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Optimization of the process conditions for biodegradation of phenolic compounds in olive mill wastewater using *Bacillus subtilis* in bioreactor cultures

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ABSTRACT

Olive mill wastewater (OMWW) poses serious environmental problems due to the presence of high phenolic compounds (PC) content. The search for new strains able to degrade pollutants in OMWW and withstand the toxic effects of the high PC concentrations is of great scientific and industrial interest. In this study, an isolated bacterium identified as *Bacillus subtilis* was selected based on its ability to grow on minimum synthetic medium based media using PC as sole sources of carbon and energy and to degrade the OMWW. The treatment conditions were optimized and included the following parameters: OMWW dilution with the addition of different nitrogen sources, shaking and incubation temperatures. The maximum abatement rate of PC and chemical oxygen demand was 98% and 97%, respectively. This result was obtained at 25% dilution of OMWW with 1 mL inoculums size in a bioreactor with150 rpm, at 37°C with the addition of the degradation media with 1 g/L yeast extract and 3 g/L urea. High-performance liquid chromatography analysis of extracts from treated OMWW showed the evolution of PC content during the degradation process and indicated the high reduction in phenolic compound concentrations.

Keywords: Olive mill wastewater; Biological treatment; *Bacillus subtilis*; Phenolic compounds; Bioremediation; Optimization process

1. Introduction

Olive-growing is one of the oldest agricultural activities in the Mediterranean basin. For these countries, the production of olive oil is a wealthy business transmitted over several generations [1,2]. Currently, the olive industry produces virgin olive oil as food with different virtues for human well-being. This olive oil is the subject of developing interest, especially on account of the results of scientific research which affirms its advantages and its basic place in the Mediterranean eating regimen [3,4]. This interest is reflected by a change in the structure of production and a more noteworthy challenge between the maker countries.

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This expansion of the olive oil production and the introduction of modern techniques for the extraction of oil have put the industry in a fragile position of a potential polluter, specifically with the advent of the continuous systems for crushing with three phases using a significant amount of water [5].

The olive mill wastewater (OMWW) is extremely loaded with organic materials particularly in phenolic compounds that apply an exceptionally high polluting action [6]. In Morocco, the OMWW discharges do not undergo treatment and are frequently spilled in nature [7]. It results in a negative impact on the environment which is reflected by the clogging of the soil, the pollution of surface waters, and groundwater and the release of bad odors [8]. This negative impact on the environment is explained by their high load in toxic organic material (particularly the phenolic compounds which have little biodegradable properties), their high chemical oxygen demand (COD) (up to 320 g/L) and biochemical oxygen demand (more than 132 g/L), their strong saline load, and their acid pH [6].

Up to now, the OMWW treatment is a complex problem for the quantity and the quality of the chemical substances they contain. Due to the pollutant load of these effluents and their potential environmental impacts, OMWW ought to be treated before being discharged. It is important to eliminate or reduce the phenolic components and COD in the OMWW. In effect, the application of a simple treatment proves to be inadequate and incomplete [9,10]. Despite the fact that there is not yet an ideal solution permitting the OMWW treatment, some processes appear to be more effective than others [11].

The search for alternative methods or complementary treatments led to the appearance of new technologies, which incorporate the utilization of microorganisms. The aim of this investigation is concentrated on the performance of a pure culture of *Bacillus subtilis* (*B. subtilis*) that can degrade the OMWW phenolic compounds (PC) and decrease its COD. This strain was selected to examine its biodegradation activity under different conditions; such as diluted OMWW, shaking, and various incubation temperatures and with or without the addition of nitrogen sources.

2. Materials and methods

2.1. Origin and physicochemical characterization

The OMWW composition and main physicochemical characterization were previously assessed, OMWW used in this work was obtained from an olive oil production plant in Chichaoua (Marrakech, Morocco), which uses a three-phase centrifugation process for the extraction of olive oil. The samples were stored at –20°C until required for analysis. The physicochemical characteristics of the raw OMWW used in this study are shown in Table 1 [7].

Table 1
Physicochemical characteristics of untreated olive mill wastewater [7]

Parameters	Unit	OMWW
pH		4.80 ± 0.04
EC	(mS/cm)	13.9 ± 0.1
TSS	(g/L)	10.0 ± 0.3
TS	(g/L)	122.90 ± 2.62
AshC	(g/L)	40.7 ± 1.0
COD	(g/L)	187.6 ± 19.1
TOC	(g/L)	73.26 ± 1.80
TKN	(g/L)	0.160 ± 0.001
Proteins	(g/L)	1.00 ± 0.02
Lipids	(g/L)	4.51 ± 0.40
Sugars	(g/L)	21.45 ± 0.40
PC	(g/L)	4.3 ± 0.1

Values are the average of three measurements ± standard error.
EC, electrical conductivity; TSS, total suspended solids; TS, total solids; AshC, ash contents; COD, chemical oxygen demand; TOC, total organic compounds; TKN, total Kjeldahl nitrogen; PC, phenolic compounds; OMWW, olive mill wastewater.

2.2. Isolation, selection and identification of bacterial strain

2.2.1. Isolation of a bacterial strain

The bacteria have been isolated from different biotopes, such as sludge from OMWW evaporating ponds, soil irrigated with OMWW. The samples were collected from 8–10 cm profundity using a sterile spatula and transferred to pre-autoclaved sterile glass bottles with rubber stoppers. The samples were brought to the laboratory and stored under refrigeration temperature. 1 g of each sample was suspended in 9 mL sterilized physiological water. The suspension was incubated at room temperature, 150 rpm for 2 h, and then a series of dilutions were prepared. For each dilution, 0.1 mL was seeded on nutrient agar containing an antifungal to inhibit the growth of molds and yeasts. The Petri dishes were incubated at 37°C for 24 h. The colonies with distinct morphological parameters such as the color, shape, and size were isolated and purified. The purified isolates were stored at 4°C.

