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BIOTECHNOLOGICAL TOOLS TO IMPROVE BIOREMEDIATION OF PHENOL BY *Acinetobacter* sp. RTE1.4

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Abstract

The use of native bacteria is a useful strategy to decontaminate industrial effluents as well as the environment. *Acinetobacter* sp. RTE1.4 was previously isolated from polluted environments and constitutes a promising alternative for this purpose due to its capability to remove phenol from synthetic solutions and industrial effluents.

In this work, this strain was identified at species-level as *A. tandoii* RTE1.4. Phenol degradation pathway was studied and some reaction intermediates were detected, confirming that this strain degraded phenol through *ortho*-cleavage of the aromatic ring. Phenol removal assays were carried out in a stirred tank bioreactor and a complete degradation of the contaminant was achieved after only 7 h, at an aeration rate of 3 vvm and at agitation of 600 rpm. Moreover, this bacterium was immobilized into calcium alginate beads and an increase in phenol biodegradation respect to free cells was observed. The immobilized cells were reused for four consecutive cycles and stored at 4 °C for 9 months, during which phenol removal efficiency was maintained. Post removal solutions were evaluated by Microtox® test, showing a toxicity reduction after bacterial treatment. These findings demonstrated that *A. tandoii* RTE1.4 might be considered as a useful biotechnological tool for an efficient treatment of different solutions contaminated with phenol in bioreactors, either using free or immobilized cells.

Key words: phenol; bacteria; bioremediation, bioreactor, immobilization, toxicity

1. INTRODUCTION

Phenol is one of the pollutants most frequently found in the environment and is considered as a priority pollutant because of its adverse effects on animal and human health, widespread diffusion and persistence in the environment.

Industrial phenol production is estimated to be over three million tons per year, being used mostly in petrochemical industry, synthesis of resins, pharmaceuticals, perfumes, as well as intermediate in the preparation of other chemicals (e.g., plastics, drugs, explosives, pesticides and detergents), solvents or lubricating oils [1]. Due to their high production and usage, phenol arises as wastes or by-products of the above mentioned industries.

With the aim of protecting human and animal health, the United States Environmental Protection Agency [2], has established limit concentrations for phenol in drinking-water less than 3.5 mg/L. In Argentina, the law of Hazardous wastes (Law 24,051, Annex II), established a guide level of 1 µg/L for protecting aquatic life in fresh surface water, and 2 µg/L for water source for human consumption [3]. However, higher concentrations than those mentioned were frequently found in contaminated environments. Therefore, the removal of such compound from water and soils is of relevant significance.

As a consequence, various physicochemical treatments such as distillation, liquid-liquid extraction with different solvents, adsorption, membrane pervaporation and membrane-solvent extraction have been proposed to remediate phenol polluted sites [4]. However, these treatments are complex, expensive and, sometimes, they produced more toxic compounds. Thus, it has become necessary to look for environmentally friendly technologies, such as biological methods, which are safer and least disruptive treatments.

At present, there are a variety of technologies developed for the remediation of contaminated water or industrial effluents, involving the use of bacteria in batch and/or continuous processes in bioreactors, using cell-free cultures or immobilized cells in various matrices [5,6]. The aims of this work were to establish the identity at specie level of a bacterium previously isolated, characterized and named as *Acinetobacter* sp. RTE1.4 strain by our research group and to study its performance on phenol degradation process in a stirred tank reactor. In addition, we were interested in determining if its immobilization into calcium alginate beads could contribute to enhance phenol removal. Moreover, a bioassay for the determination of potential toxic effects of treated solutions was also applied and evaluated.

2. MATERIALS AND METHODS

2.1 Microorganism and culture conditions

In this work, *Acinetobacter* sp. RTE1.4, isolated from an effluent derived from a chemical industry and previously characterized in detail was used [7]. Bacterium was routinely grown on TY agar medium [(g/L): 5 tryptone;

3 yeast extract; 0.65 CaCl₂; 13 agar] and kept at 4°C. For the different experiments described below, inocula were prepared by growing the strain in TY liquid medium.

2.2 Identification at the species-level

Identification of *Acinetobacter* sp. RTE1.4 strain at the species level was initially based on analysis of the sequence of the housekeeping gene recombinase A (*recA*). For that, a bacterial culture was incubated in TY medium at 28±2 °C during 12 h and bacterial genomic DNA was obtained using DNA purification kit (Wizard-Promega) according to protocols supplied by the manufacturer. This DNA preparation was used as a template for *recA* gene amplification by PCR using the primer pair rA1/rA2 described previously by Nowak and Kur [8]. The sequences of these primers were: rA1 (5'-CCTGAATCTTCTGGTAAAC-3') and rA2 (5'-GTTTCTGGGCTGCCAAACATTAC-3').

