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Chapter 3

Selection of Promising Bacterial Strains as Potential Tools for the Bioremediation of Olive Mill Wastewater

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Additional information is available at the end of the chapter

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Abstract

The main objective of this paper was the selection of promising bacterial strains to be used as potential tools to remove phenols in olive mill wastewater (OMW) or in other food wastes. Therefore, 12 OMW samples were analyzed and 119 isolates were collected. After a preliminary screening on a medium containing vanillic and cinnamic acids, three isolates were selected to evaluate their viability in presence of different compounds (cinnamic, vanillic and caffeic acids, rutin, tyrosol and oleuropein) and a possible bioremediation effect. The isolates generally survived with phenols added and exerted a significant bioremediation activity in some samples (reduction of phenols by 20%). The last step was focused on the evaluation of the combined effects of pH, cinnamic and vanillic acids on the viability of a selected isolate (13M); the combination of the acids exerted a strong effect on the target, but alkaline pH played a protective role.

Keywords: Bioremediation, phenol degradation, phenolic compounds, olive mill wastewater

1. Introduction

Olive oil production is one of the most important food sectors in the Mediterranean area as olive processing is considered a traditional industry for its countries since ancient times [1]. It is mainly produced in Spain (36% of the global production), Italy (24%), Greece (17%) followed

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by Portugal, France, Cyprus, Croatia, Turkey, Syria, and Tunisia. New producers are Argentina, Australia, and South Africa.

Olives are processed through two methods: pressing (discontinuous process) and centrifuging (continuous process, two/three phase centrifugation). The main inconvenience of these methods is the production of a polluting by-product that is a dark effluent known as olive mill wastewater (OMW) [2].

The environmental impact of olive oil production is strong due to the use of large quantities of water and the production of OMW: 1,000 kg of olives produce 0.5 m^3–1.5 m^3 of OMWs [1]. They are by-products generally considered undesirable but inevitable for every olive processing.

As defined in reference [3], OMW is "a stable emulsion constituted by vegetation waters of the olives, water from the processing, olive pulp and oil." It is characterized by a particular color (intensive violet-dark brown up to black color), odor (strong specific olive oil smell), high degree of organic pollution (expressed as biological and chemical oxygen demand (BOD and COD) values), acidic pH, high electrical conductivity, high content of polyphenols, high buffer capacity, and high content of solid matter.

OMWs are generally composed of water (83%–96%) and organic fraction (3.5%–15%) composed of 1%–8% carbohydrates, 0.5%–2.4% nitrogen compounds, 0.5%–1.5% organic acids, 0.02%–1% of fatty acids such as propionic, butyric, etc., and 1%–1.5% of phenolic compounds consisting of a hydroxyl group (-OH) bound directly to an aromatic hydrocarbon group and pectins [4].

Concerning phenols, they comprise low molecular weight compounds and polyphenols. Low molecular weight compounds are represented by caffeic, cinnamic, 2,6-dihydroxybenzoic, *p*-hydroxybenzoic, syringic, 3,4,5-trimethoxybenzoic, vanillic, and veratric acids; they have phytotoxic effects and antibacterial activity. Polymeric phenols (lignins, tannins, etc.) cause the typical brownish-black color of OMW and are the most recalcitrant fraction of this effluent.

The quantitative and qualitative composition of OMWs are variable due to climatic conditions, variety, ripeness of olives, and extraction processes; generally, they are produced in high quantities in a short time, thus their disposal represents an important problem.

As OMWs are rich in nutrients they could be used to remediate arid or semi-arid regions but their phytotoxicy affect plant growth [5]. OMWs have the highest polluting rate within the food industry due to the fact that they are recalcitrant to traditional biodegradative methods. The reduction of COD and BOD values represent an important goal for many industries but the high content in phenols complicate waste management; they exert an antimicrobial activity towards wastewater microflora thus biodegradation is slowed [6].

For these reasons phenols are considered as undesirable compounds; thus, physical, chemical, or biological treatments are used to reduce their pollutant load.

Waste remediation has been traditionally performed through some expensive methods (incineration, pyrolysis, landfill, etc.). In recent years, the increasing trends towards green economy and friendly approaches for the environment are the background to design alterna-

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tive ways. According to this point of view, numerous researchers proposed bioremediation, defined as "the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state or to level below the limits established by regulatory authorities" [7]. According to the Environmental Protection Agency (EPA), bioremediation is a "treatment that use naturally occurring organisms to break down hazardous substances into less toxic or nontoxic substances."