2.2.2. Screening of bacteria

Bacteria isolates were tested on different dilutions of OMWW (25%, 50%, 75%, and raw OMWW) for their ability to use the effluent as a source of carbon and energy. Each dilution was supplemented with 15 g of agar-agar/L, then sterilized at 120°C for 20 min. The medium is poured into sterile Petri dishes [7]. After solidification, the strains were seeded and incubated at 37°C for 24–48 h. The colonies cultivating this medium based on OMWW have been purified. This screening was followed by a culture of these strains on a liquid medium composed sterilized OMWW, to ensure that the OMWW was used as a carbon and energy source for the growth of these strains.

2.2.3. Test of use of phenolic compounds as a carbon source

The ability of the isolates to develop on phenolic compounds as a sole source of carbon was tested on a minimal medium (2 g/L sodium nitrate, 1 g/L potassium phosphate, and 0.5 g/L magnesium sulfate) with different concentrations of phenolic compounds (1,000; 500; and 250 mg/L) extracts from the OMWW [7].

2.2.4. Amplification and sequencing of the 16S rDNA of the selected strain

The strain isolated in this work was developed on Luria-Bertani medium as standard cultures of *Bacillus* sp. The isolate was incubated in filled flasks with rubber stoppers and shaking at 100 rpm. Inoculation was performed aerobically with aerobically grown overnight culture with an OD578 of 0.3. Anaerobic conditions were accomplished after a short time through the consumption of residual oxygen by the inoculated bacteria. The cells for preparations of RNA were harvested after 3 h amidst the exponential growth phase.

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using standard protocols [12] with forwarding primer 16F27 (5′-AGAGTTTGATCATGGCTCAG-3′) and reverse primer 16R1488 (5′-CGGTTACCTTGTTAGGACT TCACC-3′) (both from Pharmacia, Amersham Pharmacia Biotech, Inc.). The PCR products were purified using the Microcon QIAquick spin-gel extraction kit (Qiagen, Germantown, MD, USA). Direct sequence determinations of PCR-amplified DNAs were made with an ABI PRISM dye terminator, cycle sequencing ready-reaction kit (Perkin-Elmer, USA) and an ABI PRISM 377 sequencer (Perkin-Elmer, USA) according to the manufacturer's instructions. The resulting DNA sequences were compared to reference 16S rRNA gene sequences available in the GenBank and European Molecular Biology Laboratory databases from the National Centre of Biotechnology Information (USA) database using the Basic Local Alignment Search Tool search. Phylogenetic analyses were made using MEGA version 2.1 [13] after multiple alignments of the data by CLUSTAL X [14]. Distances and clustering were determined using the neighbor-joining and maximum parsimony algorithms. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications).

2.3. Biological treatment and factors affecting the biodegradation of olive oil mill wastewater

The biodegradation was done in mini bioreactors of 500 mL, which contained 100 mL of OMWW. To get the optimum conditions, different factors were considered. The dilutions of OMWW 25%, 50%, 75%, and raw OMWW (v/v) were gotten by using sterile distilled water with the shaking of 100 and 150 rpm. Three ranges of temperatures 25°C, 37°C, and 45°C were tested for incubation. Different nitrogen sources (ammonium nitrate, urea, and ammonium sulfate) were tested. 1 mL of the suspension of the selected bacteria, in the exponential phase, was used as inoculum. The COD and the decomposition of phenolic compounds were followed during treatment. The samples were taken every 3 d for analysis during the one month of treatment. Control experiments were done in the same condition without inoculation either the supplement nitrogen source.

3. Results and discussion

3.1. Selection of the most potent phenolic compounds degrading microorganisms

In this study, five isolates were selected as potential phenolic compounds degrading microorganisms. Table 2 presents the results of the bacteria tolerance test indicating that isolate B1 can develop better on liquid and solid media based on OMWW diluted up to 25% given their tolerance to high loads of phenolic compounds. However, the B1 isolate can grow better on the MSM based on liquid and solid phenolic compounds extract up to 1,000 mg/L (Table 3). The results suggest that isolate B1 can use phenolic compounds as the sole source of carbon, which justified its use for the bioremediation of OMWW.

Table 2
Tolerance results of bacteria strain on the solid and liquid medium based on olive mill wastewater with different dilutions

	Olive mill wastewater							
	Solid				Liquid			
	100%	75%	50%	25%	100%	75%	50%	25%
B1	–	–	–	+	–	–	–	+
B2	–	–	–	+	–	–	–	–
B3	–	–	–	–	–	–	–	–
B4	–	–	–	–	–	–	–	–
B5	–	–	–	–	–	–	–	–

B = bacteria

3.2. Identification of the selected isolate

Comparisons of the 16S rRNA gene sequences of the selected strain to those available in the GenBank database indicated that it is phylogenetically closely related to *B. subtilis* (98% sequence homology), *Bacillus mojavensis* (97% sequence homology), *Bacillus malacitensis* (97% sequence homology) and *Bacillus axarquiensis* (96% sequence homology). The phylogenetic tree, constructed using the neighbor-joining method, is depicted in Fig. 1.