Amplification reactions were carried out in 25 µl final volumes containing the following reagents: 20 mM Tris-ClH (pH 8.4)/50 mM KCl buffer, 1.5 mM MgCl₂, each triphosphate deoxynucleotide at a concentration of 0.2 mM, 0.3 µM of each primer, 5.5 µg of template DNA and 2.5 U *Taq* DNA polymerase (Invitrogen). The PCR conditions used were 3 min of denaturation at 94 °C, 35 cycles of 1 min of denaturation at 94 °C, 1 min of primer annealing at 45 °C and 2 min of extension at 72 °C, followed by a final step at 72 °C for 5 min. PCR reactions were performed in an i-Cycler (Bio-Rad) thermocycler. Aliquots (10 µl) of the PCR products were analyzed by electrophoresis on a 1% agarose gel containing 0.5 µg/mL ethidium bromide using 100 bp size marker (Invitrogen). The PCR products were purified with DNA PuriPrep-GP kit (Highway) according to the manufacturer's recommendations and submitted for sequencing to Instituto Nacional de Tecnología Agropecuaria (INTA, Castelar, Argentina). The obtained *recA* gene sequence was compared and identified using BLAST program [9] and deposited in GeneBank database under the following accession number JX258136.

A phylogenetic tree was constructed based on the *recA* gene sequences from different *Acinetobacter* species. For that, multiple alignments of different *recA* gene sequences downloaded from GenBank were performed using CLUSTAL-X software and the evolutionary distances were calculated by the Tamura-Nei model. For the phylogenetic tree construction the Neighbor-Joining method by MEGA4 software [10] was used and a bootstrap analysis of up to 1000 iterations were carried out.

In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to improve identification, using a Bruker Daltonics equipment (Germany). Samples were prepared using cells from a single colony of a fresh overnight culture following the ethanol/formic acid extraction procedure recommended by the manufacturer (Bruker Daltonik, Bremen, Germany). The procedure was done following standard procedures [11]. MALDI-TOF MS measurements were made using an Microflex III instrument (Bruker Daltonik, Bremen, Germany) for the automatic acquisition of mass spectra according to the manufacturer's instructions. The peaks from the obtained mass spectra and their matching against the reference spectra of the integrated database was performed using MALDI Biotyper Software (Bruker Daltonics). Score values ≥ 2.0 were considered appropriate for identification at species level [12]. The results were analyzed using the software Microflex 3.1. These studies were carried out at the specialized Laboratory of Clinical Microbiology of Hospital Juan A. Fernández (Buenos Aires, Argentina).

2.3 Evaluation of intermediates of phenol metabolism

For sample preparation, the bacterial strain grew in TY liquid medium until late exponential phase. Then, this culture was inoculated in 250 mL Erlenmeyer flasks (10% V/V) containing 50 mL of liquid minimum medium (MM) [(g/L): 2.8 Na₂PO₄H; 9.0 KPO₄H₂; 2.5 NaCl; 1.0 NH₄Cl] with phenol 200 mg/L as sole carbon source. The flasks were placed in a shaker at 100 rpm at controlled temperature (28 °C) until phenol was completely removed. The culture was centrifuged at 12,000 g at 4 °C for 10 min. Aliquots (10 mL) of supernatants were used to identify reaction intermediates. Samples were acidified to pH 2.5 with HCl 0.5 N, extracted with 20 mL of ethyl acetate (20 mL x 2), dried over anhydrous Na₂SO₄, filtered and evaporated under vacuum. Solids were resuspended in 1 mL of mobile phase, filtered through a 0.25 µm nylon filter to remove cell debris. Samples were analyzed by HPLC (Section 2.7.1).

2.4 Phenol biodegradation in a stirred tank bioreactor

In order to maximize the removal efficiency of higher volumes of phenol by *Acinetobacter* sp. RTE1.4, the potential of this strain for phenol degradation under controlled agitation and aeration conditions was evaluated in a bioreactor. For that, a 5 L stirred tank bioreactor (BIOFLO 2000, New Brunswick Scientific, U.S.A.) was used.

The bioreactor was operated in batch mode with an initial phenol concentration of 200 mg/L at pH 7 and 28±2°C under different rotational speeds and airflow rates: 200, 400 and 600 rpm and 1 and 3 vvm, respectively. The reactor vessel was carefully sterilized by autoclave prior all experiments, at 120 °C, 1 atm for 30 min. A volume of 2 L of MM was inoculated with 200 mL of an *Acinetobacter* sp. RTE1.4 culture, previously grown in TY medium without phenol, until late exponential phase of growth (OD_{620nm} 0.9±0.1 equivalent to 2.2±0.87×10¹¹ CFU/mL). Non-inoculated controls were also assessed. Culture samples were withdrawn each two hours by quadruplicate and tested for biomass and residual phenol concentration (Sections 2.7.2 and 2.7.3).

