italy with 277,713 tons. Morocco is classified in the fifth position with 174,400 tons, and Turkey in the sixth position with 154,326 tons [2]. The industrial processing of olives in crushing units allows the extraction of 20% of olive oil as the targeted product. But it generates 30% of solid polluting co-products called olive pomace, and 50% of liquid polluting co-products commonly referred to as olive mill wastewater (OMW) [3]. In numerical terms, this means that the annual quantities produced of these two wastes are estimated to almost 2.9 million tons of olive pomace produced globally and around 30,000 tons in Morocco [4]. The annual production of OMW is even more staggering, with over 30 million tons worldwide and nearly 685,000 tons in Morocco [5,6]. These liquid discharges which are composed mainly of water, oil emulsion, pulp, and crushed olive pits [7] are highly polluting [8]. Their toxicity is due to several factors, including their acidic pH, which degrades all soils except limestone soil, and their high mineral content, which causes eutrophication of shallow stagnant water bodies [9]. The presence of heavy metals disrupts plant growth [10], affects the entire environment and consequently human health is constantly threatened [11]. Their high organic load, especially simple sugar [12], suffocates aerobic microorganisms in aquatic environments [13], while lipids cause the gradual extinction of aerobic and photosynthetic aquatic organisms [14-15], resulting in a general disbalance in the aquatic ecosystem [16]. Moreover,

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OMWs are rich in other organic molecules called phenolic compounds, which give them their characteristic of dark color : reddish to black [17], purplish or brown [18]. These substances have antimicrobial and phytotoxic effects, preventing natural biodegradability [19]. Thus, we could say that indeed OMW are among the most dangerous industrial liquids discharges, as reported other authors in the literature [20]. Thus, given their high pollutant load, these liquids waste pose multiple environmental problems when released into the environment without prior treatment [21]. Hence, the need to find effective, practical, and immediate solutions to this environmental scourge. For this, research efforts were focused on valorization and treatment methods. Valorization involves transforming OMW residual substances into other beneficial by-products for various sectors such as agri-food, biotechnology, agriculture, construction, chemistry, and so on. For example, their transformation into very valuable products such as biopolymers, enzymes, and biofuels [22], or their reuse as natural dyes for dyeing materials in the textile field, or the manufacture of clay or terracotta building bricks [23]. Treatment methods aim at eliminating as much toxic molecules as possible which are contained in these OMW through physical, chemical, and biological processes, which can be used separately or combined. Physical treatments such as activated sludge [24] nanofiltration [25], adsorption on activated carbon [26], etc. Chemical treatments such as the use of photocatalytic membranes [27], electrohydrolysis [28], and electrocoagulation [29]. In biological treatment, several studies have been carried out on aerobic and anaerobic processes, used separately or in combination [30,31]. Though, given the complexity of the characteristics OMWs and their high polluting organic load, any single treatment method is insufficient and economically unfeasible when applied in isolation. Hence, there is a need to consider combined treatments, as reported by Ranalli [32] and mentioned by Faggiano et al. [33]. Concerned with contributing to the resolution of this environmental drama, our laboratory has been committed since 2000 to setting up biotechnological processes for aerobic biological treatment that aim at transforming OMW into biofertilizers and biostimulants for plant growth [34,35,36]. This current work focuses on the evaluation of the physicochemical and microbiological characteristics of the OMW that we collected from twelve olive crushing units in four regions of Morocco (three units from each region). Then, we introduce the technique that allowed us to isolate and purify a microflora with a strong ability to assimilate and degrade the polyphenols of OMW.

Finally, we give the results of OMW treatment in this study. It is the decolorization of liquid OMW (diluted to 1/2, 1/3, 1/5, and even raw) visible to the naked eye after nine days of treatment by a microbial association composed of seven pure different molds, at ambient temperature and without pH adjustment. This is a very suitable and promising result, as we expect even better results when we involve the action of our yeasts and few bacteria that we have also selected and purified, and which are kept in stock. We believe that we are on the right track towards real, practical, and less costly applications of an efficient and stable industrial process that depollutes these liquids discharges from the olive oil industry and recycles them into new products designed to fertigation of agricultural soils.

2 Materials and methods

2.1. Sampling and Physicochemical parameters of OMW

OMW samples were taken from four Moroccan regions (Fez, Ouazzane, Settat, and Taza) from 12 crushing units equipped with a continuous three-phase system (3 units from each region). They were collected in 5-liter plastic food containers for physico-chemical analysis. Samples for microbiological analysis were taken in sterile 2-liter bags and bottles, kept away from light at 4°C. Analyses were performed immediately upon arrival at the laboratory.

2.1.1.Measurement of pH and conductivity

For pH and conductivity measurement, representative samples were used, and the pH meter (pH-2005) was calibrated using standard buffer solutions, with stable pH readings recorded for each sample. Simultaneously, the conductivity meter (EC 214) was calibrated with standard solutions, and stable conductivity readings were recorded.

2.1.2. Nitrogen, Moisture, and Ash Content

Nitrogen content was quantified using the Kjeldahl procedure [37], which involves digestion and distillation processes for accurate measurement. Moisture content was determined by oven-drying the OMW sample at 100°C until a constant weight was achieved, aligning with the specifications provided by the Association of Official Analytical Chemists [38]. Ash content was estimated through the methods outlined by the American Association of Cereal Chemists [39], involving the incineration of the sample to determine the inorganic residue.