Thanks to their ubiquity and metabolic pathways (aerobic, anaerobic fermentation, and co-metabolism) microorganisms are able to degrade and utilize various toxic compounds as energy source. Generally, the aerobic biodegradation has a higher efficiency than anaerobic processes and it is widely used. Nevertheless, in many cases, aerobic and anaerobic processes can also be used in series to reduce the complexity or the toxicity of the contaminants.

Numerous bacteria such as *Bacillus pumilus* [8], *Pediococcus pentosaceus* [9], *Lactobacillus plantarum* [10], *Arthrobacter* sp. [11], *Azotobacter vinelandii* [12–14], *Azotobacter chroococcum* [15], *Pseudomonas putida*, and *Ralstonia* sp. [16, 17] were able to degrade and/or remove phenols from OMW.

Yeasts and molds are also able to degrade phenols, namely, *Candida tropicalis*, *Candida cylindracea* and *Yarrovia lypolitica* [18, 19, 3, 20, 4], and white-rot fungi such as *Phanerochaete chrysosporium* or the genus *Pleurotus* [21–25]. In addition, *Trametes versicolor*, *Funalia trogii*, *Lentinus edodes*, *Aspergillus niger*, and *Aspergillus terreus* have been also mentioned as phenol-degrading organisms [26]. The main objective of this paper was the selection of promising bacterial strains to be used as potential tools for bioremediation; namely, after the isolation of some strains from OMWs, they were studied in relation to their ability to grow in a medium containing two secondary phenols. Then, a validation on a lab scale was performed.

2. Materials and methods

2.1. Isolation and phenotyping of potential phenol-degrading strains

Twelve different samples of OMW were analyzed. Aliquots of 100 ml of each OMW sample were mixed with 900 ml of sterile Ringer solution (0.25×; Oxoid, Milan, Italy) and shaken at 100 rpm for 30 min at room temperature. Then, this homogenate was serially diluted with a sterile saline solution (0.9% NaCl) and plated onto a Plate Count Agar (PCA; Oxoid) at 30°C for 48–72 h (for mesophilic bacteria and *Bacillus*) and Pseudomonas Agar Base + Pseudomonas C-F-C supplement (Oxoid) at 25°C for 72 h for *Pseudomonadaceae*. The analyses were performed in duplicate over two different batches. From each batch, some colonies were randomly selected from plates, and stored at 4°C on Tryptone Soya Agar (TSA) slants (Oxoid, Milan, Italy). Phenotyping of isolates was carried out through different tests (Gram, catalase activity, oxidase test, proteolytic activity, and oxido-fermentation).

2.2. Selection of potential phenol-degrading strains

The ability of the isolates to grow with phenols added was evaluated using Mineral Salt Medium (MSM), a synthetic medium containing K_2HPO_4 (1.6 g/l), KH_2PO_4 (0.4 g/l), NH_4NO_3

(0.5 g/l), $MgSO_4$*$7H_2O$ (0.2 g/l), $CaCl_2$ (0.025 g/l), $FeCl_2$ (0.005 g/l), Agar (12 g/l), cinnamic ($C_9H_8O_2$) or vanillic acids ($C_8H_8O_4$) (0.5 or 1 g/l; Sigma-Aldrich, Milan, Italy). After streaking the isolates onto the surface of this modified medium, the plates were incubated at 30°C for mesophilic bacteria and *Bacillus* and at 25°C for *Pseudomonadaceae* for 72 h. MSM without phenolic compounds was used as control. For a second assay, the isolates were preliminary grown in MSM broth added of 0.025 g/l and 0.05 g/l of cinnamic and vanillic acids and incubated for 24 h; thereafter they were streaked onto the surface of MSM with phenols, as reported above.

2.3. Effect of phenolic compounds on the viable cell count

This step was performed on three selected strains (6P, 13M, and 44M); they were grown in TS broth incubated at 25°C (strain 6P) or at 30°C (strains 13M and 44M) for 48 h. Each culture was centrifuged at 4,000 rpm for 10 min and the pellet was re-suspended in sterile saline solution (0.9% NaCl); ca. 6–7 log cfu/ml were inoculated in MSM medium (1, 2, and 3 g/l), added with phenols (cinnamic acid; vanillic acid; caffeic acid-$C_9H_9O_4$; rutin hydrate-$C_{27}H_{30}O_{16}$ x H_2O; tyrosol-$C_8H_{10}O_2$; oleuropein-$C_{25}H_{32}O_{13}$; phenols were purchased from Sigma-Aldrich). MSM without phenols was used as control.