3.3. Factors affecting the biodegradation of OMWW by selected bacteria B. subtilis

3.3.1. Shaking and static conditions

The results in Fig. 2 indicated that the shaking condition accelerated the biodegradation process of OMWW in comparison with the static condition. The maximum degraded phenolic compounds (75%) and decreased COD (81%) were at 150 rpm, at 25% dilution of OMWW, while these values do not exceed 60% and 68% for PC and COD, respectively, in static condition. The 150 rpm shaking rate was the optimal condition for achieving the higher degradation of phenolic compounds by *B. subtilis*. This result could be attributed to increased amounts of dissolved O_2 as a result of shaking [15].

3.3.2. Incubation temperature

From Fig. 3 it could be seen that the percentage of phenolic compounds and COD reduction is influenced by temperature. The optimum incubation temperature for *B. subtilis* was 37°C with phenolic compounds removal of (90%) and COD reduction of about (89%). While increasing

Table 3
Results of the growth of bacteria on solid and liquid mineral salt medium (MSM) based on a phenolic extract

	MSM (mg/L)									
	Solid					Liquid				
	1,000	500	250	125	C	1,000	500	250	125	C
B1	+	+	+	+	–	+	+	+	+	–
B2	–	–	–	+	–	–	–	–	+	–
B3	–	–	–	+	–	–	–	–	+	–
B4	–	–	–	–	–	–	–	–	–	–
B5	–	–	–	+	–	–	–	–	+	–

B = bacteria, C = control

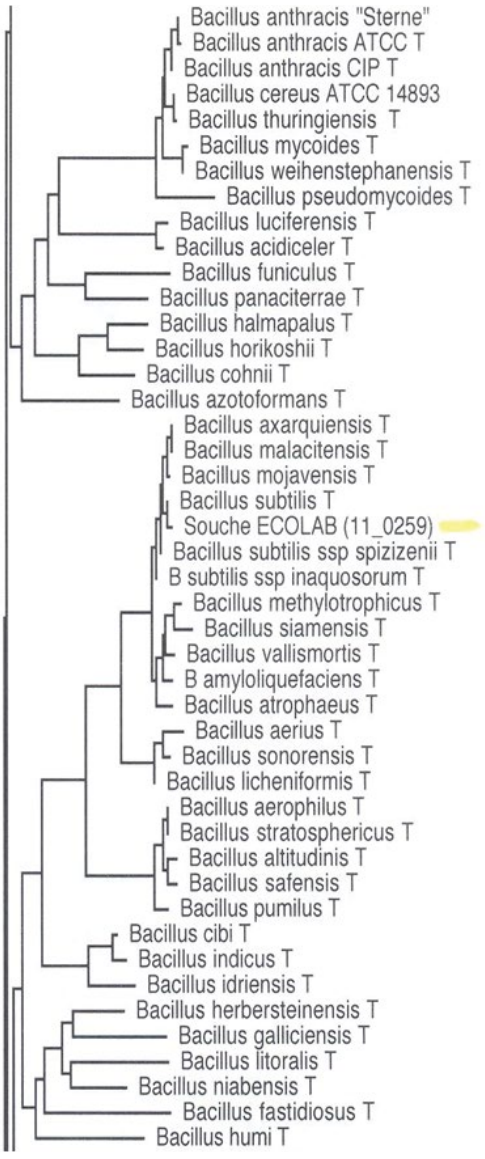

Fig. 1. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences of the selected isolate. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Bar, 1 nt substitutions per 100 nt.

or decreasing the temperature causes an inhibition in the PC removal by 75% and 65% and COD degradation by 80% and 74% at both 30°C and 45°C, respectively. Accordingly, *B. subtilis* could be specified as a thermotolerant. Hassimi et al. [16] reported that *B. subtilis* are thermotolerant and can grow inside a temperature range of 20°C to 50°C. The incubation temperature of 35°C to 37°C is the optimal conditions for achieving a higher percentage of phenol degradation by *Actinobacillus* sp. [15]. *Pseudomonas aeruginosa* growth and phenol degradation rate increased with the increase in temperature of up to 37°C, but above 37°C, it decreased [17].

3.3.3. Effect of nitrogen supplements on biodegradation of OMWW by selected bacteria B. subtilis

The effect of nitrogen sources on the biodegradation of OMWW was investigated for the concentrations of 25% (Fig. 4). The results show a reduction of the pollution load during treatment by *B. subtilis* of more than 89% of COD during one month of treatment without nitrogen sources. While this rate achieved 97% when the carbon/nitrogen (C/N) ratio is adjusted around 30 by adding urea as a nitrogen source, 150 rpm shaking and 37°C temperature, in only 15 d of treatment (Fig. 4a). However, the percentage reduction remains around zero for the uninoculated control. Indeed, there has been noted a high level of reduction during the first days, the concentration of COD has decreased to reach 44%, due to the biodegradation of organic matter such as using compounds that are easily metabolizable by *B. subtilis* including sugars, proteins, and simple phenolics. These results can be compared to those of Fakharedine et al. [18] where the phenolic compounds have been reduced by 90% by microorganisms in the soil by an aerobic bioprocess. In another study conducted by Tziotzios et al. [19], a COD reduction of the order of 82%–90% was obtained, during 30 d of treatment, in reactors by *Alcaligenes* and *Acinetobacter* isolated from the pulp of olive. In effect, Ait Baddi et al. [20] explained this reduction where the decomposition of the organic matter is changing as follows: the reduction begins by the hydrolysis of proteins, the urea and the hemicellulose freeing soluble ammonia, amino acids, sugars and aliphatic acids. As the progress of decomposition, lignin and cellulose are degraded. The sugars, the aliphatic compounds and certain phenolic compounds are used to synthesize the microbiological safety of the biomass. In a recent study, Maza-Márquez et al. [21] have cultivated many bacteria isolated from OMWW, in order to select for strains having the capacity to reduce the pollutants of the effluent. The strains *Raoultella terrigena* and *Pantoea agglomerans* were selected and used for the biological treatment of OMWW.