2.1.3. Biological oxygen demand (BOD5) and chemical oxygen demand (COD)

For the assessment of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) in

Olive Mill Wastewater (OMW), representative samples were used.
In the BOD analysis, BOD bottles were inoculated with bacteria, the OMW sample was added, and incubated in the dark at 20°C. Dissolved oxygen was measured before and after incubation to calculate BOD. For COD analysis, the OMW sample was digested using an oxidizing agent. The absorbance of the digested sample was measured, and values were converted to COD concentrations [40].

2.1.4. ICP-OES analysis of potassium and phosphorus

The determinations of potassium and phosphorus concentrations were carried out using ICP-OES, as described by Mefteh et al. [41], samples were acid-digested, and calibration standards were prepared. The ICP-OES instrument is calibrated for potassium and phosphorus analysis, and samples were introduced into the instrument. Concentrations were calculated from calibration curves, and quality control includes blank solutions and certified reference materials. Indeed, results are reported with analytical details.

2.1.5. Determination of total phenolic content (TPC)

The total phenolic content of OMW was evaluated using the Folin-Ciocalteu spectrophotometric method, following the detailed procedure described by El Kabous et al., 2023 [42]. This method involves the use of Folin–Ciocalteu reagent to quantify phenolic compounds based on their reduction of the reagent, providing a precise measurement of the total phenolic content in the OMW sample. The average values of the measurements of these physico-chemical parameters are summarized in Table 1.

2.2. Microbiological analysis of OMW

Table 2 summarizes the microbiological analyses of OMW samples taken from four regions of Morocco (Fez, Ouazzane, Settat, and Taza) from 12 crushing units equipped with a modern three-phase continuous system (3 units from each region).

2.2.1. Flora of hygienic interest

- *Enumeration of Total Aerobic Mesophilic Flora (TAMF)*

The enumeration of Total Aerobic Microorganisms (TAM) was carried out on Plate Count Agar (PCA) medium by inoculating 1.0 mL of each dilution ranging from 10^{-1} to 10^{-7}.
The readings were taken after 72 hours of incubation at 30°C [43].

- *Coliform enumeration*

Total coliforms are enumerated on Methylene Blue Eosin (MBE) agar medium, with incubation at 37 °C for 24 to 48 hours. The enumeration of fecal coliforms is carried out on MacConkey agar medium with incubation at 44°C for 48 hours [43].

2.2.2. Pathogenic and toxigenic flora

- *Staphylococcus enumeration*

The enumeration of staphylococci is carried out on a selective Chapman medium. Cultures are incubated at 37 °C for 24 to 48 hours [44].

- *Fecal Streptococcus Enumeration*

The enumeration of fecal *Streptococci* requires two consecutive tests: a Presumptive Test (ROTHE) with incubation at 37°C for 24 hours. If the result is positive (presence of bacterial turbidity), the content of the tube is streaked onto Eva Litsky Medium (Confirmatory Test). After incubation at 37°C for 24 hours, tubes showing microbial turbidity and a violet (whitish) disc at the bottom confirm the presence of fecal *Streptococci* [45].

- *Enumeration of Sulfite-Reducing Clostridiums*

Reinforced Clostridium Agar (RCA) contains cysteine as a reducing agent. Degradation of this agent by *Clostridium* releases sulfur, which combines with iron to give *Clostridium* colonies a black color. After 48 hours incubation at 37°C, we read the black colonies that appear at the bottom of the tubes [46].

- *Search for Salmonella*

The search for Salmonella is performed in three steps:
Pre-enrichment: Incubation of the mixture [1.0 mL sample + 9.0 mL buffered peptone water (BPW)] at 37°C during 24 hours.
Enrichment: 1 ml pre-enrichment medium is added to 9.0 mL selenite-cysteine (SCB) selective broth. Tubes are homogenized and incubated for 24 hours at 44°C.
Isolation: Salmonella-Shigella (SS) medium is inoculated from the pre-enrichment medium. Incubation takes place for 24 to 48 hours at 37°C [44].

- *Lipolytic enumeration*

We followed the method described by RATH, 1966 [47], which uses the fatty substance associated with Victoria blue (M.G.B.B.V).
The sample is diluted in physiological water to 10^{-4}. Each sterile petri dish is inoculated with 0.5 mL of each dilution (deep culture).
Nutrient agar and grease/dye mixture are then melted and brought to 45°C. Next, 5 ml of the mixture is introduced into the 100 mL flasks of nutrient agar. Finally, the mixture is thoroughly emulsified, then quickly poured into Petri dishes. Incubation takes place at 30°C for 48 hours. A positive test is indicated by dark blue colonies.

- *Pseudomonas isolation*

Pseudomonas spp. are aerobic, non-spore-forming, motile (one or more polar flagella), Gram-negative bacilli. They are easy to culture, as their nutritional requirements are very simple. They grow between 10°C and 42°C on ordinary media: Mueller Hinton, selective agars Drigalski, Hektoën, MacConkey. Generally, 24 to 48 hours incubation is sufficient for good growth. We isolated them on King B medium. This is a *Pseudomonas* differentiation medium, which detects the synthesis of pyoverdine, a pigment produced by these species, and enables their identification. Enumeration is carried out according to standard [48].

- *Bacillus isolation*

The *Bacillus* genus is highly heterogeneous. It includes both aerobic and facultative anaerobic species. Gram-positive *Bacillus* bacteria are purified and successively cultured on nutrient agar media (NA). Isolated *Bacillus* are identified biochemically and morphologically according to the criteria of Tortora et al. [49].