2.5 Phenol biodegradation using immobilized cells

2.5.1 Entrapment of cells in calcium alginate

Acinetobacter sp. RTE1.4 was inoculated into 100 mL TY medium in 250 mL Erlenmeyers flasks, incubated at 28±2°C and agitated at 100 rpm until optimum growth was achieved (OD_{620nm} 0.9±0.1). Next, 10 ml of this culture (cell content in terms of wet weight was equivalent to 0.6 g of cells) was added to 30 ml of a 4 % W/V sterilized Na-alginate solution, obtaining final alginate concentration of 3% (V/V). These alginate-cell suspensions were extruded into a 0.2 M CaCl₂ solution to form beads with a diameter of approximately 3 mm. This procedure was performed with continuous stirring. The beads were gelled for 20 min under stirring. Then, they were washed three times with sterile saline solution (SSS) and were maintained in the same solution. After 40-60 min, these beads were used for phenol degradation

experiments and/or stored at 4 °C. The immobilization procedure was performed under sterile conditions and all solutions were previously sterilized.

2.5.2 Phenol degradation assays

Phenol degradation assays, using *Acinetobacter* sp. RTE1.4 cells immobilized in calcium alginate beads were conducted in Erlenmeyer flasks (100 mL) containing 30 mL of MM without phosphates, supplemented with phenol concentrations between 200 and 2,500 mg/L. Each flask was inoculated with 24 g of beads.

In all experiments, controls were performed using the same medium inoculated with free cells (10% V/V), previously grown in TY medium until late exponential phase (OD_{620nm} 0.9 ± 0.1). Abiotic controls consisting of the same culture medium inoculated with calcium alginate beads without bacterial cells were also performed.

The Erlenmeyer flasks containing immobilized bacteria, free cells or beads without bacterial biomass were incubated at 28 ± 2 °C. They were stirred at 100 rpm. Medium and beads samples were taken at different time intervals (each 3 or 10 d) to evaluate phenol removal and bacterial biomass in order to analyze the possible losses of cells as a result of the broken beads.

All assays were performed three times by triplicate.

2.5.3 Reusability and stability during storage of immobilized cells

Repeated cycles of phenol (200 mg/L) biodegradation were carried out to establish the reusability of immobilized cells. After complete phenol degradation, the used medium was discarded and fresh medium containing the same phenol concentration was added to the flasks. The degradation processes were performed until the beads lost their degradation ability.

In order to evaluate the stability during storage, free and immobilized cells were stored at 4 °C, and then the cells were tested for their phenol degradation capability, at different time periods.

In both cases, phenol degradation assays were carried out as it was previously described in section 2.5.2.

2.5.4 Biomass estimation

Biomass was estimated through determination of protein content and viable cell number.

Protein content was assessed in beads, in the reaction medium of the beads and in the culture medium containing the free cells.

For protein determination in beads, the method described by Mollaei et al. [13] was used. One g of beads was sectioned into a number of pieces using a surgical knife, that were then incubated in 3 mL of sodium dodecyl sulphate (SDS) solution (10%) during 2 h for protein extraction. After centrifugation, proteins were determined by Bradford method using bovine serum albumin as a standard [14]. To determine protein in the medium containing free cells as well as in reaction media of the beads, 2 mL of culture were centrifuged at 10,000 rpm and the pellet was suspended in 3.5 ml of distilled water. Then, SDS solution was added to this solution as it was described before.

In order to determine the viable cell number contained into the beads, the method described by Sossa Urrego et al. [15] was used. Ten alginate beads were rinsed twice with a sterile 0.9 % NaCl solution and resuspended in 250 μ l of sterilized 0.16 M phosphate buffer, pH 7.4. The suspension was homogenized to achieve a complete dissolution of the alginate, and the viable cells number (CFU/ml) was determined by plating diluted cell suspensions on TY plates. The colonies were counted on agar plates after incubation during 24 h at 28 ± 2 °C. All determinations were made by triplicate. Also, appropriate dilutions of free cell cultures were used to determine viable cell number of the controls.

2.6 Toxicity evaluation of phenol biodegradation products

The toxicity of phenol solutions after treatment with *Acinetobacter* sp. RTE1.4 was evaluated using Microtox Acute Toxicity Test (Microtox®).

Removal reactions were carried out in Erlenmeyers flasks containing MM supplemented with phenol (600 mg/L) and inoculated with *Acinetobacter* sp. RTE1.4 (10% V/V, OD_{620 nm} 0.9 ± 0.1 equivalent to $2.2\pm 0.87\times 10^{11}$ CFU/mL). They were incubated at 28 ± 2 °C in an orbital shaker at 100 rpm until phenol was completely degraded. Then, they were centrifuged during 20 min at 1,000 rpm and the supernatants were used for toxicity evaluation. These solutions were named as post-removal solutions (PRS). Moreover, the toxicity of MM and MM plus 600 mg/L phenol were also evaluated as controls.

