Optimization of the Self-bioremediation procedure of industrial cork wastewaters and preliminary assessment of the toxicity of the final residue

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Abstract

Cork-boiling wastewaters are rich in phenolic compounds and have traditionally been remediated by chemical techniques. An alternative treatment, based on microbial bioremediation, is being developed. The objective of this work was to design and optimize an effective self-bioremediation system to treat polluted cork wastewater. Polyphenol tolerant cultivable bacteria were isolated from this effluent. The strains, identified by 16S rDNA, include Acinetobacter sp., Staphyloccocus sp., two Microbacterium spp. and Bacilllus sp., that were able to tolerate up to 15mM of phenol, and up to 1mM and 0.1mM of 4-chlorophenol and 2,4-dichlorophenol, respectively, using the pollutant as the sole carbon source. Degradation assays were performed using Acinetobacter sp. and the consortium formed by the five selected strains immobilized onto residual cork particles. Addition of low levels of carbon, together with micromolar concentrations of H2O2 in the presence of light were the optimal conditions for phenol removal. Under these conditions, the consortium was able to degrade more than 60% of initial phenol from cork wastewater in 10 days. The post-removal solution was tested for its toxicity applying a toxicity test based on the germination of Medicago sativa seeds. Results suggested that the treated solution was less toxic than the parent solution. Thus, self-bioremediation seems to be a promising eco-friendly technology that allows the treatment of cork boiling wastewater with low costs and the absence of collateral impacts.

Keywords: *Phenol, Chlorophenol, Bacteria, Biofilms, Cork, Wastewaters, Bioremediation.*

1 INTRODUCTION

The accelerated growth of chemical and oil industries is accompanied by the release of dangerous wastes, which have negative consequences for ecosystems and public health. These compounds are disposed indiscriminately in the environment and can often remain in it for long periods of time [1, 2, 3].

Phenolic compounds require relevant attention in the study of environmental contamination due to their toxicity and prevalence in water and soil [4, 5]. In nature, these contaminants are generated by processes of humification and degradation of proteins, as well as burning of forests [6]. However, the greatest amount of phenolic compounds is released into the environment from industrial activity, since they are widely used in the pharmaceutical industry, paper production, plastics, textiles and oil refining, among others [7].

The removal of phenolic compounds from industrial effluents before being discharged into the environment is compulsory and different alternatives have been sought for the treatment of these contaminants [8, 9]. Bioremediation allows microorganisms to transform or degrade dangerous compounds found in water and soil and represents an environmentally friendly alternative, being a tool widely used by international organizations such as EPA [Environmental Protection Agency] for the treatment of this type of waste, since it is less expensive and is relatively simple [10, 11]. The application of microbial consortia for bioremediation processes is considered beneficial because it has some advantages over pure cultures [12,13]. However, it is necessary to work with composite native microbial communities, since they can perform a complete mineralization of phenols, transforming them to carbon dioxide [CO2] and water [H2O] without any toxic residue [14, 15]. Another advantage of working with microbial consortia is to maintain stability in the bacterial culture, thus increasing their metabolic capacity [16, 17].

Cork industry is one of the most important ones in Portugal and Southern Spain, being these two countries the world first and second producers respectively [18]. Cork treatment involves boiling the cork plates in water. After this treatment, the resulting wastewater is a residue that contains high concentration of polyphenols, chlorophenols and tannins [19], whose depuration is performed basically by physicochemical methods including flocculation and precipitation, ozonation, membrane filtration, photo-oxidation, etc. [20]. In the last years, increasing publications describe the depuration of this residue by biological treatments using fungi [21, 22] and particularly bacteria, one or in combination with partial chemical degradation [23]. One of the most novel approaches included immobilization of [chloro]phenol-degrading bacteria isolated from the wastewaters onto residual cork particles in order to increase the durability and effectivity of bioremediation, a process called self-bioremediation [24, 25].

In this work, some steps further are taken in the process of self-bioremediation: a] The use of bacterial consortia in the place of individual bacterial strains has been investigated; b] The incubation conditions as well as the use of additives have been optimized to get an optimal removal of phenolics in cork wastewater, and c] Finally, by means of a phytotoxicity test, the quality of the treated water was evaluated.

2 Materials and Methods

2.1 Isolation and characterization of bacterial strains

Bacterial strains were isolated from cork-boiling wastewater. Water samples were provided by a local company and enriched with TSB medium in a 9:1 proportion [9 volumes of waterwaters: 1 volume of TSB]. The enriched wastewater was incubated for 48 h at 28° with continuous shaking and after that period the bacteria were isolated on TSA medium. Bacterial identification was made by 16S rDNA sequencing.