2.2.3. Yeasts and molds research

- *Enumeration and identification*

Enumeration is carried out on PDA (Potato Dextrose Agar) agar medium, incubated at 25°C for 5 days. According to [50].
Isolated species are determined by macroscopic and microscopic observation. Identification is based on the determination key in [51].

- *Conservation of yeast and mold isolates*

Yeast and mold isolates are stored in inclined tubes containing PDA medium. After incubation for seven days, the tubes are stored at 4°C to considerably increase the longevity of the cultures.

2.2.4. Isolation of OMW-degrading isolates

- *Sampling*

As previously indicated, samples were taken from 12 crushing units (3 different units in each town) equipped with a continuous three-phase system, located in four regions of Morocco (Fez, Ouazzane, Settat and Taza) with the aim of isolating strains capable of assimilating and degrading polyphenols. All samples (stored OMW, stored pomace and soil) were taken under strict aseptic conditions and recovered in sterile bottles and bags. Sampling was carried out at different levels as follows:

- At the level of the OMW storage basin, at a distance of 5m imposed by the local authorities.
- On the surrounding soil, where microorganisms have concentrated over the years.
- At the nearby pomace deposit, at a distance of 10m.

Once the samples have been collected, suspensions are prepared from a mother dilution: 5 g of each sample is suspended in 45 ml of sterile distilled water, then vortexed for a few seconds and left to settle for one or two minutes (this is the 10^{-4} dilution).
This was followed by a series of decimal dilutions from 10^{-2} to 10^{-4}. Each sample is assigned a code designating its origin and degree of dilution. The 10^{-4} dilution is used to inoculate the various culture media (Figure 1, step I).

- *Media used for isolation*

The media used to isolate molds and yeasts are:

- White agar.
- PDA (potato dextrose agar). After sterilization of this medium, bacterial growth is inhibited by the aseptic addition of gentamycin at a concentration of 5 mg/l.
- Liquid or solid selective medium prepared from stored OMW from Taza.
- PCA medium is used for bacterial culture. King medium is used for culturing *Pseudomonas sp*.

2.2.5. Strain isolation and purification method

Our starting point was the fact that the composition of OMW represents an important source of nutrients, both mineral and organic, and is truly both a culture medium and a selective medium. A selective medium too, since certain organic substances (polyphenols) found in OMW are inhibitors of most microorganisms. It is these phenolic compounds that give OMW its characteristic of black color [52]. However, some microorganisms can tolerate the high salinity, acidic pH and phenolic substances present in OMWs. In other words, the harsh conditions of this micro-ecosystem lead to a selectivity of microbial strains, and only those that are genetically adapted can grow and proliferate there. We took advantage of this particularity of OMWs to select strains capable of growing there. First, we asepticized liquid selective media based on OMWs with inocula (10^{-4} dilution) prepared from each of the different samples from the 4 regions of Morocco (Fez, Ouezzane, Settat and Taza). This operation allowed us to select the inocula with the highest decolorization of OMWs. In other words, they contain strains capable of assimilating and degrading polyphenols. In a second step, after streaking the best inocula on these solid selective media based on OMWs, the importance of the decolorization halos around the isolated colonies (molds, yeasts, bacteria) led us to isolate and purify the different performing strains.

- *Cultivation on liquid OMW (Figure 1 below, step II)*

Raw OMW is centrifuged at 5000 rpm for 20 min to remove suspended matter, then diluted 1/2, 1/3, 1/5. Volumes of 18 ml of raw and diluted OMW are then distributed in Erlenmeyer flasks. They are then sterilized at 120°C for 20 minutes. After cooling, these

liquids selective media are aseptically inoculated with 2ml of the 10^{-4} dilution prepared in advance from each of the different samples from the 4 regions of Morocco (Fez, Ouezzane, Settat and Taza). Finally, they were incubated at 30°C and observed daily for a week. We examined the degree of discoloration of these OMW in the Erlenmeyer flasks, and monitored the qualitative and quantitative evolution of the existing microbial flora as it grew over time in the different samples. Table 3 summarizes the results.

- *Purification of strains on OMW-based solid selective medium (Figure 1 below, steps III and IV)*

We used the selective medium described above, containing OMW at different concentrations (raw OMW and OMW diluted 1/2, 1/3 and 1/5), to which 15g/L agar had been added, and sterilized at 120°C for 20 min. When the medium is sufficiently cooled, it is aseptically poured into Petri dishes. Once solidified, the selective medium contained in each petri dish was inoculated under sterile conditions using the streak method from each of the samples from the 4 Moroccan regions that had shown massive growth on liquid selective medium. Then, for one week, we examined the petri dishes for the extension of the discoloration aureole around the individual colonies. Each isolated strain was assigned a code designating its origin and degree of dilution. In the current work, we are only interested in mold strains, though we have isolated yeasts and some bacteria. Table 4 illustrates the obtained results. These colonies are individually plated on PDA and white agar for a second purification step, and then stored on inclined tubes.

