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RESEARCH ARTICLE

Isolation and characterization of a *Rhodococcus* strain with phenol-degrading ability and its potential use for tannery effluent biotreatment

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Abstract
Introduction Wastewater derived from leather production may contain phenols, which are highly toxic, and their degradation could be possible through bioremediation technologies.
Materials, methods and results In the present work, microbial degradation of phenol was studied using a tolerant bacterial strain, named CS1, isolated from tannery sediments. This strain was able to survive in the presence of phenol at concentrations of up to 1,000 mg/L. On the basis of morphological and biochemical properties, 16S rRNA gene sequencing, and phylogenetic analysis, the isolated strain was identified as *Rhodococcus* sp. Phenol removal was evaluated at a lab-scale in Erlenmeyer flasks and at a bioreactor scale in a stirred tank reactor. *Rhodococcus* sp. CS1 was able to completely remove phenol in a range of 200 to 1,000 mg/L in mineral medium at 30 ± 2 °C and pH 7 as optimal conditions. In the stirred tank bioreactor, we studied the effect of some parameters, such as agitation (200–600 rpm) and aeration (1–3 vvm), on growth and phenol removal efficiency. Faster phenol biodegradation was obtained in the bioreactor than in Erlenmeyer flasks, and maximum phenol removal was achieved at 400 rpm and 1 vvm in only 12 h. Furthermore, *Rhodococcus* sp. CS1 strain was able to grow and completely degrade phenols from tannery effluents after 9 h of incubation.
Conclusion Based on these results, *Rhodococcus* sp. CS1 could be an appropriate microorganism for bioremediation of tannery effluents or other phenol-containing wastewaters.

Keywords Phenol removal · Bioremediation · Phenol tolerance · Catechol 1,2-dioxygenase · Tannery effluents · Bioreactor

1 Introduction

Phenol and its derivatives are environmental pollutants of worldwide distribution and high toxicity (Quiao et al. 2006; Paisio et al. 2009). They are considered as priority pollutants by the US Environmental Protection Agency (US EPA 1979) and Agency of Toxic Substances and Disease Registry (ATSDR 1998). Phenol contamination comes from many industries, such as petrochemical plants and oil refineries, chemical industries, explosives and dye manufacturing, wood preservatives, pulp and paper production, and coal conversion processes. In addition, it is well known that leather industries produce complex wastewaters containing not only chromium but also phenol at high concentrations (Reemtsma and Jekel 1997; Chandra et al. 2011). Nowadays, in Argentina, leather industry comprises more than 200 companies which represent a serious problem because they cause soil and water pollution (http://www.argentinaarde.org.ar/index.php?option=com_content&task=view&id=83&Itemid). Therefore, removal of toxic compounds like phenol and their derivatives from

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tannery effluents as well as from other wastewaters is highly relevant in order to reduce contaminant levels to environmentally tolerable limits.

Several researchers have focused their studies in searching for environmentally friendly, economical, and sustainable treatments to mitigate this problem. In this sense, bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using biological activities. As such, it implies relatively low costs and low technology techniques carried out in situ, which generally have high public acceptance. However, it may not always be suitable for a wide range of concentrations. In addition, time required for an efficient bioremediation process is relatively long, and residual contaminant levels may not always be as low as expected (Vidali 2001). Therefore, finding organisms that successfully solve these drawbacks and allow achieving a satisfactory result represents an important challenge.

In this context, microorganisms, due to their biodiversity, versatility, and great catabolic potential, have been useful for biodegradation of several pollutants. The main efforts have been usually focused on selection of microorganisms with the ability to degrade pollutants from industrial wastes and contaminated environments.

Regarding tannery wastewaters, biodegradation of its different hazardous constituents has been evaluated in several studies. In particular, those related to the biodegradation of its organic constituents have been of relevant concern (Shah and Thakur 2002; Srivastava et al. 2007; Cokgor et al. 2008).

Members of *Rhodococcus* genus were soon regarded as one of the most promising groups of organisms suitable for biodegradation of a large number of organic compounds, including some highly recalcitrant and toxic (Warhurst and Fewson 1994). However, to the best of our knowledge, there are few studies related to phenol biodegradation and, even more, to bioremediation of tannery effluents using *Rhodococcus* strains. Thus, this is an evolving field of environmental biotechnology, and its success depends on the increase in our fundamental knowledge of the main variables and optimal conditions that allow a proper growth and, hence, a high removal efficiency. In the present work, a *Rhodococcus* strain was isolated from tannery sediment and used to degrade phenol in artificially contaminated solutions as well as in complex tannery wastewaters. The identification and physiological and biochemical characterization of the isolated bacterium were carried out. The effect of parameters such as pH, temperature, and initial phenol concentration on removal capability of this strain and the possible degrading pathways were evaluated. In addition, scale up in bioreactor and effect of agitation and aeration on phenol degradation were also studied. Finally, removal efficiency of this strain was analyzed in solutions containing tannery effluents.