According to the results reported in Fig. 4b, there was a reduction of phenolic compounds after the inoculation of the OMWW (25%) by *B. subtilis*. After a latency time of nearly 3 d, this time is devoted to the adaptation of the bacterium and to acquire the enzyme arsenal necessary for the degradation of phenolic compounds, the biodegradation reached a rate of 90% after a month of treatment without nitrogen sources. Whereas when the bacterium is grown in the presence of different sources of nitrogen, to test the effect of nitrogen on the performance of biodegradation and to adjust the report C/N around 30, there is a strong reduction

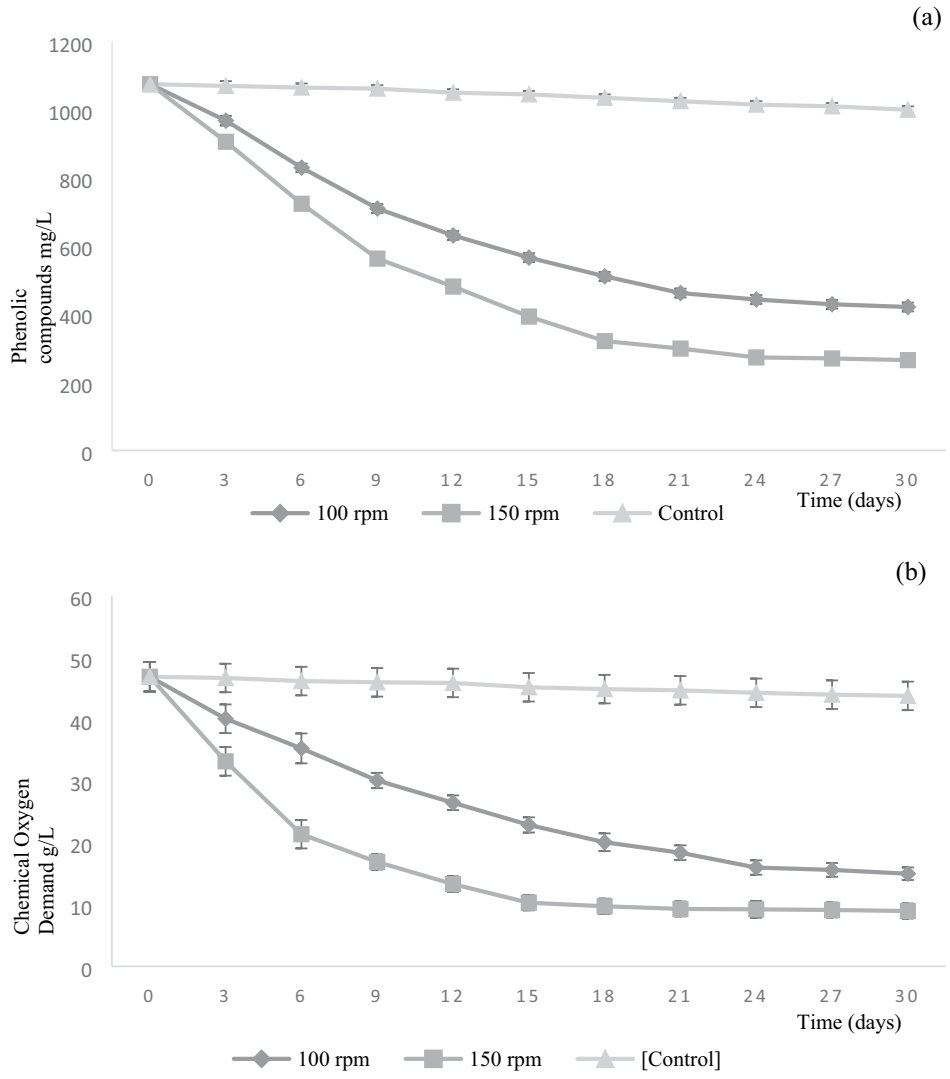

Fig. 2. Effect of different shaking rates on the olive mill wastewater phenolic compounds biodegradation (a) and chemical oxygen demand removal (b) by *B. subtilis*.

of phenolic compounds which reaches 98%, after only 15 d of treatment.

In effect, El Hajjouji et al. [8] have found that a C/N ratio which tends toward a value of 30, there is a significant reduction of phenolic compounds, with an allowance which reached 76% in the treatment where the C/N ratio adjusted with ammonium nitrate.

Several bacterial strains, in particular those of the genus *Azotobacter*, *Pseudomonas*, *Klebsiella* and *Bacillus* are able to tolerate and even degrade the phenolic compounds [22]. Mollaei et al. [23] used *Pseudomonas* spp., for the degradation of phenols, a degradation of the initial concentrations of 500 and 700 mg/L is complete after 20 and 30 h, respectively, indicating the good acclimation of the bacteria to the source of phenol. Yet, when the initial concentration is greater than 1,000 mg/L, the complete degradation requires 84 h. Whereas, for 1,200 mg/L of the strain is not able to completely degrade the phenols even after 8 d of incubation.

Recently, Banerjee and Ghoshal [24] have studied the degradation of phenolic compounds by two strains of *Bacillus cereus* MTCC 9818 AKG1 and *Bacillus cereus* MTCC 9818 AKG2. These two strains can degrade high concentrations of phenolic compounds of up to 2,000 mg/L. Treatment with *Pleurotus sajor-caju* or *Trametes versicolor* was able to remove between 8% and 76% and between 22% and 74% of phenolic compounds, respectively [25].