2.2.6. Summary of strain isolation and purification steps

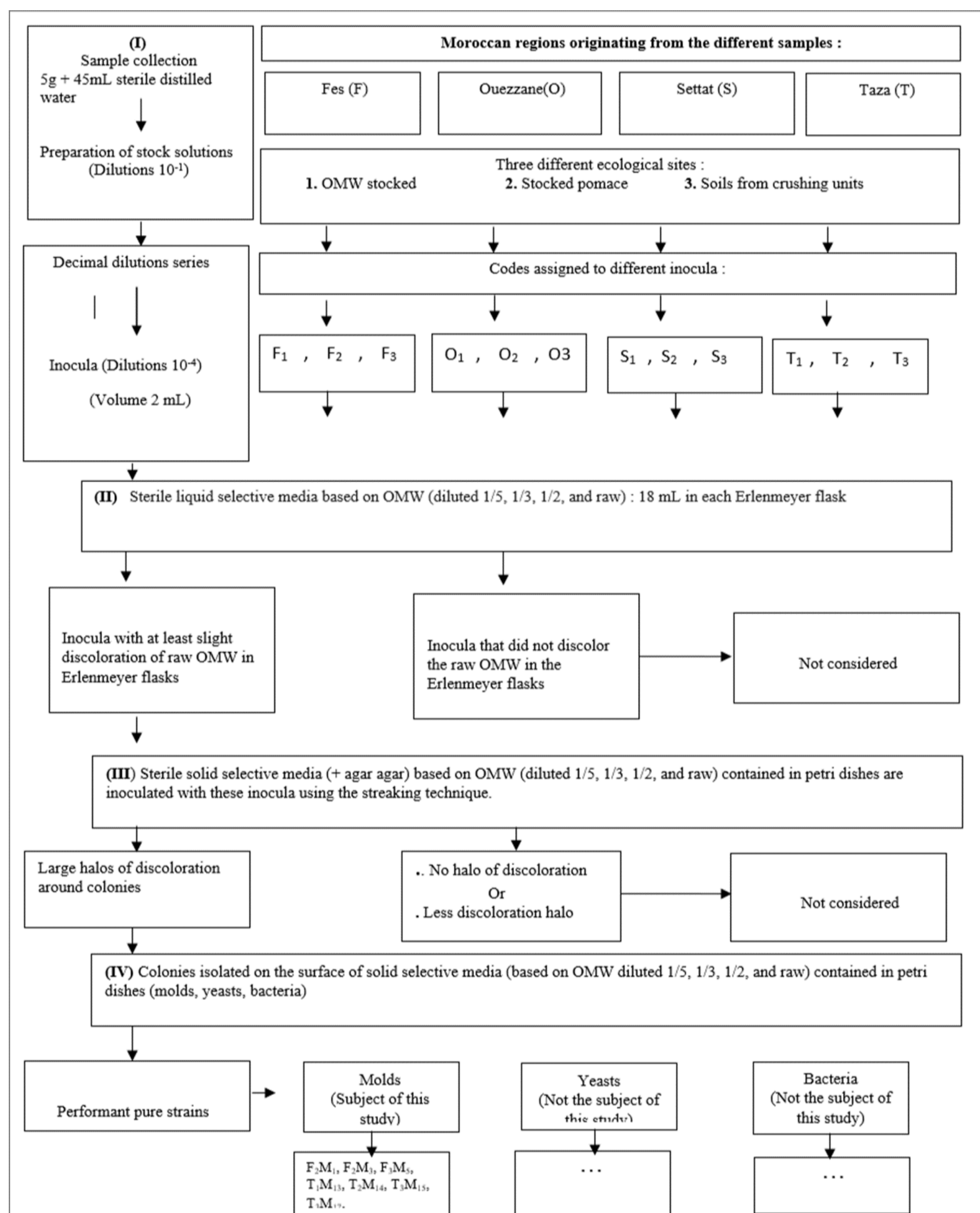

Fig. 1. Summary of the steps for isolation and purification of microbial strains

2.3. Treatment of liquid OMW from Taza by pre-cultures of purified colonies

2.3.1. Pre-cultures preparation

Under aseptic conditions, colonies that have shown discoloration from OMW on solid selective medium are aseptically inoculated into test tubes containing 5mL of sterile OMW from Taza (or sterile physiological water), then homogenized by vortexing for few seconds**.** They are then incubated at 25°C for 5 to 7 days.

2.3.2. Treatment of liquid OMW from Taza with selected mold pre-cultures

Under aseptic conditions, 5mL of pre-cultures composed of the combination of 7 different pure molds (supplied by the solid selective medium described above) are inoculated into flasks containing 45 ml of sterilized liquid OMW from Taza: raw, diluted 1/2, 1/3, and 1/5. After homogenization by shaking, the flasks were incubated at ambient temperature. Figure 2 shows results obtained after 9 days.

2.4. Measurements of physico-chemical parameters of Taza OMW before and after treatment

The results of pH, COD, BOD5 and polyphenol measurements for Taza's OMW before and after treatment are summarized in Table 5.

3 Results and discussion

3.1. Physico-chemical parameters of OMW

Table 1 shows the average values of the measured physico-chemical parameters.

Table 1. Average values of the physicochemical characteristics of OMW studied from 12 modern 03-phase continuous system crushing units in 4 regions of Morocco.

Parameters	Values
pH	4.8
Electrical conductivity EC	18.47ms/cm at 20°C
BOD_5	73.01 g O2/L
COD	103.15 g O2/L
Total dry matter (TDM)	76.5 g/L
Total nitrogen (N)	0.77 g/L
Total phosphorus (P)	0.48 g/L
Potassium (K)	5.30 g/L
Polyphenols	9.03 g/L

Data are average values for the twelve regions of Morocco.