2 Materials and methods

2.1 Collection and characterization of wastewater and contaminated sediment

Sediment and effluent samples were collected from an accessible discharge point from a tannery located in Elena, Córdoba Province, Argentina (32° 34′ South latitude and 64° 23′ West longitude) in order to isolate phenol-tolerant bacterial strains. Samples were brought to laboratory, kept at 4 °C for analysis, including quantitative determination of total phenol content, which was determined as described in the following.

2.2 Isolation of microbial strains and evaluation of phenol tolerance

Thirty grams of sediment was resuspended in sterile saline water (NaCl 0.9 %) and shaken for 30 min. Then, the suspension was sedimented, and the supernatant was filtered. A pure sample and an appropriate dilution (10^{-1}) of this supernatant were spread on agar plates with TY media [(g/L): 5 triptein; 3 yeast extract; 0.65 $CaCl_2$; 13 agar] supplemented with 100-mg/L phenol (Merck) in order to select phenol-tolerant strains. Cultivation was carried out for 48 h at 25±2 °C. Morphologically different colonies were transferred to solid mineral media [MM (g/L): 0.3 $MgSO_4$; 0.01 $FeSO_4$; 0.5 NaCl; 3 NH_4Cl; 0.01 $CaCl_2$; 1.5 K_2PO_4H; 0.5 KPO_4H_2] plates with phenol (100 mg/L) as sole source of carbon and energy. The colonies, which show a wide growth in this medium, indicated utilization of phenol by bacteria. On the basis of fastest growth in this medium, a strain was selected for further studies and named CS1.

The selected bacterium was spread in solid MM supplemented with phenol at concentrations from 100 to 1,000 mg/L in order to determine its maximum phenol tolerance. This was established as the highest concentration of the contaminant at which bacterial growth could be observed after 7 days.

2.3 Characterization and identification of the bacterial strain

CS1 strain was characterized based on its morphological, physiological, and biochemical features. The isolate was characterized by Gram staining and oxidase and catalase activities. Oxidase activity was determined using oxidase disks (Britania). Catalase activity was evaluated by transferring one single colony onto a microscopy slide and adding a drop of 3 % hydrogen peroxide solution.

Biochemical analysis was done using API 20 NE kit system (BioMerieux® SA). Protocols were followed according to the manufacturer's instructions. Results of the

biochemical test were analyzed with API web program, which allows identification of the studied microorganism.

2.4 PCR amplification of 16S rRNA and phylogenetic analysis

Identification of CS1 strain through amplification of 16S rRNA gene was done by PCR using a pair of forward (5′-CCAGCAGCCGCGGTAATACG-3′) and reverse (5′-TACCAGGGTATCTAATCC-3′) primers after genomic DNA extraction. 16S rRNA gene was sequenced by "MacroGen" company (Seoul, Korea). The sequence was compared and identified using BLAST (Altschul et al. 1997) and RDP (Cole et al. 2009) programs.

A phylogenetic tree was constructed based on the 16S rRNA gene sequences. First, multiple alignments of different 16S rRNA gene sequences downloaded from GenBank were performed using CLUSTAL-X software. Evolutionary distances were calculated by Tamura-Nei model. Phylogenetic tree was constructed by Neighbor-Joining method by MEGA4 software (Tamura et al. 2007), and a bootstrap analysis of up to 1,000 iterations was carried out.

2.5 Enzyme assays

Catechol 1,2- and 2,3-dioxygenase activities were determined as previously described (Pradhan and Ingle 2007). Briefly, CS1 strain was grown in liquid MM containing 600 mg/L of phenol until it reached 50 % of phenol removal. Then, the medium was centrifuged, and the cell pellet was suspended in Tris–HCl buffer pH 9, sonicated and finally centrifuged to obtain a supernatant which was considered as crude extract and used for determination of enzyme activities.

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were determined spectrophotometrically by measuring their reaction products cis,cis-muconic acid at 260 nm and 2-hydroxymuconic semialdehyde at 375 nm, respectively. The reaction mixture consisted of potassium phosphate buffer (0.1 M, pH 7.5), 55 μL of the crude extract, and 7.5 μL of 10-mM catechol as substrate. One unit (U) of enzyme activity was defined as the amount which catalyzed the formation of 1 μmol of product/min at 25 °C. Specific activity was calculated as units per milligram of protein. Proteins were determined by Bradford method, with bovine serum albumin as a standard (Bradford 1976).

2.6 Effects of pH, temperature, and initial phenol concentration on phenol biodegradation

Optimum pH and temperature for growth and phenol degradation of CS1 strain were determined. Then, using these optimal conditions, the effect of initial phenol concentration was evaluated.