2.2 Resistance of isolated strains towards phenol and chlorophenols

The resistance of the isolates to phenol and chlorophenols was determined in solid minimal medium [26, 27] supplemented with a minimum concentration of TSA equivalent to 10% [v/v] to provide a minimum carbohydrate dose. Different concentrations of phenolics were added to this medium: phenol [0-25 mM], chlorophenol [0-2 mM] or 2, 4-dichlorophenol [0-1 mM]. After incubating the plates at 28° C for 72-96 h, the growth was evaluated. The resistance was expressed as the maximum tolerable concentration [MTC] which is the maximum concentration of the toxic not affecting bacterial growth.

2.3. Determination of phenol/polyphenols in liquid media

The determination of phenol was done by a colorimetric method [28]. Reagents were added in the following order: 700 µL 0.25 mM $NaHCO_3$, 100 µL of the sample [supernatant of the cultures after centrifuging at 10,000rpm for 5 min], 100 µL 20 mM 4-AAP [4-aminoantipyrine] and 100 µL 80 mM potassium ferricyanide. After 5 min, the OD at 510 nm was measured and the phenol concentration was determined from a standard curve made with a stock solution of 1,000 µg phenol. mL-1.

Since wastewaters contained a mix of phenolics, in order to evaluate the removal of total polyphenols in this residue, several calibration curves were performed using different phenolic compounds [phenol, tannic acid and caffeic acid]. The concentration of polyphenols in wastewater samples was then calculated as the mean of the concentrations obtained by using the different calibration curves corresponding to every substance.

2.4. Phenol Degradation

2.4.1. Degradation of phenol in aqueous solution

Bacteria were grown in flasks containing 25 mL of minimal medium [26] supplemented with TSB [10% v/v] and phenol [2.5 mM] at 28° C with constant shaking at 200 rpm. The amount of inoculum for each strain was 100 µL of an overnight culture [about 108 CFU/mL] of the bacterial strains cultivated in the same conditions, i.e., in the presence of 0.1 mM phenol for induction of the degradation machinery. Aliquots of 2 mL were taken for a total period of 48 h. The OD at 600 nm was determined as a measure of bacterial growth. The aliquots were centrifuged at 10,000 rpm for 5 minutes and the phenol concentration was determined in the supernatant as described in section 2.3 [28].

2.4.2. Optimization of the conditions for phenol biodegradation

The conditions for optimal degradation of phenolic compounds were optimized, in particular: a] the optimal amount of additional carbon source in the form of TSB; b] the optimal amount of H_2O_2; c] optimal temperature; d] shaking/static cultures; and e]

the presence of light/dark. In all cases, the corresponding controls without bacteria were performed in parallel. After 72h incubation, the amount of total polyphenols was determined as previously described in section 2.4.1 [28].

2.5 Degradation of phenol by consortia of bacteria

Once the best phenol degradation conditions were established, the possibility of improved degradation with bacterial consortia was tested. In these consortia, the strain BAE9A -which was the best degrader- was always included. Besides, different combinations were tested. Cultures of each of the bacteria were grown individually overnight in tubes of 5 mL containing minimal medium [26] supplemented with 10% [v:v] TSB and 2.5 mM phenol in order to induce phenol degrading enzymes. Next morning, the OD at 600mn of the cultures was determined and adjusted 1.0 with sterile minimal medium. Flasks containing 25 mL of minimal medium [26] supplemented with TSB [10% v/v] and phenol [2.5 mM] were inoculated with 1mL of the overnight grown cultures. When using bacterial consortia, the volume was divided for the number of strains [previously adjusted to the same optical density].

2.6 The flasks were incubated for 72 h at 28° C with shaking at 200 rpm. At the end of the experiment, the cultures were centrifuged at 10,000 rpm for 5 minutes and the phenol concentration was determined in the supernatant as described in section 2.3. 2.6. Degradation of phenol by bacteria immobilized onto cork particles

Cork particles – a residue collected from the floor of the cork industry- were homogenized with a coffee grinder, passed through a 0.5 mm sieve, washed with 25 mL HCl [1mM] for 10 min and the pH was neutralized with an appropriate volume of NaOH [1mM]. Subsequently, 5 washes with sterile distilled water were performed. Particles were sterilized in autoclave and air dried. Cork particles [approx. 0.01 g] were mixed with 850 µL of minimal media, 100 µL of TSB medium [10% v:v with regard to final volume] and 150 µL of phenol [1mM] and inoculated with 60 µL of a bacterial inoculum [an overnight culture of bacteria grown in TSB medium, approx. 108 CFU/mL] in an eppendorf tube. A control without bacteria was performed along. The tubes were incubated at 28° C under constant shaking at 100 rpm for 10 days in order to allow the formation of a mature biofilm. After this time the cork particles were washed 5 times with sterile distilled water and passed through a sieve. The supernatant was centrifuged at 10,000 rpm for 15 min to avoid interferences in the determination of phenol by the colorimetric method [28]. Cork particles were used for microscopy studies. For that, the particles were fixed overnight in the presence of OsO4 and subsequently treated with Au and visualized by SEM using a scanning electron microscope Jeol 6460LV [24].

