Bioremediation of Organometallic Compounds by Bacterial Degradation

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Received: 9 July 2010 / Accepted: 4 August 2011 / Published online: 18 August 2011
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Abstract The use of organometallic compounds in the environment is constantly increasing with increased technology and progress in scientific research. But since these compounds are fairly stable, as metallic bonds are stable, they are difficult to degrade or decompose naturally. The aim of this work was to isolate and characterize heterotrophic bacteria that can degrade organometallic compounds (in this case ‘ferrocene’ and its derivatives). A Gram-negative coccobacillus was isolated from a rusting iron pipe draining into a freshwater lake, which could utilize ferrocene as a sole source of carbon. Phylogenetic analysis based on 16S rDNA sequence suggested that the isolated organism resembled an environmental isolate of *Bordetella*. Ferrocene degradation was confirmed by plotting the growth curve of the bacterium in a medium with ferrocene as the sole source of carbon. Further confirmation of degradation of ferrocene and its derivatives was done using Gas Chromatography Mass Spectroscopy. Since the bacterium degraded organometallic compounds and released the metal in liquid medium, it could be suggested that this organism can also be used for extracting metal ions from organo-metal containing wastes.

Keywords Ferrocene · Bioremediation · Biodegradation · Organometallic compounds · *Bordetella* sp

Increasing environmental concerns, federal restrictions and bans on the release of organic pollutants in the environment necessitates development of efficient waste treatment methods. While chemical methods of treating wastes might serve the purpose at some level the problem may still persist regarding how to dispose off the treated waste and chemical by-products. As a result, new approaches, including bioremediation, are coming in focus. Bioremediation utilizes the metabolic versatility of microorganisms to degrade hazardous pollutants [1, 2]. The indigenous microorganisms could be simulated or specially developed, to be added to the site to degrade, transform, or attenuate organic and organometallic compounds to low levels and nontoxic products [1–3].

Heavy metal pollution is becoming a major concern as the use of heavy metals is increasing in industrial applications. A number of studies are now available which focus on the bioremediation of toxic metals, which either remove the metals from the waste by bioadsorption [4–6] or convert the chemical properties of the metals by reduction [7, 8]. These methods, however, may not be sufficient for treating wastes with organometallic compounds where biodegradation of organic matter is essential. Unlike many other organic pollutants, organometallic pollutants are highly stable as they have stable metallic bonds [9, 10]. Thus, bioremediation of organometallic pollutants may require the capacity of microorganisms to break these strong metallic bonds. Farbiszewska-Kiczma and Farbiszewska [11] used phthalocyanines with different metals to isolate bacteria that can degrade organometallic compounds. However, metallic bonds in phthalocyanines are relatively weaker than many other organometallic bonds. Hence, in this study, one of the most stable organometallic compounds, called ferrocene, is used to isolate bacteria which can degrade organometallic compounds.

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Ferrocene ($\text{Fe}(\text{C}_5\text{H}_5)_2$) is a prototypical metallocene, a type of organometallic chemical compound consisting of two cyclopentadienyl rings bound on opposite sides of a central metal atom. It has a molecular weight of 186.04, boiling point of 249°C and melting point of 172.5°C [12]. Ferrocene has no unpaired electrons and all of its valence orbitals are filled and are of low energy, thus it is resistant to nucleophilic and electrophilic attack—explaining its remarkable stability. Because of their unusual structure, robustness, and redox potential, ferrocene and its derivatives have many industrial and biomedical applications [13, 14].

There are no reports on microbial degradation or photodecomposition of ferrocene. Hence, the isolation of ferrocene degrading bacteria is desirable, which could thus find its application in environment management and organo-metallurgical technology. The aim of this work was to isolate and characterize heterotrophic bacteria that can degrade ferrocene and its derivatives.