For optimum pH and temperature determination, CS1 cultures at exponential phase of growth in TY medium without phenol were inoculated in 100-mL Erlenmeyer flasks containing 30 mL of MM plus 200 mg/L of phenol as sole carbon and energy source. Initially, the inoculum had an optical density (OD) of 0.05. Cultures were stirred in a shaker at 100 rpm.

First, the experiments were carried out at several temperatures, ranging from 25 to 40 °C and keeping the pH value constant (pH=7), to find the optimum temperature for microbial growth and phenol biodegradation. In the same way, further experiments were carried out at different pH values, ranging from 5 to 11, to find out the optimum pH, maintaining constant the optimum temperature previously selected. After incubation, dry weight (DW) and residual phenol concentration were determined, as it is described in the following. Then, the potential of CS1 strain for phenol degradation and the effect of the initial concentration on its degradation ability were evaluated in liquid MM initially containing 200- to 1,000-mg/L phenol, under optimal pH and temperature conditions. DW and residual phenol concentration were determined at different time intervals until complete degradation.

All of the experiments were carried out by triplicate, and non-inoculated controls were evaluated in each assay in order to check phenol concentration (abiotic control).

2.7 Phenol biodegradation in a stirred tank bioreactor

Phenol degradation assays were carried out in a bioreactor to determine the optimal agitation and aeration conditions to maximize phenol removal by the isolated strain.

A 1.5-L stirred tank bioreactor (BIOFLO III, New Brunswick Scientific, USA) consisting of a glass vessel, four baffle plates, a multiport stainless steel head plate, and a six-bladed disc turbine impeller was used. A stainless steel sparger located below the turbine was used to aerate cultures. The bioreactor was operated in batch mode with an initial phenol concentration of 200 mg/L at pH 7 and 30±2 °C under different rotational speeds and airflow rates: 200, 400, and 600 rpm and 1 and 3 vvm, respectively. A volume of 1,000 mL of MM was inoculated with 50 mL of CS1 culture, at exponential phase of growth, in TY medium without phenol. Non-inoculated controls were also assessed. Culture samples were withdrawn every hour and tested for DW and residual phenol concentration, as it is described in the following.

2.8 Phenol biodegradation of tannery wastewater

The capability of CS1 strain to degrade phenols contained in a tannery wastewater was assayed. The effluent was

used pure (100 %) and diluted with sterile distilled water (25, 50, and 75 % V/V). Five milliliters of bacterial inoculum, at exponential phase of growth, was added to 250-mL Erlenmeyer flasks containing 100 mL of non-sterilized effluent. A non-inoculated flask was evaluated simultaneously, under the same conditions, in order to check phenol biodegradation performed by native microorganisms usually found in tannery effluent. Flasks were kept on an orbital shaker at 200 rpm and 30±2 °C. Culture samples were collected at regular time intervals to determine total phenol contents and $OD_{620\ nm}$ measurements. Experiments were done by triplicate. Proper blanks, containing pure or diluted tannery effluent, were performed in order to subtract the absorbance due to the effluent.

2.9 Analytical determinations

In all biodegradation experiments, samples of bacterial cultures were taken and evaluated for biomass estimations and for the determination of residual phenol or phenolic compound concentration.

Cell density was estimated with a Beckman spectrophotometer DU640 by measuring OD at 620 nm. For biomass estimation, $OD_{620\ nm}$ was 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 at 80 °C until constant weight.

For residual phenol determinations, culture samples were centrifuged at 10,000 rpm for 5 min, and supernatants were used. A spectrophotometric assay (Wright and Nicell 1999) based on phenol rapid condensation with 4-aminoantipyrine (20.8 mM), followed by oxidation with potassium ferricyanide (83.4 mM) under alkaline conditions (sodium bicarbonate 0.25 M, pH 8.4) to give a red color product, which was measured at 510 nm, was used. Phenol concentrations were calculated using a calibration curve, which was carried out with known concentrations of pure phenol.

In sediment samples, total phenol concentrations were determined through a Gas Chromatography-Flame Ionization Detector (GC-FID) method, by service of IACA Laboratory (Bahía Blanca, Argentina).

2.10 Statistical analysis

Statistical analysis was performed using STATISTICA 7.1 software package. All of the data were analyzed using ANOVA. In all cases, $p \leq 0.05$ was statistically significant. Dunnett test was used for comparing several treatment groups with a control.

3 Results and discussion

3.1 Characterization of tannery sediments and effluents

Analytical determination of a sediment sample collected from a wastewater discharge channel from a tannery revealed that it contained phenols (0.10 mg/kg of soil) and high levels of metals (data not shown), including chromium at levels that exceeded the acceptable environmental standards. Moreover, total phenol concentration in the effluent was 17.5 mg/L. The levels of phenols detected in the effluent as well as in the sediment were in accordance with the results obtained by several researchers (Reemtsma and Jekel 1997; Khwaja et al. 2001; Chandra et al. 2011). It is important to note that phenol concentrations, detected in our study, exceeded more than 10.000 times the guideline values given by the law of hazardous wastes (National Law from Argentina N° 24.051, Decree 831/93) and those recommended by US EPA (1979) and ATSDR (1998).