The samples were stored at 25°C–30°C for 33 days and periodically analyzed to evaluate the viable count on TSA and the content of phenols through the Folin-Ciocalteau method [27]. The analyses were performed in duplicate and the results analyzed through one-way Analysis of Variance (one-way ANOVA), using Tukey's test as the post-hoc comparison test, or t-student test for paired comparisons. The statistical analysis was performed using the software Statistica for Windows version 10.0 (Statsoft, Tulsa, OK, USA).

2.4. Combined effects of pH, cinnamic, and vanillic acids on the viable count of the strain 13M

The strain 13M was used as target; it was inoculated into MSM broth to 6–7 log cfu/ml. The amounts of cinnamic and vanillic acids and pH varied according to a 2^k experimental (Table 1). The samples were stored at 30°C and periodically analyzed to evaluate the viable count and phenol content (up to 12 days).

Combinations	pH	Cinnamic acid (g/l)	Vanillic acid (g/l)
A	7	0	0
B	7	0	2
C	7	2	0
D	7	2	2
E	9	0	0
F	9	0	2
G	9	2	0
H	9	2	2

Table 1. Combinations of 2^k design.

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The analyses were performed in duplicate and the results of viable count analyzed through a multiple regression approach by using the option DoE/2^k design of the software Statistica for Windows.

3. Results

3.1. Isolation and screening on MSM

The viable count of mesophilic and spore-forming bacteria and *Pseudomonas* was fairly high (7–8 log cfu/ml) (Table 2); thus, we selected 119 isolates (46 labeled as mesophilic bacteria, 44 and 29 belonging to *Bacillus* and *Pseudomonas* genera, respectively).

	Samples											
	1	2	3	4	5	6	7	8	9	10	11	12
M	7.95	7.77	8.30	7.60	7.48	9.30	8.30	8.30	8.00	6.78	6.30	6.70
B	7.30	6.70	7.30	6.78	6.78	7.00	6.60	6.70	5.70	6.48	6.48	6.30
P	7.70	6.70	8.00	6.90	7.60	6.30	8.30	8.00	7.84	7.00	7.48	8.78

Table 2. Viable count (log cfu/g) of mesophilic bacteria (M), *Bacillus* (B), and *Pseudomonas* (P) in OMW samples. Data are the average (n=2).

The isolates were streaked on MSM with cinnamic or vanillic acids; Figure 1 shows the results for vanillic acid. Namely, 12 isolates were able to grow in presence of 0.5 g/l of this compound (Figure 1a) and 9 with 1g/l (Figure 1b). None of the isolates grew with cinnamic acid.

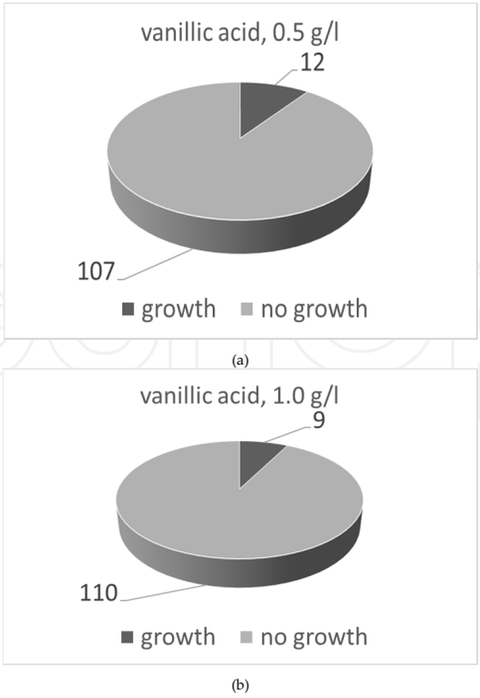


Figure 1. Screening of the isolates on MSM with vanillic acid (0.5 g/l and 1.0 g/l). The numbers on the pictures indicate if the targets are able or not able to grow.

The screening was also performed after isolate growing in MSM broth with low amounts of phenols; this step could be referred as an induction phase, aimed at inducing the resistance to phenols. Figure 2 shows the results with 1 g/l of vanillic acid. There were 32 out of 119 isolates that acquired the ability to grow in MSM with vanillic acid; at the lowest concentration (0.5 g/l) 49 strains were able to grow. The same protocol was also used for cinnamic acid, but only a single isolate was able to grow after induction both at 0.5 g/l and 1 g/l (the isolate 26M).