Microtox® test is based on measuring changes in the light emitted by a nonpathogenic naturally luminescent marine bacterium (*Vibrio fischeri*) upon exposure to toxic substances. The response to toxicants affecting the bacterial metabolism is observed as a reduction in luminescence, which is a by-product of cellular respiration. This change is measured after 30 min and can be directly correlated to toxicity. The test was performed following the Standard Protocols and the results were expressed as percentage of effective concentration (EC50 %) [16,17].

2.7 Analytical methods

2.7.1 Determination of phenol metabolism intermediates

HPLC analysis was carried out in a Perking Elmer Clarus 500 chromatographer attached to a PDA UV detector at 280 nm by injecting 10 μ L of samples onto a Waters Chrompax C18 analytical column (4.6 mm internal diameter x 150 mm length). The mobile phase consisted of water with 0.1% (v/v) of acetic acid delivered at 1.0 mL/min. The column was held at a temperature of 30 °C. The total chromatographic run time was 27.0 min. Solvents were of HPLC grade and commercial standards of phenol (Merck), catechol (Fluka AG) and *cis,cis*-muconic acid (Sigma, Aldrich, Argentina) were used to detect the compound by co-elution.

2.7.2 Biomass determination

Samples of bacterial cultures were taken and evaluated for biomass estimations with a Beckman spectrophotometer DU640 by measuring its OD_{620 nm} [18,19]. These values were converted to dry weight by a calibration curve, which was obtained by plotting dry weight of biomass vs. OD_{620 nm}. For determinations of dry weight, culture samples were centrifuged and dried subsequently at 80 °C until a constant weight was obtained.

2.7.3 Phenol determination

Phenol determinations were carried out following the method of Wagner and Nicell [20]. Aliquots of 100 µl of each sample were mixed with 100 µl of 4-aminoantipyrine (20.8 mM), 100 µl potassium ferricyanide (83.4 mM) and 700 µl of 0.25 M sodium bicarbonate (pH 8.4). After five minutes, the absorbance of the coloured compound formed was determined at 510 nm. Phenol concentrations were calculated using a calibration curve, which was performed using known concentrations of pure phenol.

2.8 Statistical analysis

Statistical analysis was performed using STATISTICA 7.1 software package. All data were analyzed using ANOVA. In all cases differences between data were considered statistically significant at $p \leq 0.05$. *Dunnnett* test was used to compare several treatment groups with a control.

3 RESULTS AND DISCUSSION

3.1 Identification of the strain at the species-level

In the present work we show complementary new results to improve the identification of *Acinetobacter* sp. RTE1.4 strain at species level, because the 16S rRNA gene is not sufficiently polymorphic to clearly distinguish all closely related genomic species of *Acinetobacter* [12 and references there in]. Thus, we analyzed the sequence of the housekeeping gene *recA*, which seems to be one of the most useful tools for the identification and taxonomic classification of various bacterial species, including *Acinetobacter* spp [21,8]. The *recA* gene was amplified and sequenced using the pair of primers rA1 and rA2. A fragment of 356 bp (Fig. 1) was obtained, sequenced and subsequently aligned using online BLAST tool. It had a 99% sequence similarity with different strains of the *tandoii* species. The gene sequence was deposited in GeneBank (accession number JX258136).

A phylogenetic tree was constructed based on sequences of this gene. Fig. 2 shows the phylogenetic relationship of *Acinetobacter* sp. RTE1.4 with other species of *Acinetobacter* genus. As it can be seen in the figure, the *recA* gene sequence of *Acinetobacter* sp. RTE1.4 showed closest relation with two strains of *A. tandoii*, CT33 and CCUG 56317, forming a cluster in which the homology between them was high (99%).

In addition, a rapid, high-throughput identification method, mass spectrometry (MALDI-TOF- MS) was used to confirm this results because it has been demonstrated to be effective for the identification of several microorganisms. The main advantages of MALDI-TOF MS are that species can be routinely identified quicker than with DNA sequence analysis, costs are not extremely high and no external conditions such as culture medium or conditions can affect the

result. As was pointed out earlier score values ≥ 2.0 were considered appropriate for identification at species level. Thus, the obtained score (>2.05) yielded high-confidence identification at the species level for the studied strain with 100% concordance with the molecular method. Therefore, the studied strain displayed identical identification at genus and species level using a combination of genetic and spectrometric analysis, resulting in a correct identification of the strain, as *A. tandoii*.

Thus, the results presented in this work, along with those of the 16S rDNA gene sequencing and biochemical tests previously performed [7], confirmed that the studied strain belongs to *Acinetobacter* genus and, particularly, corresponds to *tandoii* species. Because of this, it was named as *Acinetobacter tandoii* RTE1.4.

