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High phenol degradation capacity of a newly characterized *Acinetobacter* sp. SA01: Bacterial cell viability and membrane impairment in respect to the phenol toxicity

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ABSTRACT

An efficient phenol-degrading bacterial strain, belonging to *Acinetobacter* genus, was isolated and selected to study the impact of different environmentally relevant phenol concentrations on the degradation process. The bacterial isolate, labeled as *Acinetobacter* sp. SA01 was able to degrade the maximum phenol concentration of 1 g/l during 60 h at optimum condition of pH 7, 30 °C and 180 rpm. Aeration and initial cell density, the two important factors, were carefully examined in the optimal growth conditions. The results showed that these two variables related proportionally with phenol degradation rate. Further investigations showed no effect of inoculum size on the enhancement of degradation of phenol at over 1 g/l. Flow cytometry (FCM) study was performed to find out the relationship between phenol-induced damages and phenol degradation process. Single staining using propidium iodide (PI) showed increased cell membrane permeability with an increase of phenol concentration, while single staining with carboxyfluorescein diacetate (cFDA) demonstrated a considerable reduction in esterase activity of the cells treated with phenol at more than 1 g/l. A detailed investigation of cellular viability using concurrent double staining of cFDA/PI revealed that the cell death increases in cells exposed to phenol at more than 1 g/l. The rate of cell death was low but noticeable in the presence of phenol concentration of 2 g/l, over time. Phenol at concentrations of 3 and 4 g/l caused strong toxicity in living cells of *Acinetobacter* sp. SA01. The plate count method and microscopy analysis of the cells treated with phenol at 1.5 and 2 g/l confirmed an apparent reduction in cell number over time. It was assumed that the phenol concentrations higher than 1 g/l have destructive effects on membrane integrity of *Acinetobacter* sp. SA01. Our results also revealed that the toxicity did not reduce by increasing initial cell density. Scanning electron microscopy (SEM) examination of bacterial cells revealed the surface morphological changes following exposure to phenol. The bacterial cells, with wizened appearance and wrinkled surface, were observed by exposing to phenol (1 g/l) at lag phase. A morphological change occurred in the mid-logarithmic phase as the bacterial cells demonstrated coccobacilli form as well as elongated filamentous shape. The wrinkled cell surface were totally disappeared in mid-stationary phase, suggesting that the complete degradation of phenol relieve the stress and direct bacterial cells toward possessing smoother cell membrane.

1. Introduction

Consumption of aromatic compounds in some industries is unavoidable which has been resulted in contamination of ecosystems including soil and water. The examples of phenol applications in industry can be included but not limited to production of some materials such as dyes, plastics, pesticides, resins, pulp, papers and pharmaceutical products and its usage as organic solvent to dissolve other organic compounds such as ether, chloroform and alcohols (Barbera et al., 2013; Barnes et al., 2008; dos Santos et al., 2009; Hering et al., 2013; Prasse et al., 2018). Accumulation of phenol is not only toxic for flora, fauna and human, but it is also toxic for microbial communities (Jagani et al., 2009; Lyu et al., 2018; Prasse et al., 2018; Puupponen-Pimiä et al., 2001; Saha et al., 1999). Toxic features of phenol for its effect on microbial communities are used in sterilization of clinical instrument surfaces (Berardinelli et al., 2008). The concentration of phenol in

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wastewater of different industries varies from a few mg/l to several g/l. For example, the phenol concentration in wastewater of coal processing plants varies from 9 to 6800 mg/l. This range of phenol concentration can vary from the germistatic activity of phenol to its germicide activity which can interfere with other microbial processes either in environment or activated sludge (Berardinelli et al., 2008; Duan et al., 2018; Pizzolitto et al., 2015; Pradeep et al., 2015).

Among the microorganisms, some of them not only can bear toxicity of phenol but also can consume it as an energy and carbon source (Krastanov et al., 2013; Thomas et al., 2002). Consequently, these microorganisms are engaged in biological wastewater treatment. For instance, it has previously been reported that *Pseudomonas* sp. SA01 can degrade up to 700 mg/l of phenol. The optimization of cultivation conditions improved the phenol degradation process up to 1000 mg/l (Shourian et al., 2009). In general, cultivation of bacterial cells with higher concentrations of phenolic compounds leads to prolonged duration of lag phase. In a previous work, Kroll and Anagnostopoulos (1981) studied the continuous monitoring of K^+ leakage from *Serratia marcescens* exposed to phenol and described the potassium leakage as a lethality index of phenol (Kroll and Anagnostopoulos, 1981). Exposure to phenolic compounds may cause either structural membrane modifications or increasing its permeability, leading to cell death due to loos of vital components (Keweloh et al., 1990). As an example, the lipid-protein ratio of the plasma membrane of *E. coli* is changed after exposure to ethanol stress (Dombek and Ingram, 1984). In other studies, the ratio of lipid–to-protein of both cytoplasmic and outer membranes of *E. coli* is reduced in the presence of phenol (Keweloh et al., 1990; Léonard and Lindley, 1999). Therefore, the viability of bacteria could potentially be influenced by toxic effects of phenolic compounds along with the delay in cell growth. Increasing the inoculum size has been shown to reduce the length of the bacterial lag phase (Beshay et al., 2002; Shourian et al., 2009). It was also revealed that cell density alongside phenol concentration and reaction time are important factors for optimization of phenol degradation by the microalga *Chlorella pyrenoidosa* (Priyadharshini and Bakthavatsalam, 2016).