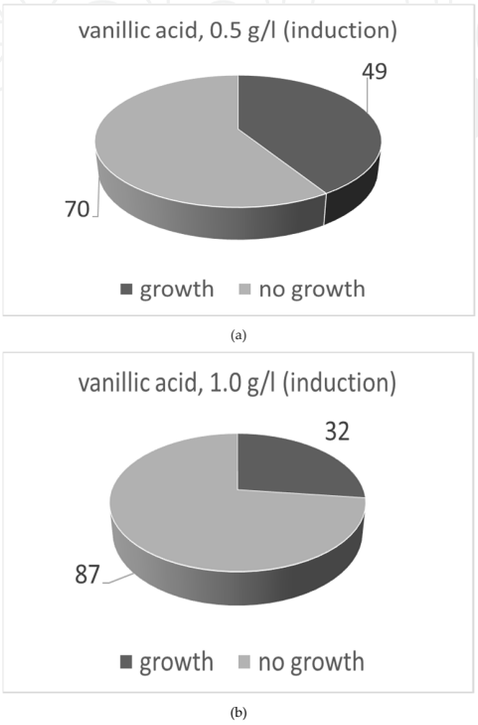


Figure 2. Screening of the isolates on MSM with vanillic acid (0.5 g/l and 1.0 g/l) after the induction. The numbers on the pictures indicate if the targets are able or not able to grow.

3.2. Viability and phenol reduction by some selected isolates

Three isolates (6P, 13M, and 44M; see Table 3 for their phenotypic traits) were selected and used as targets to assess their viability in the presence of various phenolic compounds (caffeic, cinnamic and vanillic acids, oleuropein, and rutin and tyrosol) at different concentrations (1 g/l, 2 g/l, and 3 g/l); moreover, we focused also on microorganism ability to reduce phenol content. Figure 3 reports the viability of the isolate 6P in the presence of caffeic acid; the initial cell number was 7 log cfu/ml. Then, it underwent a strong reduction within 5 days (ca. 1.5 log cfu/ml at 1 g/l and 2 log cfu/ ml at 3 g/l); in the last days of storage we found a tailing effect, with a residual cell count of 5 log cfu/ml. Similar results were found in the presence of cinnamic acid, tyrosol, rutin, and oleuropein (data not shown).

Vanillic acid at 2 g/l reduced the viable count by 3 log cfu/ml in 5 days with a final tailing effect and a residual cell count of 3–4 log cfu/ml. The lowest concentration (1 g/l) resulted in a slower death kinetic, with a similar residual viable count (Figure 4).

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Phenotyping	Isolates		
	6P	13M	44M
Gram	-	+	+
Catalase	+	+	+
Oxydase	-	+	+
Proteolitic activity	-	+	+
O/F	F	F	F

Table 3. Phenotyping of the isolates selected for the second step of the research. F, metabolism under aerobic and anaerobic conditions.

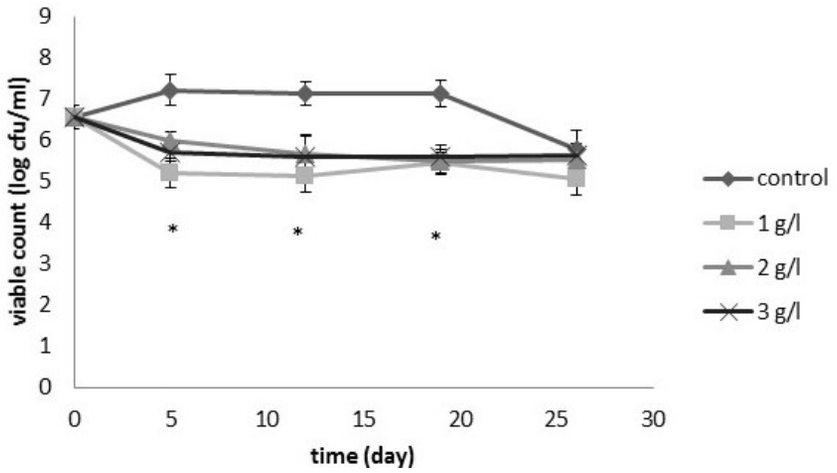


Figure 3. Viability of the isolate 6P in MSM+caffeic acid (mean values ± standard deviation). *, viable count in MSM +caffeic acid are significantly different from control.