The bacterial samples were collected from Pashan Lake ($18^\circ31'59''\text{N}$ and $73^\circ47'16''\text{E}$), Pune. The samples were scraped from the surface of partly submerged rusted iron pipes in the lake. The lake consisted of some amount of floating organometallic wastes as it had outlets from adjacent small-scale industries. Enrichment was carried out using mineral salts medium (pH 7) containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.185 g/l), Na_2HPO_4 (0.047 g/l), KH_2PO_4 (0.06 g/l), MgSO_4 (0.097 g/l), KCl (0.4 g/l), NaCl (8.0 g/l) and distilled water 1000 ml, supplemented with ferrocene (5.0 g/l; ferrocene being the sole source of carbon). As ferrocene is insoluble in water, yellowish orange clumps of ferrocene could be seen floating on the surface of the medium. Nevertheless, vigorous shaking led to the settling of some ferrocene at the base of the glass flask. Even though ferrocene was used for primary isolation, other ferrocene derivatives, namely ferrocene-4, ketobutanoic acid and ferrocene-1,1' ethanone, were also used for comparisons. Sample was inoculated in mineral salts medium and was incubated aerobically with shaking, at 37°C for 2 weeks. Enriched organisms were streaked on nutrient agar medium for obtaining the pure culture. The isolated pure culture was again inoculated in mineral salts medium with ferrocene as sole source of carbon for a conformation of ferrocene degradation. The pure culture was deposited in National Collection of Industrial Microorganisms (NCIM), Pune.

Biochemical characterization of the pure culture was done using Himedia Biochemical Identification Test Kits KB002 and KB009. DNA isolation was done using Promega Wizard[®] genomic DNA purification kit (Cat. #A1120). Universal primers were used for amplifying 16S rDNA sequence (forward 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 5'-ACGCTACCTTGTTACGACTT-3') and a

partial 16S rDNA sequence was obtained. Partial 16S rDNA sequence was deposited in GeneBank (<http://www.ncbi.nlm.nih.gov/genbank/>). BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed to find similar sequences in the database [15]. Plasmid isolation was performed using alkaline lysis method [16].

Degradation studies were setup for ferrocene and its derivatives: ferrocene-4 ketobutanoic acid and ferrocene-1,1' ethanone, by growing the organism in basal salt medium with ferrocene or its derivative as a sole source of carbon. In the case of ferrocene, two methods were performed to study its degradation. First, by performing growth curve of organism in basal salt solution with ferrocene as a sole source of carbon and, second by performing GCMS (Gas chromatography mass spectroscopy) of samples for different time steps of growth curve. For the growth curve of the organism, 20 test tubes with 10 ml basal salt solution and ferrocene were inoculated with equal density of culture. One tube was kept as a negative control. One tube was analyzed immediately after inoculation and dry weight of cells in 1 ml of the suspension was determined. This was considered as 'day zero' reading. After every 2 days three tubes were analyzed and the dry weight of biomass was determined per ml of the sample. This was done for 12 days from incubation. One remaining tube was sacrificed on 20th day for confirming the stationary stage of the growth. During the growth curve experiment, some part of the sample was used for GCMS analysis. The sample was prepared by extracting the ferrocene and its degraded products in chloroform. Chloroform was evaporated in vacuum evaporator at 40°C . Dry powder was dissolved in methanol and 2 μl was loaded in the GCMS machine (GCMS-QP 2010 Plus, Shimadzu Corporation). For ferrocene derivatives, organism was grown in basal salt solution with ferrocene derivative as a sole source of carbon. Presence of growth was qualitatively determined by comparing density of medium against control tube without the organism. Degradation of derivatives was confirmed by GCMS of the control and the 7 day incubated tube.

In the enrichment culture an organism was isolated, which could utilize ferrocene as its sole source of carbon. This organism was submitted to NCIM. The NCIM accession number for the isolate is NCIM 5372. NCIM 5372 is a Gram-negative motile coccobacillus (1.60 μm long and 0.45 μm wide). It produced small (0.7 mm diameter), low convex, round, translucent and butyrous colonies with smooth edge on Nutrient agar after 24 h incubation at 37°C . It was biochemically characterized as a citrate utilizing and nitrate reducing organism. It could utilize a variety of carbohydrates such as lactose, xylose, maltose and D-arabinose. Plasmid extraction using three independent attempts revealed that NCIM 5372 did not

Fig. 1 Growth curve of the isolate NCIM 5372 in basal salt solution with ferrocene as a sole source of carbon. Circles are average values of three replicates and *error bars* are standard errors. *Solid line* is logistic model ($y = 0.0319/(1 + 1.5224 \times \exp(-0.3754x))$) fit to the average values ($r = 0.9765$, $P < 0.0001$)

contain any plasmid. Phylogenetic analysis based on 16S rDNA sequencing (HM593901) revealed that the organism belong to class Betaproteobacteria, order Burkholderiales and family Alcaligenaceae. In the BLAST analysis, the organism showed 97% similarity with the environmental isolate of genus *Bordetella*, namely *B. petrii*.