3.2 Isolation of phenol-tolerant bacteria from tannery sediments

Isolation of phenol-tolerant bacteria from a sediment sample was carried out. Four different isolates were able to rapidly grow on solid TY medium plates containing 100 mg/L of phenol. Then, to determine whether these isolates were also able to metabolize the contaminant, they were spread in MM agar plates supplemented with 100 mg/L of phenol, as sole carbon and energy source. Only one isolate, denominated CS1, was able to grow fast, and it was selected for further studies. In addition, CS1 strain was able to tolerate phenol concentrations as high as 1,000 mg/L after 48 h at 25±2 °C, in MM.

Microorganisms living in environments contaminated with tannery wastes have been often isolated, and they have shown high tolerance to the more common pollutants found in these sites, such as chromium and phenolic compounds (Shah and Thakur 2002; Cokgor et al. 2008; Chandra et al. 2011). Therefore, in the present work, it is not surprising that several phenol-tolerant bacteria could be isolated from this sediment. Moreover, CS1 strain was able to tolerate high phenol concentrations in MM (1,000 mg/L), indicating a strong tolerance for this compound. This finding is promising because it indicates that the strain is not only able to use this contaminant as carbon source but also to tolerate and survive at phenol concentrations higher than those found in the sediment sample.

3.3 Characterization and identification of CS1 strain

Morphological, physiological, and biochemical assays showed that CS1 strain was an aerobic, Gram positive,

oxidase-negative, and catalase-positive actinobacteria, which forms small and beige colonies.

Complete details of the results obtained using API 20 NE kit are given in Table 1. Generally, API web system allows us to identify a studied strain based on biochemical characteristics; however, in this case, CS1 biochemical profile showed no concordance with any bacterium included in API web database. This result could be due to the fact that API kits were designed for analysis of clinical rather than environmental samples.

3.4 PCR amplification of 16S rRNA and phylogenetic analysis

In order to identify CS1 strain, 16S rRNA gene was amplified and sequenced. A fragment of 1,305 bp was obtained and subsequently aligned using online BLAST and RDP tools. It had a 99 % similarity with species of *Rhodococcus* genus. The gene sequence was deposited in GenBank (accession number JF521654).

A phylogenetic tree was constructed based on 16S rRNA gene sequences of *Rhodococcus* strains and CS1 strain (Fig. 1). As can be seen in the figure, phylogenetic analysis showed high similarity of CS1 strain with various species of the genus *Rhodococcus*, indicating a close relationship with two species of this genus, *R. erythropolis* ATCC djl-11 and 4277 T, and *R. qingshengii* ZJB-09153. These bacterial species were included in the same cluster. Thus, CS1 strain was consider as a typical member of *Rhodococcus* genus.

Members of the genus *Rhodococcus* represent an abundant part of indigenous bacterial communities occurring in contaminated environments with aromatic pollutants (Fahy et al. 2006; Leigh et al. 2006). Moreover, *Rhodococcus* strains have shown resistance to several toxic xenobiotics and ability to degrade many of these pollutants, including phenolic compounds (Margesin et al. 2005; Rehfuss and Urban 2005).

Table 1 Physiological and biochemical characteristics of CS1 strain. Positive reaction (+); Negative reaction (−)

Characteristics	CS1 strain
Nitrate reduction	+
Indole production	−
D-glucose fermentation	−
Enzyme production	
Oxidase	−
Catalase	+
Arginine dihydrolase	+
Urease	+
β-glucosidase	+
Protease (Hydrolisis of gelatin)	−
β-galactosidase	+
Sugar assimilation	
Glucose	+
Arabinose	+
Mannose	+
Mannitol	+
N-acetyl-glucosamine	+
Maltose	+
Potassium gluconate	+
Acid assimilation	
Capric acid	−
Adipic acid	+
Malic acid	+
Trisodium citrate	+
Phenylacetic acid	+

3.5 Enzyme assays

It is well known that phenol can be metabolized by two major pathways: *ortho* and *meta* cleavage. Phenol hydroxylase is the first enzyme in the metabolic pathway of phenol degradation and catalyzes phenol oxidation to form catechol. In the next step, two enzymes can be induced, catechol 1,2- or 2,3-dioxygenase, which belong to the *ortho* and *meta* ring fission pathways, respectively (Dagley 1971; Harayama and Renik 1989). Thus, to distinguish between *ortho* and *meta* pathways of aromatic ring cleavage, catechol 1,2- and 2,3-dioxygenase activities were measured. A catechol 1,2-dioxygenase activity of 0.20 U/mg could be detected in a crude extract of *Rhodococcus* sp. CS1 cells, indicating that catechol ring fission was performed through the *ortho* pathway. Catechol 2,3-dioxygenase activity was not detected.