3.2 Evaluation of reaction intermediates of phenol metabolism

Previously, we proposed that *Acinetobacter* sp. RTE1.4 degrade phenol by the *ortho*-pathway [7]. In the present work we try to find out the presence of specific metabolites of this path by HPLC analysis of the supernatants of samples, in which phenol was almost degraded.

A single chromatographic condition, showing high signal-to-noise ratio, was optimized in order to detect the presence of catechol (tR 10.76 min), *cis,cis*-muconic acid (tR 15.51 min) and phenol (tR 22.78 min) in the above mentioned matrix. An example chromatogram is shown in Fig. 3 where peaks 1, 3 and 5 correspond to catechol, muconic acid and phenol, respectively, while peaks 2 and 4 are unidentified products, assumed to be other intermediate compounds produced during phenol cleavage.

In our previous studies, we detected only catechol 1,2-dioxygenase activity in *A. tandoii* RTE1.4, suggesting that this strain would metabolize phenol by the *ortho*-pathway [7]. In the present work, the presence of *cis,cis*-muconic acid and catechol in the metabolized media confirmed this hypothesis.

This result is in agreement with those described by Saxena et al. [21], which showed the presence of catechol as a phenol degradation product in culture medium of *Acinetobacter* strains. However, these authors could not demonstrate the presence of *cis,cis*-muconic acid, as it was observed in the present work. In this sense, the *cis,cis*-muconic acid production has been reported for different microbial strains that degrade phenol by *ortho*-pathway [22, 23,24] but, to our knowledge, the finding of this specific intermediate as result of phenol degradation by *Acinetobacter* strains was not reported so far.

3.3 Phenol biodegradation in a stirred tank bioreactor

Phenol degradation by *A. tandoii* RTE1.4 was also evaluated in a 5 L-stirred tank bioreactor operating in a batch system. In these experiments, different aeration and agitation conditions were assayed in order to improve removal efficiencies of higher volumes (2 L) of solutions containing phenol compared with those obtained in Erlenmeyers flasks [8].

When aeration of 1 vvm was used (Fig. 4a), no difference was observed in the removal efficiency between the three agitation rates used (200, 400, and 600 rpm) until 4 h of cultivation. However, after 6 to 8 h, different phenol removal efficiencies ($p \leq 0.05$) were found and complete phenol degradation was attained after 7 h of treatment at 600

rpm whereas at 200 and 400 rpm the remaining phenol was completely removed after 8 h. The growth of the microorganism increased as phenol biodegradation occurred, reaching similar dry weight values for the three assayed agitation rates at the end of these assays ($p>0.05$) (Fig. 4b).

When aeration was increased to 3 vvm (Fig. 4c), 93.5% of degradation was observed after only 6 h under 600 rpm, reaching complete phenol biodegradation after 7 h under this condition. When agitation rate was decreased (200 and 400 rpm), complete removal of the contaminant was achieved (8 h). DW was similar at end of the assay (0.2 g/L) for 200 and 400 rpm and was highlight higher when 600 rpm of agitation were used, although this difference was not statistically significant ($p>0.05$).

Phenol removal was also evaluated in a bioreactor operating without bacterial inoculation under all agitation and aeration conditions used. Only an insignificantly loss of the contaminant (means of 8%) was observed as a result of evaporation ($p>0.05$).

These assays demonstrated the possibility of process scale, allowing the efficient removal of 2 L of phenol contaminated solution. As it was shown, complete phenol (200 mg/L) degradation by *A. tandoii* RTE1.4 was detected in short periods of time under the assayed experimental conditions, however the process was faster (in only 6 h) at high agitation rates (600 rpm) and air flows (3 vvm). Similar results were found by Agarry et al. [25], since the optimum process conditions for maximizing phenol (100 mg/L) removal by a *Pseudomonas fluorescens* strain were: aeration 3.0 vvm and agitation 300 rpm. However, this bacterium was able to reach lower percentage of phenol degradation (60.7%) compared with that reached with *A. tandoii* RTE1.4 for 200 mg/L of phenol. Using other kind of bioreactor such as batch jet loop bioreactor (JLB) with activated sludge and mixed culture, a complete degradation of phenol up to the initial concentration of 500 mg/l occurred in less than 5 h [26].