3.1.1. Hydrogen potential (pH)

The average pH value of the OMW collected is 4.8. This is within the range of (3 and 6) quoted by [20]. Their high acidity explains the scarcity of bacteria compared to the relatively higher numbers of molds and yeasts we isolated. This is in line with the authors' statement [53] that molds and yeasts are much more resistant to the acid pH, salinity and phenolic compounds of OMW.

3.1.2. Electrical conductivity (EC)

The average electrical conductivity of the OMW collected is around 18.47ms/cm at 20°C. This is within the range of [18 to 50 ms/cm] reported by [54]. This EC value indicates that our OMWs are relatively high in mineral elements. This could be explained by the excessive salting of olives for their preservation when the duration of their storage is extended in certain units. This excess of salinity would explain the low number of bacteria isolated in comparison with that of molds and yeasts, and thus, confirms the quotation from [53] mentioned above.

3.1.3. Oxygen demand (COD) and biological oxygen demand (BOD5)

Our collected OMW have average of COD and BOD values of 103.15 g O2/L and 73.01 g O2/L, respectively. These values are within the range of (40 g/L to 200 g/L) for COD, and (23 g/L to 100 g/L) for BOD [55], indicating that they are moderately loaded with organic pollutants. The COD/BOD5 ratio, commonly called the biodegradability index Ib, is used as an indicator of the biodegradability of the pollutant. According to a study [56] :

- If Ib < 3, the pollutant is highly biodegradable.
- If 3 < Ib < 6, the pollutant is less easily biodegradable.
- If Ib > 6, the pollutant is difficult to biodegrade.

The average value of the biodegradability index (Ib =1,41). Therefore, we can conclude that the OMW from these four Moroccan regions are generally highly biodegradable.

3.1.4. Total dry matter (TDM) concentration

The average TDM value is equal to 76.5g/L. This is well above the value of 17.9 g/L reported by [57] However, it remains slightly below the 87 g/L concentration found by [58] in OMW from 3-phases extraction processes. The observed differences in the recorded values could be explained by the influence of various parameters, including the geoclimatic conditions of the region, the extraction system used, and the ripening stage of the olives [59].

3.1.5. Nutrient content (NPK)

In our OMW from a continuous 3-phase system, the average values of these three elements are as follows: N (0.77 g/L); P (0.48 g/L); K (5.30 g/L). These values are almost identical to those quoted by [14]. On the other hand, comparing our results to those obtained by the authors [60] who also worked on OMWs from three-phase extraction systems, we can say that our values of N, P and K are roughly in agreement with theirs for P and K, but that their OMWs are almost twice as rich in N as ours. Indeed, these authors obtained the following values: N (1.6 g/L); P (0.3 g/L); K (4.6 g/L). This high NPK content explains why OMW is used as a fertilizer for agricultural soils [61].
These effluents therefore represent a source of nutrients that could replace conventional fertilizers. OMW from the 3-phase continuous system could therefore be used for ferti-irrigation of agricultural soils.

3.1.6. Phenolic compounds

The average phenolic content is around 9.03 g/L. This is within the range of [1.6 to 10.7 g GAE/L] (GAE: gallic acid equivalent) quoted by [62].
This high value indicates that our OMWs are rich in these substances. It confirms the stronger antimicrobial effect of these molecules towards bacteria, as mentioned above by [53].

3.2. Microbiological characterization of OMW

Table 2 shows the results of microbiological analyses of OMW collected from 12 modern three-phase continuous system crushing plants in 4 regions of Morocco, and their average values.

Table 2. Microbiological analyses of different OMW samples collected from 12 modern 03-phase continuous system crushing units from 4 regions of Morocco.

OMW origin region	Sample	Germs							
		TAMF (cfu/g)	T. colif (cfu/g)	*Staphy* (cfu/g)	*Strep* (cfu/g)	*Clost* (cfu/g)	*Lipo. Bact* (cfu/g)	*Pseudom spp* (cfu/g)	*Bacil. spp* (cfu/g)
Fes (F)	F_1	$11.\ 10^5$	10^2	$0.2.\ 10^2$	Absence	$0.67.\ 10^2$	$3.\ 10^2$	$2.\ 10^2$	$2.5.\ 10^3$
	F_2	$5.5.\ 10^5$	Absence	Absence	Absence	$0.20.\ 10^2$	$3.5.\ 10^4$	$1.5.\ 10^2$	$3.\ 10^5$
	F_3	$9.2.\ 10^5$	10^2	$0.1.\ 10^2$	Absence	$0.37.\ 10^2$	$2.2.\ 10^4$	$2.\ 10^2$	$3.1.\ 10^5$
Ouezzane (O)	O_1	$4.5.\ 10^5$	0	0	Absence	$0.52.\ 10^2$	$4.2.\ 10^4$	$4.3.\ 10^2$	$1.1.\ 10^6$
	O_2	$5.\ 10^5$	0	0	0	$0.67.\ 10^2$	$3.3.\ 10^4$	$3.8.\ 10^2$	$3.\ 10^4$
	O_3	$4.2.\ 10^5$	0	0	0	$0.20.\ 10^2$	$3.1.\ 10^4$	$9.\ 10^2$	$4.7.\ 10^4$
Settat (S)	S_1	$7.\ 10^5$	0	0	0	$0.21.\ 10^2$	$3.3.\ 10^4$	$2.\ 10^2$	$3.\ 10^5$
	S_2	$9.\ 10^5$	$0.1.\ 10^2$	0	0	$0.75.\ 10^2$	$2.3.\ 10^4$	$3.5.\ 10^2$	$2.7.\ 10^5$
	S_3	5.10^5	0	0	0	$0.54.\ 10^2$	$3.5.\ 10^4$	$1.2.\ 10^2$	$1.1.\ 10^5$
Taza (T)	T_1	$3.5.\ 10^5$	0	0	0	$0.32.\ 10^2$	$4.2.\ 10^4$	$0.7.\ 10^2$	$7.\ 10^5$
	T_2	$4.2.\ 10^5$	0	0	0	$0.43.\ 10^2$	$2.5.\ 10^4$	$1.1.\ 10^2$	$4.1.\ 10^5$
	T_3	$3.2.\ 10^5$	0	0	0	$0.32.\ 10^2$	$2.3.\ 10^4$	$2.5.\ 10^2$	$2.2.\ 10^5$
Average		$5.94.\ 10^5$	$0.17.\ 10^2$	$0.02.\ 10^2$	0	$0.44.\ 10^2$	$2.87.\ 10^4$	$2.8.\ 10^2$	$0.31.\ 10^6$