These results agree with those described by Margesin et al. (2005) which also showed that four strains of *Rhodococcus* only have catechol 1,2-dioxygenase activity.

3.6 Phenol biodegradation assays

Industrial wastewater treatment plants usually employ microbial oxidative processes. Nevertheless, phenolic compounds can inhibit microbial growth, even in the case of species which are capable of metabolically utilizing these compounds as growth substrates. In this sense, optimization of degradation conditions is of great importance to use this bio-oxidative process in industrial and environmental applications. For this purpose, the effect of pH, temperature, and initial phenol concentration on phenol degradation by *Rhodococcus* sp. CS1 was investigated.

Rhodococcus sp. CS1 strain was able to grow and to utilize phenol in liquid mineral medium, under a wide range of pH and temperature (5–11 and 25–40±2 °C, respectively) (Table 2). This strain showed high removal efficiency (85–100 %) at pH 5–9 and 25–30±2 °C. Optimal pH value for

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequence analysis (1,000 bootstrap for the confidence level) showing the relationship between CS1 strain and representative species of *Rhodococcus* genus. The bacterial species are indicated in the figure by their taxonomical identification followed by the accession number between parentheses. The *scale bars* represent 5 substitutions per 100 nucleotides

maximum growth and phenol removal was found to be near neutral, as it has also been reported for several bacterial strains (Reda and Ashraf 2010; Chakraborty et al. 2010; Banerjee and Ghoshal 2011). However, this strain also showed high removal efficiencies between pH 10 and 11 and at 37 °C, which is an important feature, taking into account that pH and temperature values may vary in different industrial effluents.

According to these results, the selected conditions to perform subsequent assays were a pH of 7 and a temperature of 30±2 °C.

Table 2 Effect of pH and temperature on phenol removal efficiency of CS1 strain

Variable		Removal efficiency (%) ± SE
pH	5	100
	6	100
	7	100
	8	100
	9	97±4.1
	10	70±20.0
	11	60±15.8
Temperature (°C)	25	85±18.0
	30	100
	37	89±10.0
	40	21.5±3.5

Batch tests for evaluation of phenol biodegradation were conducted in Erlenmeyer flasks containing MM with initial phenol concentrations of 200–1,000 mg/L at the selected pH and temperature. For all phenol concentrations, the time required for complete degradation increased as a function of pollutant initial concentration. For instance, at initial phenol concentrations of 200, 400, and 600 mg/L, phenol was totally degraded after 18, 22, and 24 h of incubation, respectively (Fig. 2a). And, what is more, complete degradation of phenol concentrations as high as 800 and 1,000 mg/L was observed after 4 and 5 days, respectively (Fig. 2c). In these figures, it can also be observed that an increment in cell growth (DW) was registered simultaneously with phenol degradation (Fig. 2b, d). Non-inoculated controls showed that only 3 % of phenol was evaporated during the assays.

Therefore, *Rhodococcus* sp. CS1 was able to efficiently degrade phenol concentrations as high as 1,000 mg/L. This degradation ability is very relevant, taking into account that this concentration exceeds more than 50 times phenol level found in the collected effluent. Moreover, these results clearly show that the strain was not only able to tolerate phenol but also to use it as carbon and energy source. Similar results were described by Margesin et al. (2005), which determined that two *Rhodococcus* strains degraded phenol at concentrations of up to 1,000 mg/L reaching high biomass values, as it is shown in our experiments.

In addition, *Rhodococcus* sp. CS1 could tolerate some compounds structurally related to phenol, such as guaiacol,