3.4. High-performance liquid chromatography profile of the phenolic compounds content of OMWW

The identification of phenolic compounds has been completed by correlation of their retention times with those of standards (Fig. 5). The examination of the high-performance liquid chromatography (HPLC) of the OMWW phenolic extract shows that effluent made up of 5 compounds most of which absorb at 280 nm. Hydroxytyrosol, tyrosol,

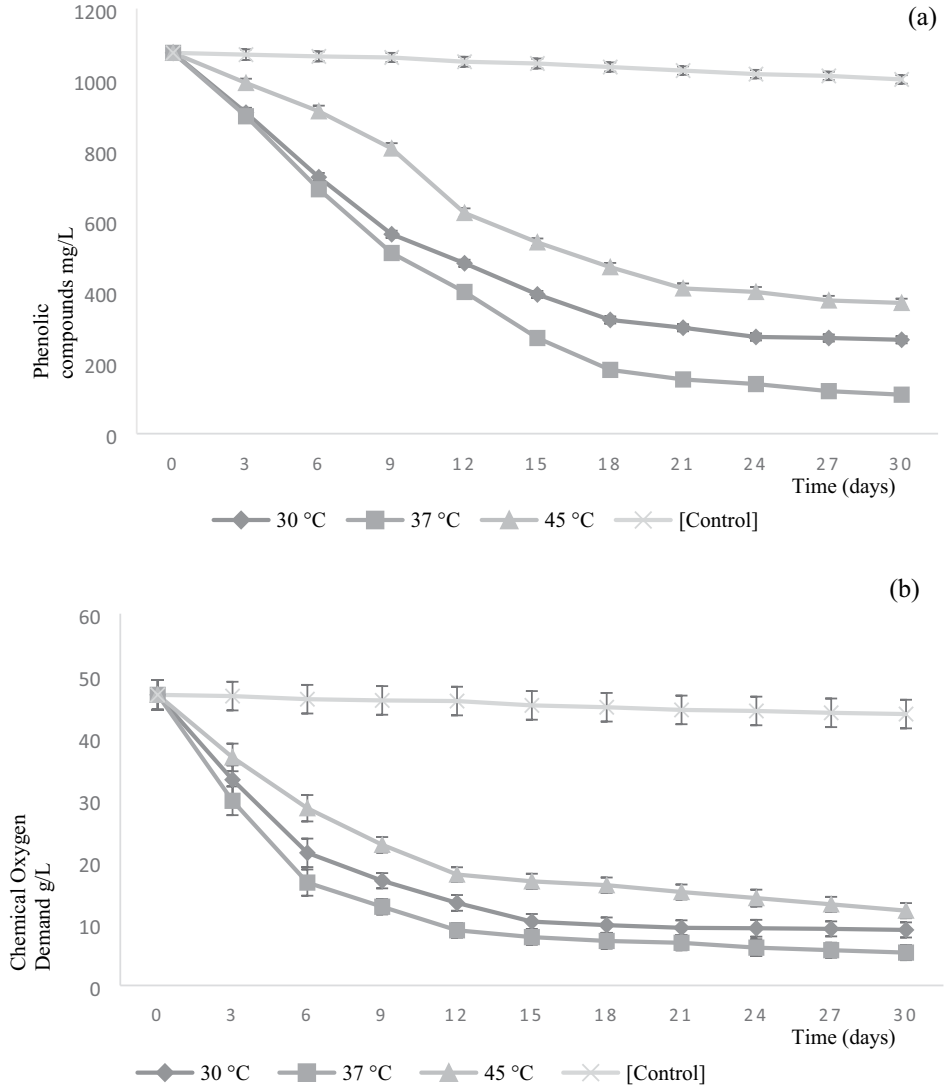

Fig. 3. Effect of different incubation temperatures on the olive mill wastewater phenolic compounds biodegradation (a) and chemical oxygen demand removal (b) by *Bacillus subtilis*.

p-coumaric acid, caffeic acid, and oleuropein. The identified phenolic compounds have already been reported by Fakharedine et al. [26] in the effluent from a crushing unit located in Marrakech.

In effect, the list of phenolic compounds identified in extracts of the OMWW has never been exhaustive; it varies from one area to the other. The results of this investigation show that the hydroxytyrosol is the major compound among the PC present in the effluent, it represents approximately 80% of total phenolic compounds. In the study of De Marco et al. [27], the HPLC analysis has shown that hydroxytyrosol is the most plentiful phenolic compounds in the OMWW. The phenolic fraction was found to be rich in hydroxytyrosol and derived secoïridoïdes and is characterized by a high degree of complexity [27–30].

All the compounds identified in the crude extract disappeared after 30 d of treatment except the compound hydroxytyrosol, which persists with a low concentration in the order of 33% (Fig. 6). This can be explained by the biodegradation of this phenol by the selected strain as well as by the bioconversion of the oleuropein in hydroxytyrosol [31].

4. Conclusion

The present study has focused on the optimization of the conditions such as temperature, cell culture shaking and nitrogen source during biodegradation of OMWW by isolated strain *B. subtilis*. The results show a high degree, of about 98%, 97% of PC and COD abatement, respectively, at 37°C, 150 rpm and C/N ratio around 30. The HPLC investigation demonstrates that tyrosol, *p*-coumaric acid, caffeic acid and oleuropein have disappeared with a conversion of oleuropein to hydroxytyrosol phenomenon. The overall results demonstrate that the isolated strain *B. subtilis* has able to grow on OMWW and to degrade and dephenolize several types of phenolic compounds present in the starting material.