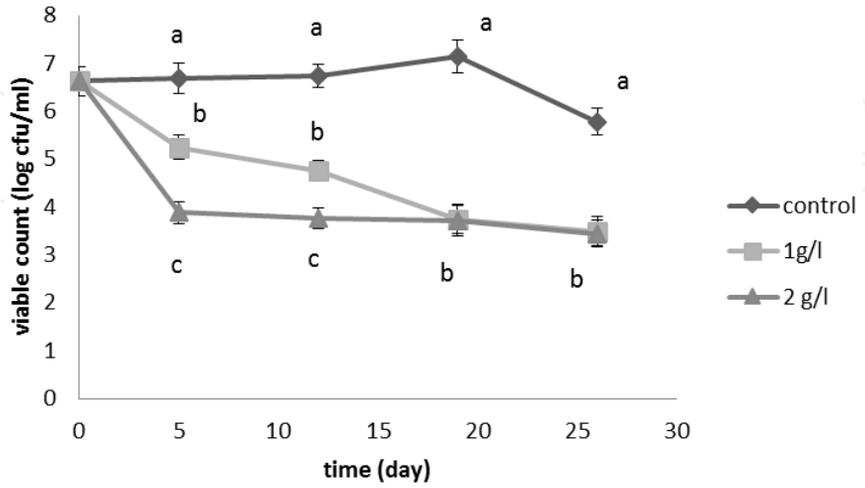


Figure 4. Viability of the isolate 6P in MSM+vanillic acid (mean values ± standard deviation). Letters indicate significant differences (one-way ANOVA and Tukey's test; $P<0.05$).

Phenols did not affect the viability of the strain 44M and the viable count was at 6–7 log/ml for the entire running time (data not shown). Some interesting results were found for the isolate 13M. Tyrosol at 3 g/l reduced the viable count by 3 log cfu/ml within 5 days, thereafter cell number increased up to 6–7 log cfu/ml (Figure 5); this trend could be the result of a kind of induction and adaptive evolution with phenols added. Caffeic acid at 1 g/l and 2 g/l caused a slight viability loss (1–2 log cfu/ml), while cell number was below the detection limit after 25 days at the highest concentration (3 g/l), thus suggesting a possible dose-dependent bactericidal effect (Figure 6). The other phenols did not affect the viable count (data not shown).

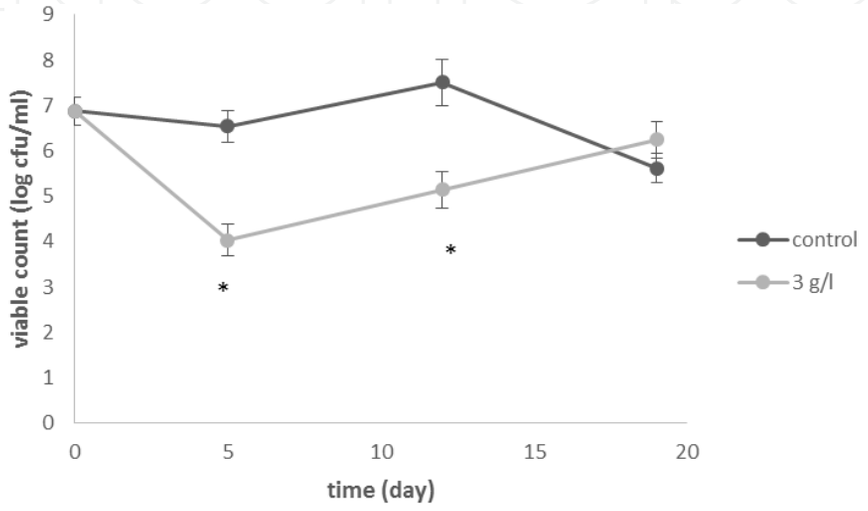


Figure 5. Viability of the isolate 13M in MSM+tyrosol (mean values ± standard deviation). *, significantly different from control (t-student test, P<0.05).

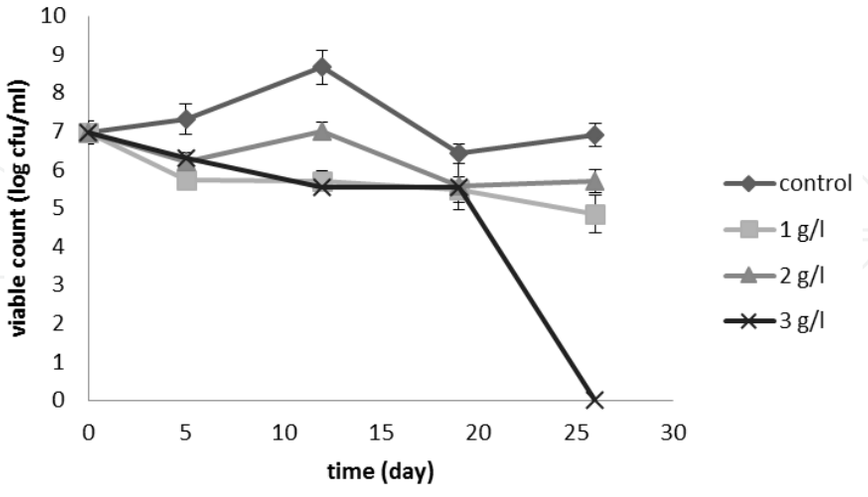


Figure 6. Viability of the isolate 13M in MSM+caffeic acid (mean values ± standard deviation).