2.7. Toxicity assays based on germination of Medicago sativa seeds

The phytotoxicity of solutions after the removal process was evaluated and compared to that of the original wasterwater. For that, a test based on the germination of alfalfa seeds was performed. Sterile water was used as external control of the germinating capacity of the seeds. Alfalfa seeds were previously sterilized with several washes of a 0.001% v/v HCl solution and washed with distilled water. The assay was performed with 4 replications of n=25 seeds with for each treatment [total seeds n = 100] in Petri dishes with a diameter of 90 mm. To sterilize the wastewaters, both the original residue and the post-treatment residue,

the solutions were filtered through 0.45 µm filters [sterilization by autoclaving was avoided in order not to alter the composition of the solutions]. 3 mL of the solutions to be evaluated [sterile distilled water, sterilized original wastewater or sterilized post-treated wastewater] were placed in the bottom of the plate and groups of 25 previously sterilized seeds [4 plates= 100 seeds] were carefully placed on each plate with the aid of a clamp. Plates were protected from light and incubated for 5 days at 25 ± 2° C. After this period, the number of germinated seeds was recorded, considering the visible appearance of the radicle as the germination criterion.

3 Results

3.1. Identification of bacteria from cork-processing wastewaters

In a previous work [del Castillo et al., 2012], the strains BAE9A and BAC4 were previously selected for bioremediation of cork wastewater based on the results obtained from phenol resistance and degradation rate, as well as the ability to form "biofilms" on the cork surface [del Castillo et al., 2012]. In addition, in this work three new strains [VR1, VR2 and VR3 VN1, VH1 and VH2] were selected since they also displayed good phenol degrading properties and they were included in the study.

Gram staining revealed that the bacteria corresponded to one gram-negative rod [BAE9A], three gram-positive rods [VR1, VR2 and VR3 VN1, VH1 and VH2] and one Gram positive coccus [BAC4]. The results of 16S rDNA sequencing and comparison of the obtained sequences with databases [Ez-Taxon, RDP, BLAST] indicated that BAE9A corresponded to Acinetobacter soli; VR1 and VR2 belonged to the genus Microbacterium, in particular M. oxydans and M. hydrocarbonoxydans respectively; whereas VR3 was identified as Bacillus weihenstephanensis and BAC4 was ascribed to the species Staphylococcus saprophyticus [Table 1].

Table 1. Identification and tolerance against phenolic compounds of isolated strains

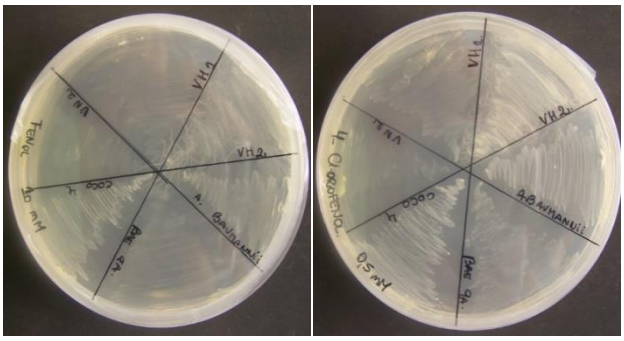
STRAIN	Maximun identity [16S rDNA]	MTC [mM]		
		Phenol	4-Chlorophenol	2,4 dichlorofenol
BAE9A	Acinetobacter soli 99%	15 mM	1 mM	<0.1 mM
BAC4	Staphylococcus saprophyticus 99%	15 mM	0.5 mM	<0.1 mM
VR1	Microbacterium oxydans 99%	10 mM	0.5 mM	<0.1 mM
VR2	Microbacterium hydrocarbonoxydans 99%	15 mM	0.5 mM	<0.1 mM
VR3	Bacillus weihenstephanensis 98%	15 mM	0.5 mM	<0.1 mM

3.2. Resistance of bacterial strains to phenol and chlorophenols in solid medium

The resistance of the strains to phenol, 4-chlorophenol and 2, 4-dichlorophenol was tested on plates containing minimal medium supplemented with 10% [v:v] TSA and the contaminants. BAE9A, BAC4, VR2 and VR3 strains showed the highest resistance to phenol [15 mM], whereas VR1 was able to tolerate up to 10 mM phenol. BAE9A strain showed the highest resistance to 4-chlorophenol [1mM]. None of the strains was able to tolerate

concentrations of 2, 4-dichlorophenol above 0.1 mM [Table. 1 and Fig. 1].