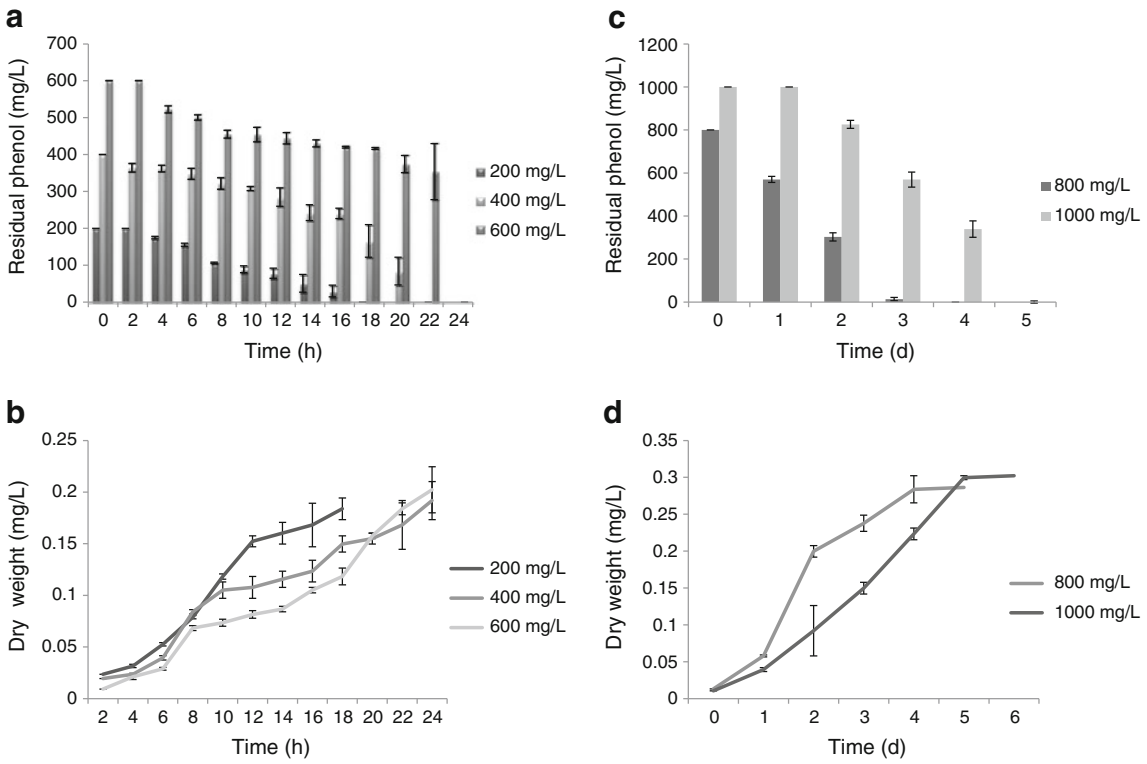

Fig. 2 Phenol biodegradation (**a** and **c**) and growth (**b** and **d**) of *Rhodococcus* sp. CS1 at initial phenol concentrations varying between 200–600 mg/L (**a** and **b**) and 800–1,000 mg/L (**c** and **d**) in Erlenmeyer flasks

2,4-dichlorophenol, and pentachlorophenol at concentrations of 1,000, 500, and 5 mg/L, respectively (data not shown). These results may indicate that the bacterium could be used to treat effluents with a complex composition. However, not all *Rhodococcus* strains have the ability to tolerate chlorophenols. For instance, Sun et al. (2011) reported that *Rhodococcus* strain Chr-9 could not grow in the presence of 2,4-dichlorophenol and *p*-chlorophenol in spite of its high phenol degradation capability.

The fact that the strain was able to grow and to degrade phenol under a wide range of pH and temperature is an advantageous characteristic because these factors play an important role in the establishment and maintenance of microbial communities, involved in the removal of pollutant constituents from effluents or other contaminated environments (Kibret et al. 2000). In this sense, Tadesse et al. (2004) demonstrated that tannery effluents had seasonal and diurnal fluctuations of pH, dissolved oxygen, and temperature. Therefore, it is of great interest to establish the effect of these and other variables, such as oxygenation and agitation, since they can contribute to optimize and enhance the removal process.

3.7 Phenol biodegradation in a stirred tank bioreactor

Phenol degradation by *Rhodococcus* sp. CS1 was also evaluated in a stirred tank bioreactor operating in a batch system, where good containment and environmental control may allow a fast, complete, and cost-effective treatment of contaminated solutions. Different aeration and agitation conditions were assayed because optimization of these process variables is an essential aspect in order to maximize phenol removal efficiency in a bioreactor.

When aeration of 1 vvm was used (Fig. 3a), no difference was observed in the removal efficiency between the three agitation rates used (200, 400, and 600 rpm) until 11 h. However, up to this time (12–14 h), different phenol removal efficiencies were found, and complete phenol degradation at 200, 400, and 600 rpm was attained after 13, 12, and 14 h of treatment, respectively. The growth of the microorganisms increased while phenol biodegradation occurred, and at the end of these batch assays, when phenol was consumed, dry weight values varied between 0.13 and 0.18 g/L for all agitation conditions used (Fig. 3b).

Growth and phenol removal efficiency were also evaluated under 3 vvm of aeration (Fig. 3c, d). No difference in phenol biodegradation was found between agitation conditions of 200 and 400 rpm, until 12 h of treatment. However, complete phenol degradation was achieved after 14 and 13 h at 200 and 400 rpm, respectively. When all phenol was degraded, biomass remainder was around 0.18 g/L for the two agitation conditions used. In contrast, under 600-rpm agitation and 3 vvm of aeration, only 38 % of phenol removal was observed after 14 h, and biomass as low as