We focused also on phenol content; Figure 7 shows the removal of vanillic acid by the isolate 13M and 44M (the initial content of the compound was 1 g/l). Both the strains were able to

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reduce its concentration in the broth, although the isolate 13M showed higher removal efficiency (ca. 18% after 33 days). The isolate 44M was also able to reduce cinnamic and caffeic acids by 24%–27% after 33 days (Figure 8). Removal was always found at 1g/l of phenols; higher initial amounts completely depleted bioremediation, probably due to a possible saturating action on the membrane.

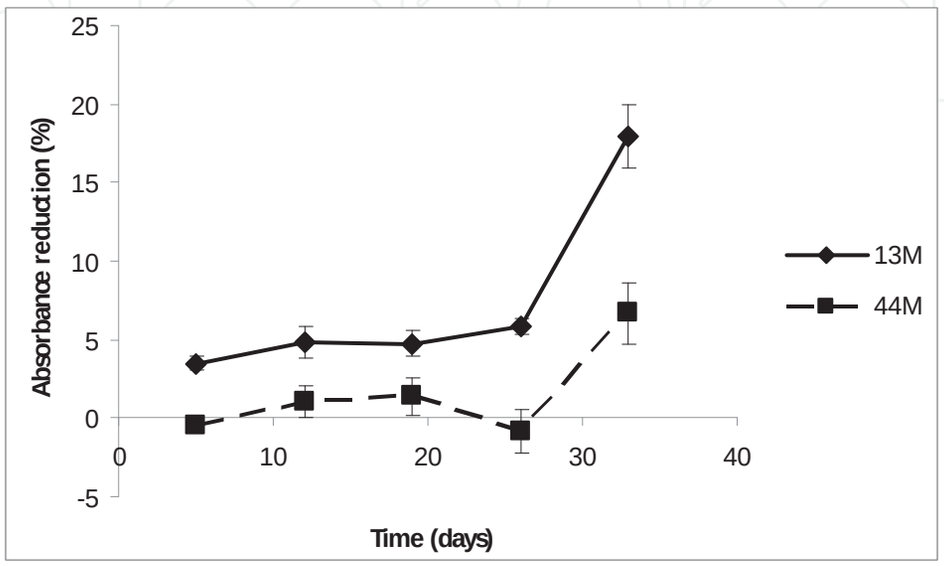


Figure 7. Removal of vanillic acid (initial content, 1 g/l) in MSM inoculated with the isolates 13M and 44 M; data are reported as absorbance fall. Mean values ± standard deviation.

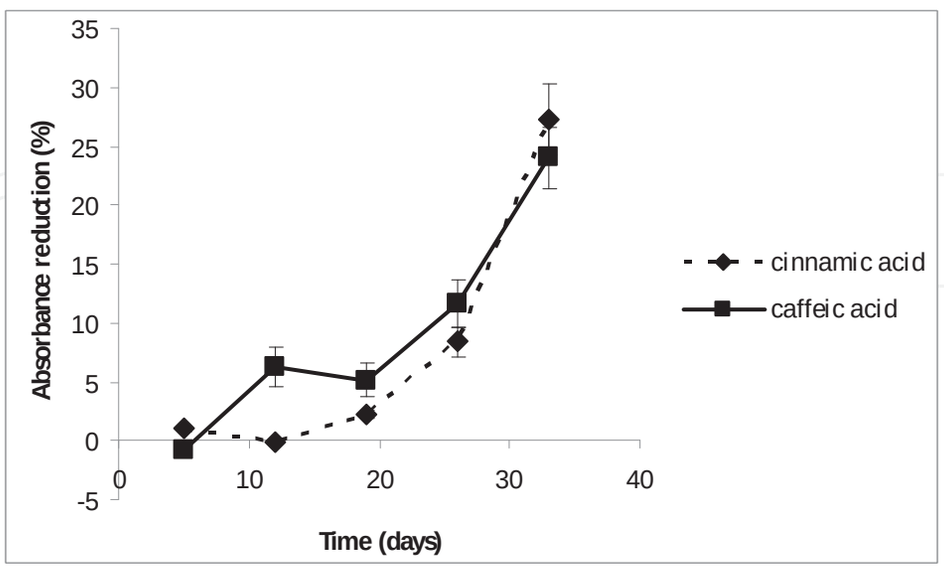


Figure 8. Removal of cinnamic and caffeic acids (initial content, 1 g/l) in MSM inoculated with the isolate 44 M; data are reported as absorbance fall. Mean values ± standard deviation.