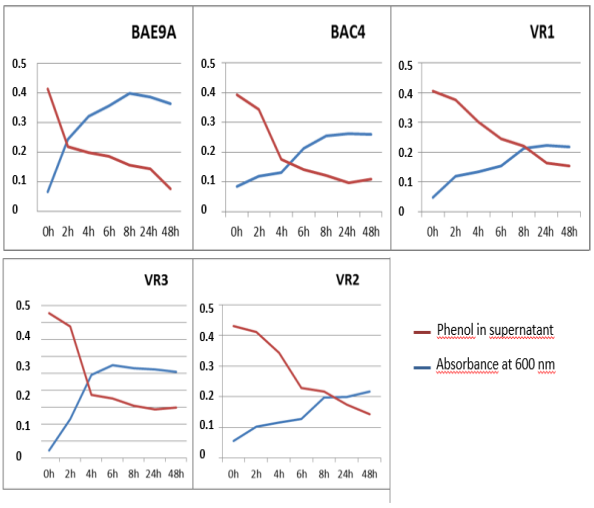
Fig. 1. Plaques of different bacterial strains resistant to 10 mM phenol and 0.5 mM 4-chlorophenol.



3.3. Evaluation of phenol removal by individual strains in liquid medium

The strains were grown in minimal medium supplemented with 10% TSB medium and containing 2.5 mM phenol. DO600 nm was measured as a bacterial growth parameter. The disappearance of phenol in the culture medium was evaluated. The ability to grow with phenol varied among strains. The strains that showed the fastest growth rate in the presence of phenol were BAE9A and VR3. The remaining strains [BAC4, VR1 and VR2] showed a growth retardation of approximately 4h. Although all strains showed good phenol degradation abilities, BAE9A and BAC4 strains appear to be the most efficient phenol degraders, since the remaining percentage of phenol in the medium was the lowest [see Fig. 2].

Fig. 2. Growth and consumption of phenol by bacterial strains isolated from cork-processing wastewaters in presence of 2.5 mM phenol. Bacterial strains were grown in 1/10 TSB medium supplemented with 2.5mM phenol. Bacterial growth [OD at 600nm] and phenol in the supernatant of the cultures were evaluated for a total period of 48 hours.



3.4. Optimization of conditions for phenol degradation by Acinetobacter soli BAE9A

Different conditions were tested in order to evaluate the phenol consumption in liquid medium when consortia are formed. The conditions evaluated are shown in Table 2.

Table 2. Evaluated treatments in minimal medium supplemented with 2,5 mM of phenol

TREATMENTS	MM	PHENOL	BAE9A*	BAC4*	VR1*	VR2*	VR3*
CONTROL	+	+	-	-	-	-	-
T1	+	+	+	-	-	-	-

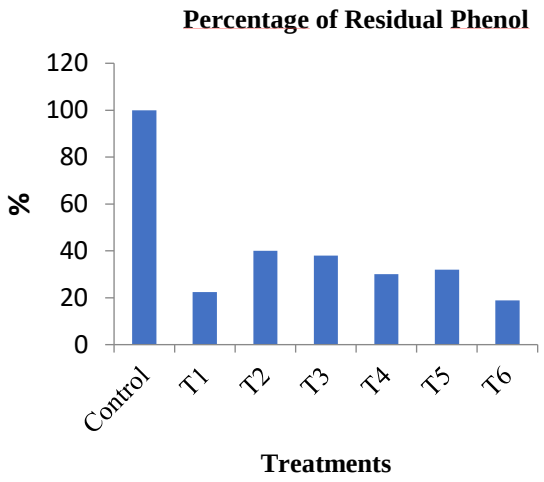
T2	+	+	+	+	-	-	-
T3	+	+	+	-	+	-	-
T4	+	+	+	-	-	+	-
T5	+	+	+	-	-	-	+
T6	+	+	+	+	+	+	+

*Bacterial strains selected

3.5. Phenol removal by bacterial consortia: optimization of variables

In bacterial consortia, BAE9A was used as the main strain because of its high rate of phenol degradation [Hernández, 2011; Del Castillo et al., 2012]. Besides, other bacteria were used in combination with BAE9A. The consortium formed by the five strains: BAE9A, BAC4, VR1, VR2 and VR3 showed the lowest percentage of residual phenol [18.8%], followed by the strain BAE9A [22.4%] cultured alone. The remaining conditions showed a percentage of residual phenol between 30 and 40% [Figure 3].