TAMF (Total Aerobic Mesophilic Flora); T. colif (Total coliforms); ***Staphy*** (*Staphylococci*) ***Strep*** (*Streptococci*); **Clost** (*Clostridium*); ***Lipo. Bact*** (*Lipolytic bacteria*); ***Pseudom spp*** (*Pseudomonas spp*); ***Bacil. Spp*** (*Bacillus spp*); **cfu** (colony-forming unit); **g** (gram)

The enumeration of total aerobic mesophilic flora (TAMF) in samples of Olive Mill Wastewater (OMW) reveals a significant TAMF count, with a total microbial load on the order of (5.94 ± 0.58) x 10^5 CFU/g. To check for potential fecal contamination by fecal bacteria, we conducted a search for total coliforms (TC) at a level of (0.17 ± 0.17) x 10^2 CFU/g, *staphylococci* with an almost negligible rate of (0.02 ± 0.8) x 10^2 CFU/g, an absence of total *streptococci*, and a very low presence of *Clostridium* at (0.44 ± 0.27) x 10^2 CFU/g. The total absence of these germs in OMW from the regions studied indicates that these effluents present no danger to the receiving environment. This absence is linked to the physico-chemical characteristics of OMW, which prevent their growth through the presence of antimicrobial substances (phenolic compounds, tannins) that inhibit and affect the activity of the extracellular enzymes they secrete. Almost the same results were obtained by [63-64]. However, several microorganisms manage to thrive, primarily lipolytic bacteria at (2.87 ± 0.75) x 10^4 CFU/g, *Pseudomonas spp.* at (2.8 ± 2.05) x 10^2 CFU/g, and *Bacillus* at (0.31 ± 0.24) x 10^6 CFU/g. Furthermore, our results have indicated a yeast count of 1.4 x 104 CFU/mL and a mold count on the order of 1.08 x 105 CFU/mL. These values are lower than those reported by authors [65], who state that yeast concentration can reach 106 CFU/mL according to [66]. The same authors estimate that mold concentration can reach 104 CFU/mL, while [67] suggests it could go up to 5 x 108 CFU/mL. Our flora is essentially composed of the genera *Aspergillus sp*., *Penicillium sp*. and

Alternaria sp. for molds. Yeasts are dominated by the *Rhodotorula sp*., *Candida sp*. and *Saccharomyces sp*.

3.3. Biotechnological study

4.3.1. Selection of strains with the ability to decolorize OMW

- *Growth of microorganisms in OMW-based liquid selective media*

Table 3 shows the variation in germ types, growth and decolorization of OMW in liquid selective media after treatment with inocula from 4 regions of Morocco.

After incubation at 30°C for one week, qualitative analysis by microscopic observation reveals that in all samples the microbial load consists essentially of bacteria, molds, and yeasts. Quantitatively, it is observed that for all inocula, the growth of germs is relatively more important in OMW diluted 1/5. It weakens as the OMW becomes more and more concentrated in the liquid selective medium. For inocula F_1, it stops in the raw OMW. The germs of the inocula (O_1, S_1, S_2) develop only in OMW diluted 1/5 and 1/3. It is also noted that the growth of germs of the inocula F_2, F_3, O_2, O_3, S_3, T_1, T_2, and T_3 took place in all selective liquid media based on diluted OMW and raw OMW. However, there are very significant differences between the samples from the studied regions. Indeed, the samples from the regions of Fez (F_1, F_2, F_3) and Taza (T_1, T_2, T_3) show an expressive growth on liquid selective medium based on OMW compared to those from the two other regions (Ouzzane and Settat).

Table 3. Variation in germ types, their growth, and Olive Mill Wastewater (OMW) decolorization in liquid selective media as a function of the inocula used (10^{-4} dilution)