3.3. Combined effects of cinnamic and vanillic acids and pH on cell count of the isolate 13M

This phase focused on the evaluation of pH, cinnamic and vanillic acids (combined through a 2^k experimental design) on the viability of the isolate 13M. Figure 9 shows the evolution of cell count in some selected combinations of the design; when MSM medium was adjusted to pH 7.0 in presence of 2 g/l of the phenols (combination D) the viable count (7 log cfu/ml) was drastically reduced to ca. 4.73 log cfu/ml. On the other hand, an alkaline pH played a protective role, as the viable count was not reduced both in absence and with phenol added (combinations E and H).

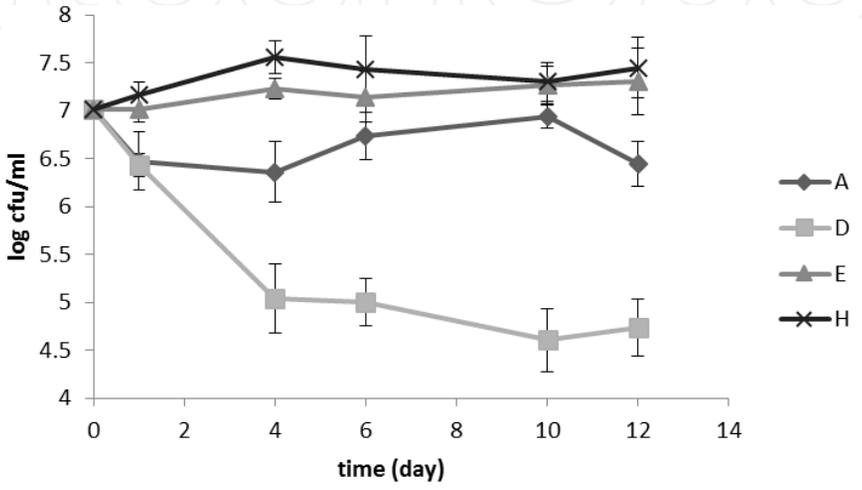


Figure 9. Viable count of the isolate 13M in some selected combinations of 2^k design (see Table 1).

Finally, Figure 10 shows the increase/decrease of cell count after 12 days; a positive value indicates a significant increase in the viable count, while a negative value indicates a death kinetic. These values were used as input data to run a multiple regression procedure and pinpoint the weight of each factor of the design; the results of this statistical analysis are listed in Table 4. The individual effects of phenols were not significant, while their interactive term played a negative role, i.e., it was the leading factor of death kinetic; on the other hand, the statistical analysis pinpointed a positive mathematical effect of pH, thus confirming its protective role toward viable count.

Terms	Statistical effect
pH	3.46
Cinnamic acid	ns
Vanillic acid	ns
pH/Cinnamic acid	ns
pH/Vanillic acid	ns
Cinnamic acid/vanillic acid	-3.06
R^2_{ad}	0.776

Table 4. Standardized effects of vanillic and cinnamic acids and pH on the reduction of the viable count of the isolate 13M after 12 days in MSM. Ns, not significant. R^2_{ad}, determination coefficient corrected for multiple regression.

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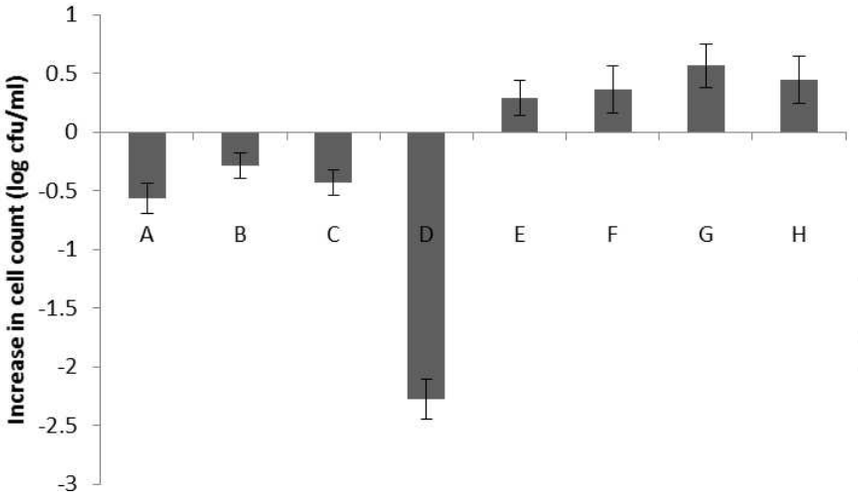