The higher removal at high agitation values and air flows found for *A. tandoii* RTE1.4 could be attributed to the high requirements of forceful agitation and oxygenation of the strain for an efficient performance of the biochemical process. In this sense, Hoq et al. [27] indicated that an increase in agitation rates and air flows may lead to a high biodegradation rate due to a better mass transfer, allowing more dissolved oxygen available for microorganism metabolism. Furthermore, it has been described that a low agitation may result in the formation of regions of poor nutrient content or inadequate temperature or pH. Therefore, as a general result, the productivity of the process declines [28]. It is noteworthy that biomass resisted the hydrodynamic stress produced by high turbulence of the media, which is relevant because it has been described that several strains are affected by this conditions and die during the process [25,29,30]. However, it is important to remark than the difference in the required time for a complete phenol removal comparing all the conditions (different rpm and vvm) was 1 to 2 hours between the more and less favorable condition, thus this gain in time would be less important considering the high energy consumption. The energy consumption of a bioreactor system should be minimized to enable a sustainable operation, thus 1 vvm and 200 rpm could be selected to reach a complete phenol removal of 2 L contaminated solution in only 7 h using *A. tandoii* RTE1.4 with lower energy cost compared with the other probed conditions.

The behavior of *A. tandoii* RTE1.4 in the bioreactor differed from those previously obtained in Erlenmeyer flasks [7] since the degradation of 200 mg/L of phenol was faster in bioreactor, allowing a reduction of 85% in the time required for complete degradation comparing with Erlenmeyers. This result clearly shows the importance of a proper selection of agitation and aeration conditions, as was previously demonstrated for other bacterial strains [31,32].

Therefore, the results obtained in the bioreactor allowed us to know the conditions of agitation and aeration to maximize growth and degradation metabolism. These findings are relevant taking into account that this bacterium was also able to remediate different industrial effluents [33], which are frequently treated using bioreactors. However, other more easily scalable alternative and of practical use can be packed bed reactor using bacterial associated in biofilm or immobilized in different matrix to reach high contaminant removal from wastewater [34]. In this sense, operation variables of a batch reactor such as impeller speed and aeration rate, that can make it difficult to scale the process and limit its use should not be a problem.

3.4 Phenol biodegradation using alginate immobilized cells

3.4.1 Phenol removal evaluation

Phenol biodegradation by immobilized bacteria was studied and compared with the catalytic efficiency of free cell cultures (Fig. 5).

Immobilized cells completely degraded 200, 600 and 1,000 mg/L of phenol in 3 d while free cells removed only 83, 70 and 45% of initial phenol respectively, even after 6 d of incubation ($p \leq 0.05$) (Fig 5a). Due to these results, it was decided to increase the concentration of the contaminant to 2,000 and 2,500 mg/L where immobilized cells removed 64 and 22.6% of phenol, respectively, after 20 d of treatment ($p \leq 0.05$) whereas free cells were unable to degrade significant amount of the contaminant (Fig 5b).

Protein content was also evaluated at the beginning and at the end of each assay (Table 1). The protein concentration of the beads containing *A. tandoii* RTE1.4 cells as well as of the reaction medium did not show significant changes ($p > 0.05$) at the end of the experiment for all the evaluated concentrations. Contrarily, in free cell cultures a significant increase ($p \leq 0.05$) in protein content was detected for all studied phenol concentrations.

Viable cell number was also determined in alginate beads and free cell cultures. Inoculated beads contained 2×10^{12} CFU/mL while the free cell inocula contained 2.2×10^{11} CFU/mL. These results correlate with protein content previously determined, because biomass was higher in beds than in free cells extracts. Protein content and viable cell number were not detected in the reaction media of the beads indicating that there was not cell leakage from them.

No significant phenol loss ($p > 0.05$) by calcium alginate adsorption or by evaporation was detected in abiotic controls.

The results obtained using immobilized *A. tandoii* RTE1.4 were consistent with previous reports which indicate that bacterial cells immobilized in different matrices have advantages compared with free cell cultures such as higher tolerance and removal efficiencies to different contaminants [35,36,37]. Besides, alginate has been described as an appropriate matrix for bacterial cells immobilization [13,38,39], as it was also observed in this work. Immobilized *A. tandoii* RTE1.4 was able to degrade phenol even at high concentrations as 2,000 mg/L, concentration at which free cells were unable to significantly degrade phenol. Similarly, Pishgar et al. [40] found that removal efficiency of phenol at 1000 mg/L was enhanced from 10 to about 40% in the presence of immobilized cells compared with free cells, although the percentage of removal was lower compared with immobilized *A. tandoii* RTE1.4 cells.

In this sense, different authors have demonstrated the reduction in phenol degradation due to its toxic effects at high concentrations when free cells are used, which can be reduced when bacteria are immobilized. Probably, it can be

attributed to the high cell density contained in beads as well as to the protection given by the polymeric matrix [41]. In the present work, a higher cellular density was detected in alginate beads compared to free cell cultures, which could explain the high phenol degradation observed. Moreover, protein content in beads and in reaction medium remained constant, suggesting that the beads maintain their integrity during the biodegradation process, without release of cells to the reaction medium. Thus, immobilization represents an advantage for bioremediation processes because cells remain entrapped in the polymeric matrix that provides protection to the microorganisms, ensures their prolonged viability and allows the repeated use of their catalytic activity.