Fig. 3. Percentage of Residual Phenol in liquid medium. The treatments evaluated are shown in Table 2.



3.6. Polyphenols removal from cork-boiling wastewater [bacterial consortium]

The conditions evaluated in this assay are showed in Table 3. Treatment with BAE9A strain alone showed the highest degradation of total polyphenols, with a percentage of removal of 70.66% [total polyphenols remaining 12.94 mg/L].

Table 3. Evaluated treatments in cork-boiling wastewater supplemented with TSB [10% v/v] and H2O2 [0.01% v/v]

TREATMENTS	WASTEWATER [WW]	TSB	H_2O_2	BAE9A*	BAC4*	VR1*	VR2*	VR3*
CONTROL	+	+	+	-	-	-	-	-
T1	+	+	+	+	-	-	-	-
T2	+	+	+	+	+	-	-	-
T3	+	+	+	+	-	+	-	-
T4	+	+	+	+	-	-	+	-
T5	+	+	+	+	-	-	-	+
T6	+	+	+	+	+	+	+	+

*Bacterial strains selected

All bacterial consortium tested showed a significant removal of polyphenols in cork water samples; however, the most effective bacterial consortia in the removal of polyphenols were the consortium formed by all strains [BAE9A, BAC4, VR1, VR2 and VR3] with a 63.5% removal percentage [total remaining polyphenols 16.09 mg/L], followed by the consortium formed by the BAE9A/BAC4 strain, with a 60.8% removal percentage [total polyphenols remaining 17.28 mg/L] [see Fig. 4]. The addition of H2O2 decreases color, improving the appearance for possible future uses [see Fig. 5].

Fig. 4. Concentration of total polyphenols remaining in cork processing wastewater after 10 days of treatment

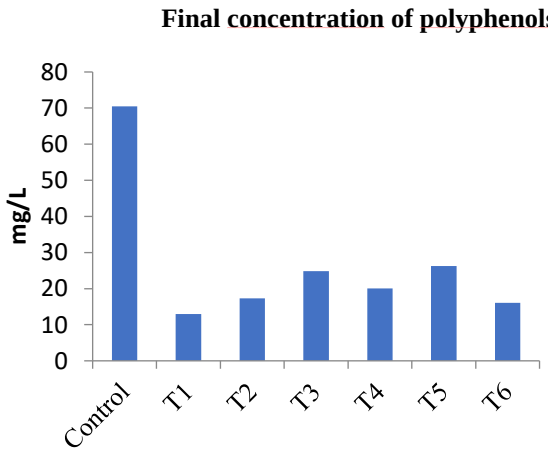
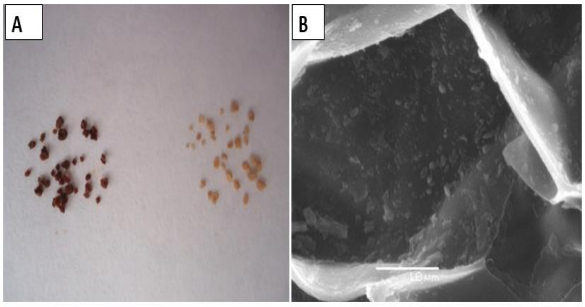


Fig. 5. Cork processing wastewater before and after the treatment with bacterial consortium.

3.7. Formation of "biofilms" onto cork particles

In the cork manufacturing process, small residual particles are released, which can serve as adhesion surfaces for the formation of biofilms. The formation of biofilms on the surface of cork particles was evaluated. The formation of biofilms was observed by SEM. It was observed on the cork particles, the presence of several bacterial morphologies corresponding to the strains that formed part of the consortium. It is clearly seen that the strains BAE9A, BAC4 and VR2 are able to colonize and grow on the surface of particles of residual cork, with a very high surface cover [see Fig. 6].

Fig. 6. Evaluation of biofilms formation on the surface of cork particles. A. Cork particles before and after the incubation with de bacterial consortium [BAE9A, BAC4, VR1, VR2 y VR3]. B. Scanning electron micrograph of a cork particle colonized by the bacterial consortium. Notice the different bacterial morphologies [cocci and rods of different sizes].



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