Figure 10. Increase/decrease of the viable count of the isolate 13M in MSM with phenols after 12 days. Mean values ± standard deviation (for the combinations see Table 1).

4. Discussion

The disposal of wastewaters represents a major problem. Namely, OMWs have the highest polluting rate within the food industry, especially for the high concentration of phenolic compounds. An increased interest in environmental issues has favored the introduction of new technologies as alternative ways to traditional methods. A promising approach is represented by bioremediation, which reduces the pollution load of various by-products using the phenol-degrading ability of some microorganisms. In a previous research, we selected some promising yeasts (*Pichia holstii* and *Candida boidinii*) able to reduce phenol content in OMW [26, 28]. Hereby, we evaluated bacterial ability to grow in a phenol-enriched medium; after isolation and phenotyping, we preliminary identified the most promising strains as *Bacillus* and *Pseudomonas* spp. These bacteria are normal constituents of wastewater microflora [29, 30]. After the screening with vanillic and cinnamic acids (chosen as representative of the most important phenolic moieties: coumaric and hydroxybenzoic compounds), we focused on viability in the presence of these compounds, as well as in a caffeic acid, rutin, oleuropein, and tyrosol enriched medium. Caffeic acid and rutin are representative of secondary phenolic compounds, while oleuropein is a high molecular weight phenolic glycoside responsible for the bitter taste in olive fruits. Oleuropein is present in olive mill wastewater as a result of debittering treatments and the extraction process of table and olive oil, respectively. Tyrosol is a product of hydrolysis of oleuropein. We used higher concentrations (1 g/l, 2 g/l, and 3 g/l) than those generally present in OMWs to evaluate bacterial viability in extreme conditions.

In some samples, phenols exerted a bactericidal action, due probably to their ability to form hydrogen bonds with proteins and/or enter cells. The resistant microorganisms generally convert phenols into carbolic acids; these latter compounds are transported through a trans-

membrane shuttle system into the cytoplasm. Carbolic acid is converted to catechol, and after three enzymatic steps, oxaloacetate is formed; the final step is the conversion of oxalacetate to acetaldehyde and pyruvate. The enzymes involved are oxygenase, hydroxylase, peroxidase, tyrosinase, and peroxidase [31, 32]. These products, finally, follow the main metabolic pathway up to their complete mineralization by mitochondrial chain [33, 34]. The oxalacetate can also be used in other cellular activities. The isolates 13M, 44M, and 6P strains were catalase positive and this trait is an important requirement as it is related to phenol degradation [28].

The isolates showed a good metabolic capacity towards simple phenolic compounds, as they were generally able to survive, with some exceptions to this generalized statement. In addition, some isolates (e.g., 13M) significantly reduced the concentration of some compounds in the broth and these are desired traits to select a promising microorganism acting as a bioremediation tool [35].

Concerning the second step of the research (combined effects of phenols and pH), the most important result was the effect of alkaline pH, as it seemed to exert a protective role on cell viability, thus we could suggest that phenolic metabolism at basic pHs is favored because the enzymes might have an optimal pH of 9. In these conditions a high presence of hydroxyl ions is ensured and it is very important as they represent a fundamental substrate used in the first step of the catabolic pathway of phenol to obtain catechol. These assumptions, however, require a confirmation. Finally, the protective effect of alkaline pH suggests the potential use of these isolates for the bioremediation of alkaline washing water of table olives.

5. Conclusions

Bioremediation could be considered as the promising solution for numerous food industry wastes, and to date, several works are in progress to isolate new phenol-degrading strains. This work concurs to confirm the importance of microorganisms to degrade pollutants; we selected some promising bacterial isolates, showing some desired traits in lab media. Further investigations are required to improve our work, i.e., evaluation of waste, evaluation of the effect on BOD and COD, and a focus on the role of alkaline pH on the removal.

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