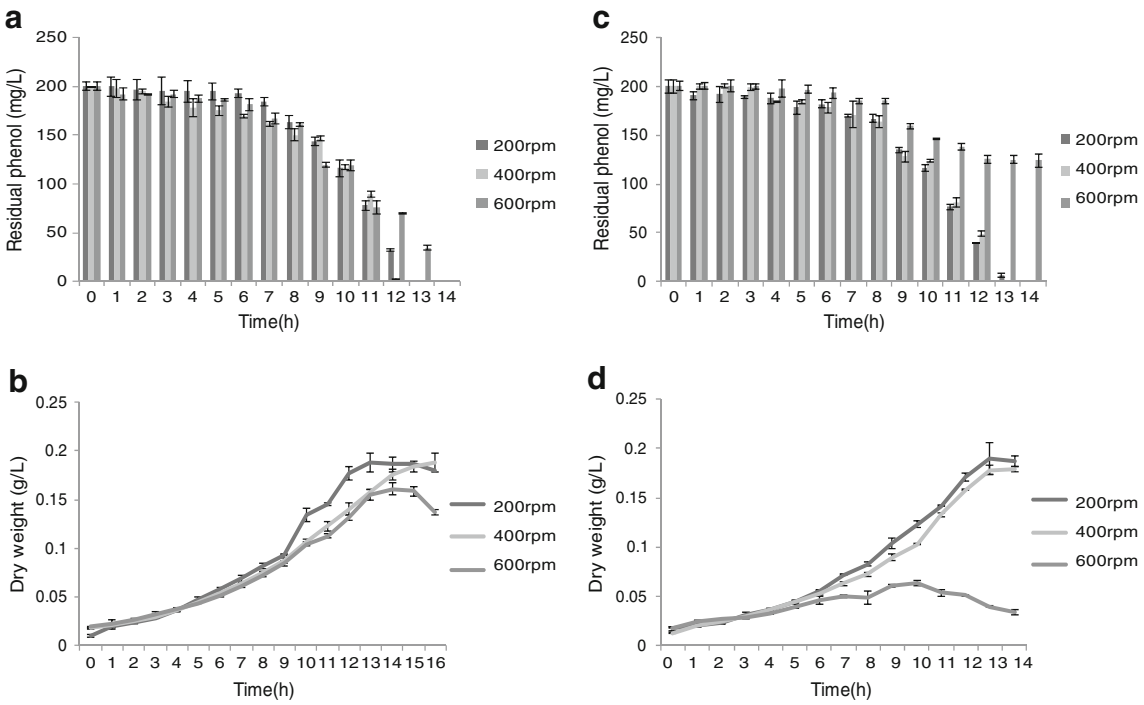

Fig. 3 Degradation of 200 mg/L of phenol (**a** and **c**) by *Rhodococcus* sp. CS1 and their growth (**b** and **d**) in a stirred tank bioreactor under different agitation (200–600 rpm) and aeration: 1 vvm (**a** and **b**) and 3 vvm (**c** and **d**)

0.03 g/L was achieved. This result could indicate that the aforementioned condition of aeration and agitation produced stress in bacteria and probably may result in cell rupture, which inhibited their metabolism and growth and consequently decreased phenol removal efficiency. In this sense, some researchers suggested that excessively high agitation rates should be avoided to prevent attrition and metabolic stress in bacterial population (Enfors et al. 2001; Gonzalez et al. 2003). In addition, Hodaifa et al. (2010) described that conditions of high hydrodynamic stress, in stirred tank bioreactors, could cause cell damage, diminishing the specific growth rate and biomass productivity. However, insufficient agitation may lead to limitations in transfer operations and the appearance of regions of insufficient nutrient content or inadequate temperature or pH (Agarry et al. 2010). For instance, *Ewingella americana* was able to degrade 300 mg/L of phenol at 200 and 250 rpm, with the same efficiency, whereas phenol degradation diminished at lower agitation rates, such as 50 and 75 rpm (Khleifat 2006).

On the other hand, the most adequate aeration must be carefully determined to provide oxygen as demanded by the cell population, but avoiding excessive flow rates that can lead to impeller flooding and cause unnecessarily high operating costs.

Based on the results obtained in the bioreactor, it could be assumed that the best conditions for complete degradation of 200 mg/L of phenol were 1 vvm and 400 rpm, although other assayed conditions showed slight differences in the time required for phenol complete degradation (13–14 h), with the exception of the last condition evaluated (600 rpm and 3 vvm). Thus, *Rhodococcus sp*. CS1 could be able to degrade phenol at a wide range of agitation rates and aeration conditions, which is a promising result for future application in bioremediation processes.

As it could be seen, bioreactor performance differed from that obtained in Erlenmeyer flasks. In this sense, degradation of 200 mg/L of phenol was slower in Erlenmeyer flasks (18 h). However, it is important to take into account that in