In addition, immobilization can also be considered for the scale up of the process in a bioreactor. Gonzalez et al. [42] applied calcium-alginate gel beads of pure strain of *Pseudomonas putida* for degrading phenol in a fluidized bed reactor. Over 98% of phenol with concentration of 250-2000 mg/l was removed by immobilized bacteria. Among the immobilized cell bioreactors, no doubt the fluidized or semi-fluidized bed bioreactor is a novel and efficient one, which can be adopted for the treatment of industrial wastewater containing phenolic compounds and other pollutants even at lower concentration. However, a proper choice of immobilized culture and careful consideration of various design parameters should be considered in order to make the remediation process cost effective in the long run [43].

3.4.2 Reusability and stability during storage of immobilized cells

Taking into account that one of the advantages of immobilized cells is their reusability, immobilized *A. tandoii* RTE1.4 cells were used for repeated cycles of phenol biodegradation. The beads were used for 4 consecutive cycles, in which the rate of the process was significantly reduced ($p \leq 0.05$) over time from 15.4 ± 1.6 to 12.4 ± 4.7 mg/L h.

On the other hand, phenol biodegradation capability of *A. tandoii* RTE1.4 immobilized cells was evaluated after different storage times at 4 °C. It was observed that immobilized bacteria retain their catalytic activity for 9 months, in which the maximum degradation efficiency was kept (100%). This high stability during storage at 4 °C indicates that the enzymatic activity was maintained for many months.

The reusability and stability of immobilized cells during storage are essential factors for their applicability in a bioremediation system. It is important to note that the removal rate of the contaminant by *A. tandoii* RTE1.4 immobilized cells was progressively reduced during the experiment, indicating a decrease of the enzymatic activity and, possibly, of the cellular viability. These results can be probably attributed to the interference of some reaction products adsorbed to the matrix, which modify its mechanic stability, altering the diffusion of the contaminant or reducing the catalytic activity of the involved enzymes as was also described for other immobilized bacteria [41]. Thus, it would be the reason for the reduction in the process rate. However, it is noteworthy that, after four cycles, all phenol was completely degraded even at low rate.

Thus, immobilized cells of *A. tandoii* RTE1.4 could be applied for efficient remediation of high phenol concentrations that free cells could not remove. They could also be reused by four consecutive cycles and be stored at 4 °C by 9 months, which emphasizes the advantage of bacterium immobilization in calcium alginate for phenol removal.

3.5 Toxicity evaluation of phenol biodegradation products

Following the phenol biodegradation process, it is interesting to evaluate the toxicity of the PRS in order to establish if they are safe and can be released into the environment. These studies were carried out through the Microtox test which is a standard method for determining the toxicity of aqueous wastes before and after biological treatments. The obtained results are shown in Table 2.

Culture media showed an EC_{50} higher than 90%, which indicates a low reduction of the luminescence of *V. fischeri* and, consequently, a low toxicity. When these media were supplemented with 600 mg/L of phenol, a high reduction of the luminescence was observed reaching values of EC_{50} of only 3.1%. However, when these solutions were treated with *A. tandoii* RTE1.4, a significant increase in the EC_{50} was achieved, reaching similar values than the culture medium without the contaminant.

These results clearly indicated that PRS did not produce toxic effects on the exposed organism, which highlight the detoxificant effect of *A. tandoii* RTE1.4 treatment. In addition, it was possible to suggest that during degradation reactions, toxic intermediates would not be produced, at least for *V. fischeri*. The toxicity of remaining solutions, after a bioremediation processes, may differ depending on the studied organism. Hence, a set of toxicity assays including different organisms could be used for the evaluation of the toxic impacts of PRS derived from *A. tandoii* RTE1.4 treatment. These studies are now being carried out in our laboratory to confirm if this treatment could be applied, without negative impacts on the environment.

Clearly, the integration of chemical analysis, ecotoxicity, and remediation potential data is required to properly assess ecological risk in the management and treatment of contaminants. Despite this, the results obtained in the present work until now could be considered sure since Microtox[®] is a validated technique for bioremediation studies and it is currently recommended by the US EPA [44,45,46].

4. CONCLUSION

In this work, a combined approach of molecular tools and MALDI-TOF MS strategy was used to accurately identify the studied strain at species level as *Acinetobacter tandoii* RTE1.4. This identification was achieved through the analysis of the housekeeping gene *recA*, one of the most useful tools for the identification and taxonomic classification of various bacterial species, including *Acinetobacter* sp and, also, by MALDI-TOF MS, which is increasingly used in the field of bacterial taxonomy.