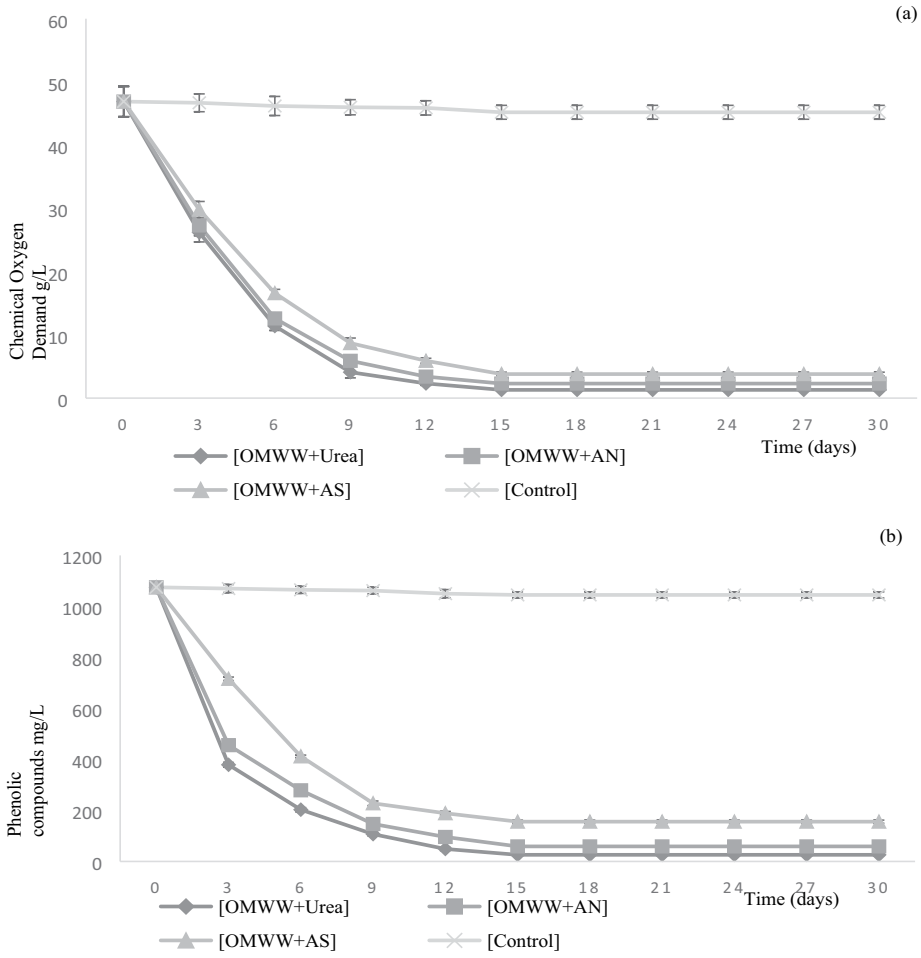

Fig. 4. Effect of different nitrogen sources on the olive mill wastewater chemical oxygen demand removal (a) and phenolic compounds biodegradation (b) by *Bacillus subtilis*. (Urea; AS: ammonium sulfate; AN: ammonium nitrate)

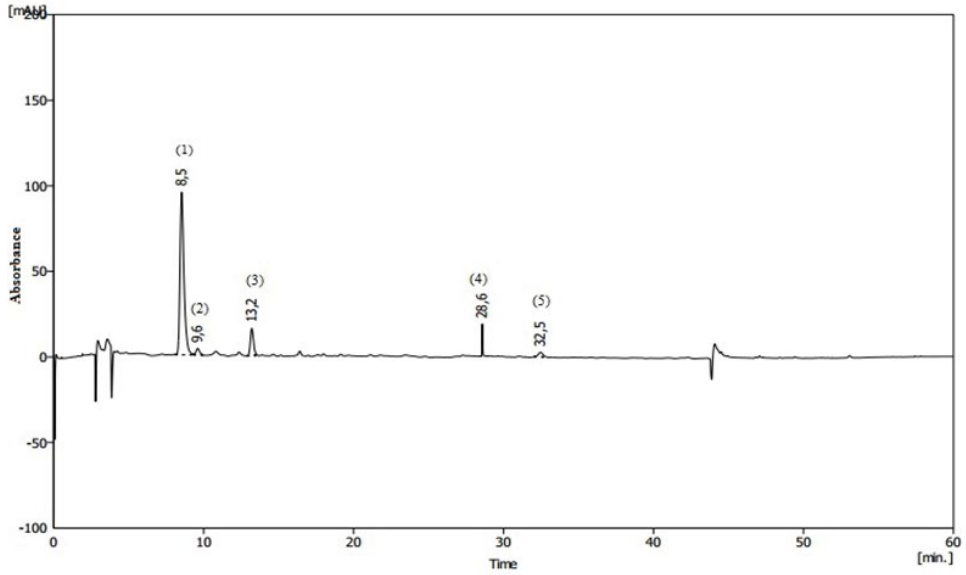

Fig. 5. HPLC chromatogram of the phenolic extract: (1) hydroxytyrosol, (2) tyrosol, (3) caffeic acid, (4) *p*-coumaric acid, and (5) oleuropein.