Although there are a variety of strategies used to enhance phenol degradation, but each have its own performance that can improve a certain level of phenol decomposition. Additionally, each strategy has its own specific human and financial burden and degradation of phenol at higher concentrations are often costly (Jiang et al., 2006; Mao et al., 2015; Mollaei et al., 2010; Mrozik et al., 2011; Prpich and Daugulis, 2005; Rosenkranz et al., 2013). Therefore, finding new bacterial strains with decomposing ability of high concentrations of phenol and a better understanding of their mechanism of action is of great importance in order to use an effective strategy, reducing the costs arising from phenol decomposition process. There are plenty of researches concerning the biochemical and cellular adaptation in response to the phenol stress, but to our knowledge, there is insufficient evidence about the potential of natural tolerance and viability of bacterial cells when confronted with toxic doses of phenol. In recent years, the development of instruments and new methods has led to the assessment of cell viability more convenient than before. FCM data analysis approaches using different fluorescent dyes for staining living cells, is a way to get reliable and accurate data (Kummrow et al., 2013).

Herein, we conducted the pertinent experiments to isolate and determine the capacity of some indigenous bacterial strains, isolated from oil and pesticide contaminated effluent, for phenol degradation. Among all isolates examined, an efficient phenol-degrading bacterium was selected and identified by 16 S rRNA gene sequencing as *Acinetobacter* sp. SA01. The efficiency of the selected isolate was further examined with analysis of degradation kinetics and pathway. To find out the optimal conditions for phenol degradation, several important physicochemical factors were examined. Afterward, the effect of phenol toxicity on cultivability and bacterial viability was evaluated using direct microscopy, viable cell count (plate count method) and FCM examination (single and double cFDA/ PI staining), gaining a deeper understanding of phenol degradation and bacterial viability.

2. Materials and methods

2.1. Enrichment and isolation of the microorganism

To isolate the efficient bacterial strains with regard to phenol biodegradation, samples were collected from a river in the south of Tehran which was contaminated with farmland pesticides and oil refinery pollutants. For this purpose, 10 ml of the collected sample was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of M9 minimal medium (pH 7). The medium composition was as follows: KH_2PO_4 (22 mM), Na_2HPO_4 (42 mM), NH_4Cl (37 mM), NaCl (8.5 mM), $MgSO_4{\cdot}7H_2O$ (162 μM), $CaCl_2{\cdot}2H_2O$ (170 μM), $FeSO_4{\cdot}7H_2O$ (90 μM), $CuSO_4{\cdot}5H_2O$ (160 μM), $ZnSO_4{\cdot}7H_2O$ (695 μM), $MnSO_4{\cdot}4H_2O$ (1.8 mM). Then, the filtered thiamin (22.6 μM) added into the medium. Phenol was filtered and added into the medium as the carbon source at the final concentration of 1 g/l. The flasks were incubated at 30 °C and 180 rpm. After observing the growth, the culture was transferred to the M9 minimal medium agar, containing 1 g/l phenol (as the carbon source). The process was repeated sequentially until obtaining enriched single colonies. To obtain single colonies of each bacterial species, each colony was purified and cultivated separately and tested for its phenol biodegradation potential.

2.2. Morphological and 16S rRNA sequence analysis

The bacterial isolates were investigated for their morphological features visually and with light microscopy. DNA extracted from each isolate and 16 S rRNA gene amplified using 27 F and 1492 R, as the forward and reverse primers. Single bands of PCR products were purified using a Qiagen gel extraction kit and subjected to sequence analysis using the same primers as used for PCR. 16 S rRNA gene sequences were matched and edited with forward and reverse sequences. Similarity, searching and sequence comparison was examined using the BLAST program (Basic Local Alignment Search Tool) (Altschul et al., 1990). ClustalW program was used to perform for the multiple sequence alignment and draw the phylogenetic tree, based on 16 S rRNA of the isolates. Phylogenetic analysis was accomplished using MEGA software version 6.0.6 and the neighbor-joining tree was constructed by this software (with bootstrap percentage values for 1000 replicates) (Tamura et al., 2013).

2.3. Phenol biodegradation studies

The bacterial isolates were inoculated into the M9 minimal medium containing 0.4 g/l phenol, as the sole carbon source (pH 7.0), and the bacterial cultures were then incubated at 30 °C and 150 rpm. Samples were collected at defined time intervals to obtain the rate of cell growth and phenol degradation. The residual phenol was determined using 4-aminoantipyrine colorimetric test. Briefly, the optical density of the samples obtained at 510 nm, and the results compared with the previously provided standard curve (Der Yang and Humphrey, 1975).

2.4. Degradation study as a function of phenol concentration

The conical flasks containing 50 ml of M9 minimal medium was inoculated with an initial cell concentration of ~ 10^7 CFU/ml and supplemented with different concentrations of phenol (0.2, 0.4, 0.7 and 1 g/l) adjusted to pH 7.0. The killed-cell for each sample (as the control) was prepared, separately under a similar condition. The cultures were incubated at 30 °C and 180 rpm. Time course sampling was performed to determine the rate of cell growth and phenol degradation every 6 h. Cell growth was determined at OD_{600} and the residual phenol defined by using 4-aminoantipyrene test.