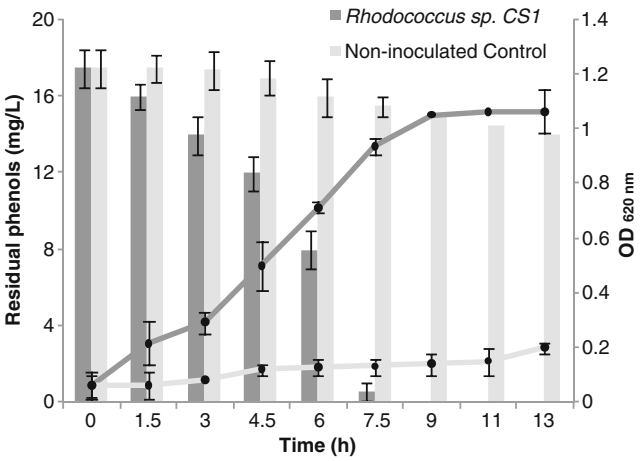

Fig. 4 Phenol degradation and growth of *Rhodococcus* sp. CS1 in a tannery effluent. *Bars* represent the residual phenol concentration, and *lines* correspond to OD values

this system, agitation was maintained constant (100 rpm), whereas oxygenation was not controlled. Contrarily, in bioreactor, the agitation was increased from 200 to 600 rpm, and hence, a reduction in the time required for the complete phenol degradation was observed (12–14 h). Moreover, the effect of aeration on phenol degradation was also demonstrated (degradation was faster at 1 vvm than at 3 vvm). Therefore, agitation and oxygenation control could improve the removal process.

Based on the results obtained in this work, it is possible to highlight that *Rhodococcus* sp. CS1 showed good growth and high phenol degradation efficiency, and hence, it is a promissory strain for treatment of phenol-containing effluents at reactor scale.

3.8 Biodegradation assays in tannery wastewater

In spite of the demonstrated capacity of *Rhodococcus* sp. CS1 to degrade phenol in synthetic culture medium, these results do not ensure that this strain has the ability to biodegrade phenol in tannery wastewaters. Thus, CS1 strain was tested for its ability to grow and to degrade phenolic compounds in a tannery wastewater (pH 9), containing total phenol concentration of 17.5 mg/L, as was previously determined.

The effluent used was pure (100 %) and diluted 25, 50, and 75 % V/V. After 24 h of incubation, CS1 strain grew and degraded all phenolic compounds contained in the tannery wastewater. The achieved OD values were 0.34, 0.47, 0.69, and 1.0 for 25 %, 50 %, 75 % dilution, and pure effluent, respectively. As it was shown, CS1 strain was able to tolerate the pure effluent, showing the highest growth under this condition.

Then, a new experiment was performed using pure tannery effluent to assess phenol degradation during the incubation time (Fig. 4). Complete phenol degradation, by *Rhodococcus* sp. CS1, was achieved after only 9 h of incubation. OD values of 1.06 were observed at the end of the experiment, whereas phenol biodegradation by native microorganisms, at this time, was only 20 %.

Tannery effluents and other industrial wastewaters are frequently the subject of several studies, including those related to bioremediation, with the aim to find a proper removal method. Nevertheless, a few reports on tannery effluent biotreatment are available. Chandra et al. (2011) revealed that most of the organic pollutants detected in tannery effluent could be diminished by bacterial communities in treatment plant. Similarly, Srivastava et al. (2007) showed that a bacterial consortium and a fungal strain were capable to remove chromium and PCP contained in tannery effluents.

To our knowledge, studies related to phenol removal from tannery wastewater by *Rhodococcus* strains have not yet been carried out. However, some *Rhodococcus* strains have been used to treat wastewaters derived from other industries, for instance, hydrocarbon-rich (Gaurgori et al. 2011) and resin manufacture wastewaters (Hidalgo et al. 2002).

It is important to remark that tannery effluents generally contain a complex mixture of phenolic compounds, and their pH usually varies during industrial process (Reemtsma and Jekel 1997; Chandra et al. 2011). In the present study, significant removal of phenols from tannery wastewater, which has a pH value as high as 9, was achieved using *Rhodococcus* sp. CS1. Moreover, this strain showed the ability to degrade high phenol concentrations in artificially contaminated solutions, and this ability was enhanced when the assays were developed in a bioreactor. These findings suggest that this strain could be very useful, from a biotechnological point of view, to treat leather industry effluents, containing high phenol concentrations with high efficiency.

4 Conclusion

In the present study, a bacterial strain isolated from phenol-containing tannery sediments was evaluated for its potential phenol-degrading capability. The strain was identified as *Rhodococcus* sp., and it showed the ability to tolerate and degrade phenol through *ortho*-cleavage pathway with high efficiency. Temperature, pH, aeration, and agitation significantly affected its phenol biodegradation capability. Furthermore, phenol removal by *Rhodococcus* sp. CS1, in a stirred tank bioreactor, was faster than in Erlenmeyer flasks, which can be attributed to an optimization of aeration and agitation conditions. Moreover, the studied strain was able to grow and remove phenols from a tannery effluent. Therefore, this study demonstrates that *Rhodococcus* sp. CS1 could be a promising candidate for rapid and efficient bioremediation of phenol from industrial wastewaters. A more exhaustive investigation on its ability to degrade other contaminants will contribute to establish its potential use in treatment of different industrial effluents. These studies are being undergone in our laboratory.

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