Region	Inocula origin (10^{-4} dilution)	Controlled parameters	OMW-based liquid selective media			
			Diluted 1:5	Diluted 1:3	Diluted 1:2	Raw OMW
Fes (F)	**OMW stocked (F_1)**	germ types	B++, MS+, Y+	idem	idem	-
		germs growth	++	+	+	-
		OMW decolorization	++	+	-	-
	Pomace (F_2)	germ types	B++, M++, Y+	idem	idem	idem
		germs growth	+++	+++	++	+
		OMW decolorization	+++	++	+	+
	Soil (F_3)	germ types	B++, MM+, MS+, Y+	idem	idem	idem
		germs growth	++	++	+	+
		OMW decolorization	++	++	+	+
Ouezzane (O)	**OMW stocked (O_1)**	germ types	B+, MS+, Y+	idem	-	-
		germs growth	++	+	-	-
		OMW decolorization	-	-	-	-
	Pomace (O_2)	germ types	B++, M++, Y+	idem	idem	idem
		germs growth	+++	++	++	+
		OMW decolorization	++	++	+	+
	Soil (O_3)	germ types	B++, M+, MS+, Y+	idem	idem	idem
		germs growth	++	++	+	+
		OMW decolorization	++	+	+	+
Settat (S)	**OMW stocked (S_1)**	germ types	B++, MM+, MS+, Y+	idem	-	-
		germs growth	++	+	-	-
		OMW decolorization	+	-	-	-
	Pomace (S_2)	germ types	B+, MM+, MS+, Y+	idem	-	-
		germs growth	+	+	-	-
		OMW decolorization	+	-	-	-
	Soil (S_3)	germ types	B++, MM+, MS+, Y+	idem	idem	idem
		germs growth	++	++	+	+
		OMW decolorization	++	++	+	+
Taza (T)	**OMW stocked (T_1)**	germ types	B+, MM+, MS+, Y+	idem	idem	idem
		germs growth	++	++	+	+
		OMW decolorization	++	++	+	+
	Pomace (T_2)	germ types	B+, MM+, MS+, Y+	idem	idem	idem
		germs growth	++	++	+	+
		OMW decolorization	++	+	+	+
	Soil (T_3)	germ types	B+, M+, MS+, Y+	idem	idem	idem
		germs growth	+++	+++	++	+
		OMW decolorization	+++	++	+	+

Germ types: B (bacteria), M (mold), MM (mold mycelia), MS (mold spores), Y (yeasts), ++ (dominant),+ (present), - (no germs)
Germ growth: +++ (high), ++ (medium), + (low), - (no growth)
OMW decolorization: +++ (strong), ++ (moderate), + (slight), - (absent)

We also note that decolorization occurred in all samples where OMW is diluted to 1/2, 1/3 and 1/5. It increases as the OMW is more diluted and vice versa. In raw OMW : it is slight for inocula F_2, F_3, O_2, O_3, S_3, T_1, T_2, and T_3. It is absent for inocula F_1, O_1, S_1, and S_2. It is also observed that decolorization is relatively moderate to strong in samples from Fes and Taza regions, while it is slight or absent in samples from the Ouezzane and Settat regions.

- *Isolation and purification of strains on OMW-based solid media*

We will proceed with the selection and purification of potent strains possessing the ability to degrade polyphenols, which is visually evident through the decolorization of OMW. For this reason, we continued our research only with the inocula that caused even a slight discoloration of the raw OMW (inocula: F_2, F_3, O_2, O_3, S_3, T_1, T_2, T_3 as listed in Table 3). This reflects that they contain microorganisms retaining their capability, even if minimal, to assimilate and degrade polyphenols despite the relatively high concentration of these compounds in the raw OMW. In other words, these inocula are likely to contain the most effective microorganisms. The following Table 4 summarizes the obtained results.

Table 4. Variation in OMW decolorization around molds isolated on OMW-based solid selective media according to these germ types

Inocula source region		Code assigned to each mold	OMW-based solid selective media			
			diluted 1:5	diluted 1:3	diluted 1:2	raw OMW
Fes (F)	F_2	F_2M_1	++	+	+	+
		F_2M_2	+	+	-	-
		F_2M_3	+++	++	+	+
	F_3	F_3M_4	++	+	-	-
		F_3M_5	+++	++	+	+
Ouezzane (O)	O_2	O_2M_6	++	+	-	-
		O_2M_7	+	-	-	-
		O_2M_8	-	-	-	-
	O_3	O_3M_9	-	-	-	-
		O_3M_{10}	-	-	-	-
Settat (S)	S_3	S_3M_{11}	+	-	-	-
Taza (T)	T_1	T_1M_{12}	++	+	-	-
		T_1M_{13}	++	+	+	+
	T_2	T_2M_{14}	++	++	+	+
	T_3	T_3M_{15}	+++	++	++	+
		T_3M_{16}	+	-	-	-
		T_3M_{17}	+++	++	+	+

OMW decolorization: +++ (strong), ++: (moderate), + (slight), - (absent)

Given that we only considered the inocula that led to even slight decolorization of the raw OMW, the final result of the best-performing molds supplied to us by these four Moroccan regions is as follows: The Fes region (F) gave us three different molds: (F_2M_1), (F_2M_3), et (F_3M_5). The two regions Ouezzane (O) and Settat (S) did not provide any mold. As for the Taza region (T), it offered us four different molds (T_1M_{13}), (T_2M_{14}), (T_3M_{15}), and (T_3M_{17}). We could explain the growth of some molds on these solid selective media without decolorizing the OMW by the fact that these germs would take their source of Carbon and the energy necessary for their metabolic activities by catabolizing organic molecules other than polyphenols. Therefore, we have selected seven of the most efficient isolates that assimilate and degrade polyphenols and other toxic compounds in OMW: (F_2M_1), (F_2M_3), (F_3M_5), (T_1M_{13}), (T_2M_{14}), (T_3M_{15}), and (T_3M_{17}).