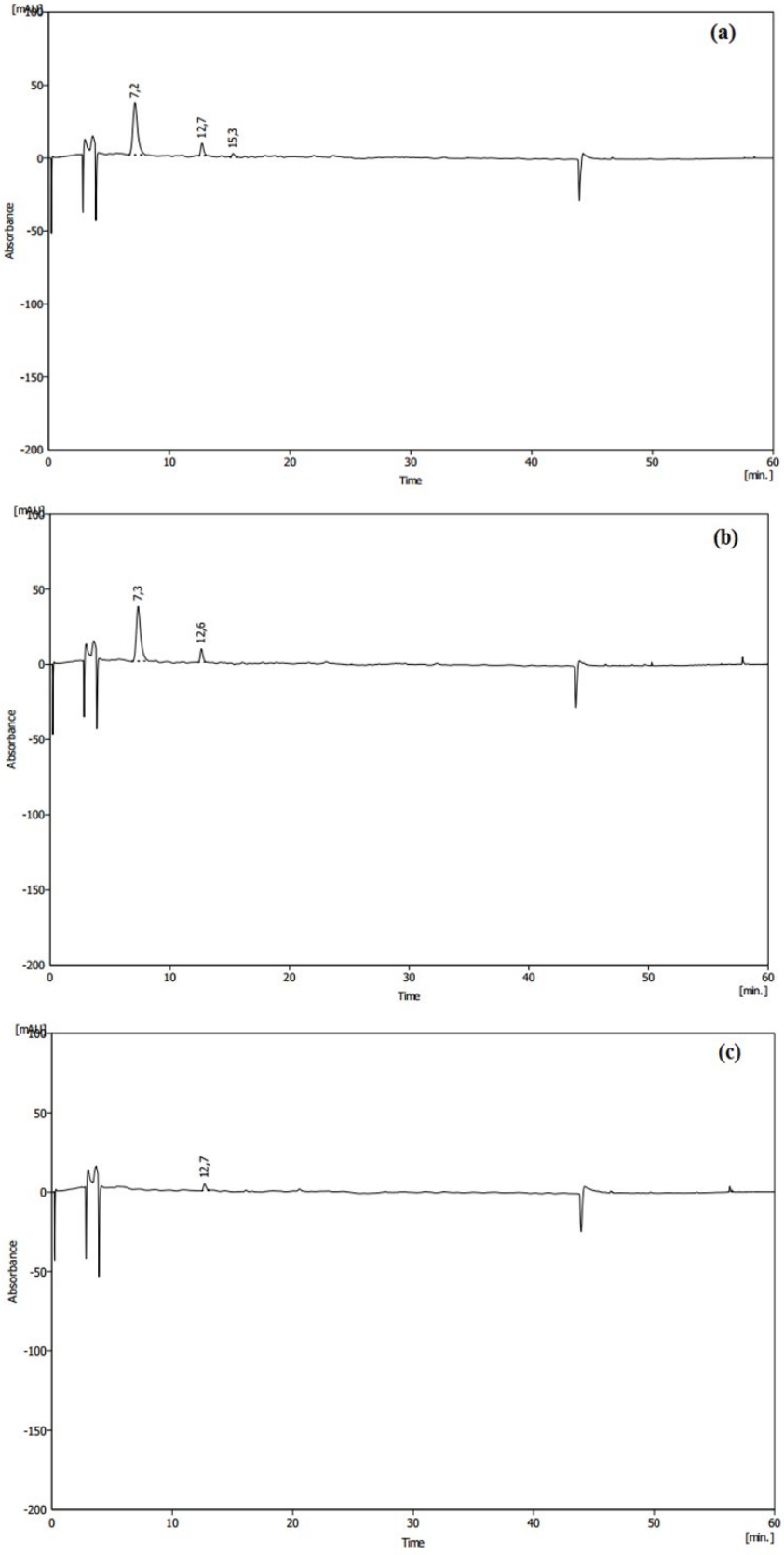

Fig. 6. HPLC chromatogram of phenolic compounds extracted from olive mill wastewater after treatment, (a) shaking (150 rpm), (b) incubation temperatures (37°C), and (c) nitrogen sources (urea).