This strain was capable to efficiently degrade phenol by *ortho* fission of the aromatic ring, producing intermediates such as catechol and *cis,cis*-muconic acid. The results showed that bioremediation process could be scaled up in a 5-L bioreactor reaching complete phenol removal of 2-L contaminated solution (phenol 100 mg/L) in short time. However, the air flow and agitation speed conditions determined as optimal for an efficient phenol removal in the batch reactor should be selected considering a lower energy cost.

Immobilization of *A. tandoii* RTE1.4 was shown as a possible strategy to reach efficient remediation of high phenol concentrations, even with the advantage that beads of calcium alginate containing the bacterium could be reused by four consecutive cycles and stored at 4 °C by a long period (9 months) without losing activity.

Considering that toxicity testing should be an integral part of a bioremediation process we evaluate the presence of toxic intermediates after phenol removal. The toxicity test (Microtox[®]) revealed that phenol treated solutions with *A. tandoii* RTE1.4 were not toxic, ensuring the safety of the treated solutions.

The performance shown by *A. tandoii* RTE1.4 makes this strain a good candidate to efficiently remove great volumes of phenol in bioreactors and highly concentrated solutions, even at those concentrations than free cells were not able to remove, with immobilized cells. The good reusability and long life of immobilized cells represent an interesting advantage for the possible application to real contaminated samples remediation, as wastewater, and to be considered for bioreactor design in order to develop a sustainable biotechnology process.

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Figure legends

Fig 1. Electrophoretic analysis of amplified product of *recA* gene by PCR. Lane 1, *Acinetobacter* sp. RTE1.4 genomic DNA; lane 2, molecular weight marker

Fig. 2. Phylogenetic tree based on *recA* gene sequence analysis (1000 bootstrap for the confidence level) showing the relationship between *Acinetobacter* sp. RTE1.4 strain and representative species of *Acinetobacter* genus. The bacterial species are indicated in the figure by their accession number between parentheses followed by the taxonomical identification. The scale bars represents 5 substitutions per 100 nucleotides.

Fig. 3. Chromatographic profile of supernatants of phenol spiked solutions after treatment by *A. tandoii* RTE1.4 culture. Conditions: C18 column; water with 0.1% (v/v) of acetic acid; flow rate: 1.0 mL/min; temperature: 25°C; injection volume 10 µL.

Fig. 4. Degradation of 200 mg/L of phenol (a and c) by *A. tandoii* RTE1.4 and its growth curves (b and d) in a stirred tank bioreactor under different agitation (200-600 rpm) and aeration: 1 vvm (a and b) and 3 vvm (c and d).

Ref.: (a) indicates significant differences ($p \leq 0.05$) of the indicated treatment respect to 400 rpm

(b) and (c) indicate significant differences ($p \leq 0.05$) of the indicated treatments respect to 200 and 400 rpm

Fig. 5. Phenol degradation by free and immobilized cells of *A. tandoii* RTE1.4. a) Phenol 200 to 1,000 mg/L; b) Phenol 2,000 and 2,500 mg/L

Ref.: (*) indicates significant differences ($p \leq 0.05$) between free and immobilized cells for each phenol concentration

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Figure 1

Figure 2

Figure 3

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Figure 4

Figure 5

Table 1. Protein content in beads, reaction medium and free cell cultures of *A. tandoii* RTE1.4 at the beginning and at the end of phenol degradation experiments

Phenol	Protein			
	Time	Beads (mg/g)	Reaction media (mg/mL)	Free cells (mg/mL)
200	Initial value	16 ±1	0.05 ±0	0.51 ±0.04
	Final value	16 ±2	0 ±0	1.03 ±0.1*
600	Initial value	18 ±6	0.03 ±0.009	0.56 ±0.007
	Final value	22 ±4	0.23 ±0.12	2.33 ±0.1*
1,000	Initial value	18 ±7	0 ±0	0.55 ±0.1
	Final value	20 ±3	0.18 ±0.007	2.66 ±0.45*
2,000	Initial value	17 ±5	0.07 ±0.01	0.49 ±0.06
	Final value	20 ±2	0.21 ±0.03	1.9 ±0.34*
2,500	Initial value	19 ±6	0.06 ±0.009	0.58 ±0.08
	Final value	17 ±4	0.20 ±0.09	1.5 ±0.35*

Ref. The values are the media and standard deviations of three replicates

(*) indicates significant differences ($p \leq 0.05$) between initial and final values for each phenol concentration

Table 2. Microtox test for culture medium, with and without phenol, and of PRS

Medium	EC ₅₀ (%)
MM	>90
MM plus phenol (600 mg/L)	3.1*
PRS	>90**

Ref. The values are the media of the three replicates of the assay.

* indicates significant differences ($p \leq 0.05$) between the indicated value and MM

** indicates significant differences ($p \leq 0.05$) between the indicated value and MM plus phenol