4.3.2. Treatment of liquid OMW from Taza with selected mold pre-cultures

At ambient temperature and without pH adjustment, we treated sterilized liquid Olive Mill Wastewater (OMW) from Taza with the combination of these 7 purified molds: (F_2M_1), (F_2M_3), (F_3M_5), (T_1M_{13}), (T_2M_{14}), (T_3M_{15}), and (T_3M_{17}). After 9 days, we obtained the results shown in Figure 1. On the other hand, the variations of some physico-chemical parameters of raw and 1/5 diluted OMW from this region before and after treatments are summarized in Table 5.

Table 5. Variation in physico-chemical parameters of the Taza OMW after 9 days of treatment

	Raw OMW		Diluted OMW to 1/5	
	Before	After	Before	After
pH	4.73	4.77	5.19	5.37
COD (g/L)	111.93	76.07	74.08	21.15
BOD5 (g/L)	53.16	35.56	40.48	12.18
Polyphenols (g/L)	10.41	6.85	7.67	1.21

As shown in Figure 2 below, we observe that decolorization of the Olive Mill Wastewater (OMW) has occurred in all samples, from raw OMW to those diluted by 1/5. It intensified proportionally with increasing OMW dilution and vice versa.

Fig. 2. Decolorization of Taza's OMW after nine days of treatment with a combinaison of seven different pure molds selected from four regions of Morocco

We can explain these results by the analytical data shown in Table 5 above. First, we note that untreated raw OMW has an acidic pH of 4.73, which falls within the pH range from (3 to 6) [20]. In fact, this acidity hinders regular microbial activity, impeding effective biological treatment [68]. The acidic pH of raw OMW acts as an inhibitor of the activity of these molds, resulting in minimal degradation of phenolic compounds. This is visually reflected in minimal decolorization. On the other hand, OMW diluted to 1/5 has a pH close to neutrality (pH = 5.19), which leads to a partial reduction of its inhibitory effect on the activity of these molds. Indeed, the acidic pH (equal to 4.73) of the raw OMW slows down the activity of the treatment molds which have weakly degraded the phenolic compounds, hence a less significant phenolic reduction, which is equal to 34.19%. And when the pH tends towards neutrality in the OMW diluted to 1/5 (pH equal to 5.19), the molds become more and more active. This leads to an ever greater phenolic reduction (84.22%), which is expressed by a more apparent decolorization of the OMW. Raw OMW has COD and BOD_5 values of 111.93 g/l and 53.16 g/l, respectively. These values are within the range of (40g/L to 200g/L) for COD, and (23g/L to 100g/L) for BOD_5 [55], which indicates that Taza OMW are moderately loaded with organic pollutants. the biodegradability index Ib (COD/BOD_5) that we previously defined is equal to 2.10. This indicates that the polluting organic load of the OMW of Taza are highly biodegradable. This is also supported by the significant reduction values of COD (71.44%) observed in OMW diluted to 1/5, which also appears more discolored after treatment. On the other hand, the reduction rate of COD of the treated raw OMW is only 32.03%, which results in less decolorization. This result could be explained by the effect of pH. Indeed, since it is acidic (equal to 4.73), it has partially inhibited the activity of these seven different pure molds. Thus, they degraded less organic matter, especially the polyphenols responsible for the characteristic color of these OMW. Also, the 69.91% BOD5 removal indicates that a good proportion of organic pollutants were degraded by these seven different pure molds. Our treatment by mixed culture shows a 71.44% reduction of COD for OMW diluted to 1/5. Compared to other aerobic biological treatments, the result we obtained is almost identical to those reported by [69], who obtained by treatment with pure monoculture 63% reduction using the strain *Coriolus versicolor*, 70% with *Funalia troggi*, and 65.77% with the mold *Aspergillus terreus*. And the polyphenol reduction that we obtained (84.22%) is almost identical to that of [70] who achieved a reduction of 83% using the mold *Phanerochaete chrysosporium*. It exceeds the 78.99% phenolic reduction that we obtained with a marine white mold, recently discovered at the Mehdia marine coast near the city of Kenitra in Morocco. This mold discolored the OMW at pH 5.13 [36]. It also exceeds the 76.12% phenolic reduction obtained by [71] who treated the OMW at a pH of 3 with an enzyme called laccase. This is an extracellular lignolytic enzyme from a white mold called *Trametes versicolor*, which they produced by fermentation.

4 Conclusion

Characterized by a high pollution load, OMWs represent a major environmental problem, as their toxicity affects the fauna and flora of terrestrial and aquatic environments, with major risks of harm to human health. Under aerobic conditions, at room temperature, and without pH adjustment, we biologically treated these liquid discharges with a culture composed of seven different pure molds. The biological treatment we carried out proved to be a promising solution for depolluting these effluents. Indeed, the results obtained, namely the decoloration of OMW and the significant reduction in COD, BOD5 and polyphenols, demonstrate the effectiveness of this approach. Above all, the absence of pH adjustment and the fact that the treatment is carried out at room temperature offer additional advantages in terms of simplicity and cost. Our study represents an important step forward in the search for sustainable solutions for OMW management, while contributing to environmental protection and the preservation of human health. In addition to its environmental effectiveness, this approach could also bring economic benefits by valorizing these OMWs into bioproducts for fertigation in agriculture. On the other hand, exploiting this potential would contribute to the creation of a circular and sustainable economy in the olive industry. In this respect, further studies are needed to optimize the process and assess its applicability on a large scale. In conclusion, the use of pure mold combinations for the aerobic biological treatment of OMW is a promising avenue that deserves to be explored in greater depth.

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