The study by Farbiszewska-Kiczma and Farbiszewska [11] has reported 10 species of bacteria belonging to 5 genera, namely *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Brevibacillus* and *Bacillus*, as potential degraders of organometallic compounds. Further, Craig et al. [17] have listed a number of bacteria, except *Bordetella*, that are involved in biotransformation and degradation of organometallic compounds. Thus, our report of *Bordetella* species as a potential degrader of organometallic compounds is in itself a novel finding. Also, since no plasmid was found in NCIM 5372, it could be suggested that the property of organometallic degradation could be present in the genomic DNA of the organism.

Bordetella was previously known only from pathogenic isolates. However, recently von Wintzingerode et al. [18] isolated *Bordetella petrii* from freshwater lake and showed that this environmental isolate has the metabolic versatility required for environmental existence [19]. The fact that *Bordetella* could have an environmental existence and has the required metabolic versatility to survive in the environment suggests that the identification of NCIM 5372 as *Bordetella* is not completely out of tune. NCIM 5372 also showed some morphological and biochemical characters common with *Bordetella* sp.

The growth curve of NCIM 5372 in the basal salt solution with ferrocene as a sole source of carbon is displayed in Fig. 1. Logistic model, $y = 0.0319/(1 + 1.5224 \times$

Fig. 2 GCMS plots for ferrocene degradation. **a** Initial readings, **b** readings after 2 day incubation and **c** readings after 14 days of incubation

$\exp(-0.3754x)$), was fitted to the average values ($r = 0.9765$, $P < 0.0001$). In the basal salt solution with ferrocene as a sole source of carbon, NCIM 5372 grew very slowly and reached a stationary phase after 10 days of incubation. GCMS plots of the extracted ferrocene and its degraded products showed decrease in ferrocene content with progression of growth and increase in the Fe content of the medium (Fig. 2). There was also a visible change in the color of the medium as it was orange at the start of the experiment and as the ferrocene was degraded, it turned yellow and the intensity of orange color decreased steadily. NCIM 5372 could also grow in the medium containing ferrocene derivatives, namely ferrocene-4 ketobutanoic acid and ferrocene-1,1' ethanone. GCMS plots confirmed the degradation of these derivatives as well, with liberation of iron (Fig. 3).

Fig. 3 Degradation of ferrocene-4 ketobutanoic acid and ferrocene-1,1' ethanone. GCMS plots of **a** control for ferrocene-4 ketobutanoic acid, **b** ferrocene-4 ketobutanoic acid degradation after 7 days

incubation, **c** control for ferrocene-1,1' ethanone and **d** ferrocene-1,1' ethanone degradation after 7 days incubation

An interesting finding from the GCMS analysis of the degradation of ferrocene and its derivatives was that during degradation, the organism utilized the organic compound completely and release iron in the medium, which keeps on accumulating. This has potential uses in waste treatment and extraction of costly metals from industrial wastes.

Even though the current study was confined to degradation of ferrocene and its derivatives, it could be stated that the organism could also be potential degrader of other organometallic compounds as well. One reason for this belief is that ferrocene is a very stable compound and thus an organism that could degrade the strong metallic bonds in ferrocene is likely to degrade other metallic bonds as well. Nevertheless, it is anticipated that Fe is a heavy metal but its toxicity is not as much as those of other heavy metals. Therefore, it needs to be established whether NCIM 5372 is resistant to toxicity of other organometallic compounds.

Acknowledgments The authors are thankful to Amit Patwa for providing ferrocene and ferrocene derivatives in the early experiments. The authors are also thankful to Amar Mohite for helping us with GCMS. Mandar Paingankar and Charushila Kumbhar helped us variously. The authors thank two anonymous referees and Manawa Diwekar for comments on the earlier draft of the manuscript.

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