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References

[1] A. Jail, F. Boukhoubza, A. Nejmeddine, J.C. Duarte, S. Sayadi, L. Hassani, Treatment of olive mill wastewater by a process combining an intensive treatment (Jet-Loop Reactor) followed by an extensive treatment (stabilization ponds), Environ. Technol., 31 (2010) 37–41.
[2] N. Meksi, W. Haddar, S. Hammami, M.F. Mhenni, Olive mill wastewater: a potential source of natural dyes for textile dyeing, Ind. Crops Prod., 40 (2012) 103–109.
[3] B. Jean-louis, The world olive oil economy, OCL, 21 (2014) 1–4.
[4] R. Ghanbari, F. Anwar, K.M. Alkharfy, A.H. Gilani, N. Saari, Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.)-a review, Int. J. Mol. Sci., 13 (2012) 3291–3340.
[5] H. Zbakh, A. El Abbassi, Potential use of olive mill wastewater in the preparation of functional beverages: a review, J. Funct. Foods, 4 (2012) 53–65.
[6] S. Dermeche, M. Nadour, C. Larroche, F. Moulti-Mati, P. Michaud, Olive mill wastes: biochemical characterizations and valorization strategies, Process Biochem., 48 (2013) 1532–1552.
[7] A. Boutafda, Y. Zegzouti, L. El Fels, Y. Ouhdouch, A. Lebrihi, F. Bekkaoui, M. Hafidi, Olive mill wastewater biological assessment: optimization and identification of compounds degrading yeasts, Desal. Water Treat., 149 (2019) 129–137.
[8] H. El Hajjouji, G. Ait Baddi, A. Yaacoubi, H. Hamdi, P. Winterton, J.C. Revel, M. Hafidi, Optimisation of biodegradation conditions for the treatment of olive mill wastewater, Bioresour. Technol., 99 (2008) 5505–5510.
[9] E. Ouabou, A. Anouar, S. Hilali, The treatment of olive oil mill wastewater by distillation followed by neutralization with lime, J. Appl. Biosci., 79 (2014) 6867–6872.
[10] G. Sbai, M. Loukili, Olive oil mill wastewater treatment by a process coupling electrochemical coagulation, Eur. Sci. J., 11 (2015) 158–169.
[11] L. El Fels, A. Boutafda, Y. Zegzouti, N. Laghmiri, M. Neffa, L. Kouisni, M. Taourirt, M. Hafidi, Fenton oxidation using $Fe^{2+}/Fe^{3+}/H_2O_2$ to improve the DCO removal and to degrade the phenolic compounds in olive oil mill wastewater, Desal. Water Treat., 152 (2019) 252–260.
[12] R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, H.A. Erlich, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, Science, 239 (1988) 487–491.
[13] S. Kumar, K. Tamura, M. Nei, MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment, Brief Bioinform., 5 (2004) 150–163.
[14] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res., 25 (1997) 4876–4882.
[15] K.M. Khleifat, Biodegradation of phenol by *Actinobacillus* sp.: mathematical interpretation and effect of some growth conditions, Biorem. J., 11 (2007) 103–112.
[16] M.S. Hassimi, H. Hamdali, Y. Ouhdouch, E. Pinelli, G. Merlina, R.J. Claude, M. Hafidi, Moroccan rock phosphate solubilization during a thermo-anaerobic grassland waste biodegradation process, Afr. J. Biotechnol., 12 (2013) 6859–6865.
[17] S.E. Agarry, T.O.K. Audu, B.O. Solomon, Optimisation of process variables for microbial degradation of phenol by binary mixed culture of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* using response surface methodology, Int. J. Environ. Pollut., 43 (2010) 161–176.
[18] N. Fakharedine, H. El Hajjouji, G. Ait Baddi, J.C. Revel, M. Hafidi, Chemical and spectroscopic analysis of organic matter transformation during aerobic digestion of olive-mill waste-waters, Process Biochem., 41 (2006) 398–404.
[19] G. Tziotzios, S. Michailakis, D.V. Vayenas, Aerobic biological treatment of olive mill wastewater by olive pulp bacteria, Int. Biodeterior. Biodegrad., 60 (2007) 209–214.
[20] G. Ait Baddi, J.A. Alburquerque, J. Gonzálvez, J. Cegarra, M. Hafidi, Chemical and spectroscopic analyses of organic matter transformations during composting of olive mill wastes, Int. Biodeterior. Biodegrad., 54 (2004) 39–44.
[21] P. Maza-Márquez, M.V. Martínez-Toledo, J. González-López, B. Rodelas, B. Juárez-Jiménez, M. Fenice, Biodegradation of olive washing wastewater pollutants by highly efficient phenol-degrading strains selected from adapted bacterial community, Int. Biodeterior. Biodegrad., 82 (2013) 192–198.
[22] Y. Rharrabti, M. EI Yamani, Olive Mill Wastewater: Treatment and Valorization Technologies, C.M. Hussain, Ed., Handbook of Environmental Materials Management, Springer International Publishing AG, 2018, pp. 1–28. Available at: https://doi.org/10.1007/978-3-319-58538-3_91-1.
[23] M. Mollaei, S. Abdollahpour, S. Atashgahi, H. Abbasi, F. Masoomi, I. Rad, A.S. Lotfi, H.S. Zahiri, H. Vali, K.A. Noghabi, Enhanced phenol degradation by *Pseudomonas* sp. SA01: gaining insight into the novel single and hybrid immobilizations, J. Hazard. Mater., 175 (2010) 284–292.
[24] A. Banerjee, A.K. Ghoshal, Phenol degradation by *Bacillus cereus*: pathway and kinetic modeling, Bioresour. Technol., 101 (2010) 5501–5507.
[25] C. Justino, A.G. Marques, K.R. Duarte, A.C. Duarte, R. Pereira, T. Rocha-Santos, A.C. Freitas, Degradation of phenols in olive oil mill wastewater by biological, enzymatic, and photo-Fenton oxidation, Environ. Sci. Pollut. Res., 17 (2010) 650–656.
[26] N. Fakharedine, M. Ouadghiri, M. Amar, P. Winterton, M. Hafidi, Y. Ouhdouch, Isolation and identification of a yeast strain involved in the degradation of Marrakech olive mill wastewater, EurAsian J. Biosci., 137 (2011) 127–137.
[27] E. De Marco, M. Savarese, A. Paduano, R. Sacchi, Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters, Food Chem., 104 (2007) 858–867.
[28] H.K. Obied, D.R. Bedgood Jr., P.D. Prenzler, K. Robards, Bioscreening of Australian olive mill waste extracts: biophenol content, antioxidant, antimicrobial and molluscicidal activities, Food Chem. Toxicol., 45 (2007) 1238–1248.
[29] P. Ramos, S.A.O. Santos, Â.R. Guerra, O. Guerreiro, L. Felício, E. Jerónimo, A.J.D. Silvestre, C.P. Neto, M. Duarte, Valorization of olive mill residues: antioxidant and breast cancer antiproliferative activities of hydroxytyrosol-rich extracts derived from olive oil by-products, Ind. Crops Prod., 46 (2013) 359–368.
[30] D. Daâssi, L. Belbahri, A. Vallat, S. Woodward, M. Nasri, T. Mechichi, Enhanced reduction of phenol content and toxicity in olive mill wastewaters by a newly isolated strain of *Coriolopsis gallica*, Environ. Sci. Pollut. Res., 21 (2014) 1746–1758.
[31] M.M. Santos, C. Piccirillo, P.M.L. Castro, N. Kalogerakis, M.E. Pintado, Bioconversion of oleuropein to hydroxytyrosol by lactic acid bacteria, World J. Microbiol. Biotechnol., 28 (2012) 2435–2440.