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Isolation of Potential Manganese Reducing Bacteria from Submerged Soil of open Mine Pit

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ABSTRACT: Manganese reducing bacterial isolates from mine pit were subjected to further screening and out of which three potential isolates IRB-1, IRB-2, and IRB-3 were identified for applications related to degradation of xenobiotic compounds. The organisms were isolated by subjecting the soil sample to serial dilution (10^{-4} and 10^{-5}) and plating on enrichment medium. The isolates were grown under anaerobic culture conditions by roll tube technique. All the isolates were identified to be *Bacillus* sp. were subjected to growth on minimal medium with xenobiotic compounds and the isolates were identified with potential ability to degrade textile dyes and aromatic hydrocarbon. Among the isolates the IRB-2 was found to degrade both hydrocarbons and the dye Methylene Blue although there was growth and absorbance of Crystal Violet, Methyl Orange, Safranin and Malachite Green.

Introduction

Bacteria that can reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) are generally referred to as iron reducing bacteria. This group is further classified into dissimilatory iron reducing bacteria and assimilatory iron reducing bacteria. Dissimilatory refers to iron reduction by respiration process (under anaerobic conditions) and assimilatory is a fermentative type metabolism. The microbial group broadly includes *Proteobacteria*, *Acidobacterium*, *Nitrospira*, most gram positive bacteria and some of the prominent species are *Thiobacillusferroxidans*, *Geobacter*, *Desulfuromonas*sp and *Aeromonas* sp. in case of iron reducing bacteria, Ferric ion acts mainly as a terminal electron acceptor and generally exists as highly insoluble ferric oxide minerals by nature. Iron exists in solid form and microbial cell contact is essential for iron oxide reduction. Such iron reducing bacteria occupying a niche in environment can play a potential role in bioremediation of hydrocarbon polluted soil. One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry [(5)].Metal reducing organism can also be a good candidate for bioremediation of heavy metal contaminated ecosystem [(2)]. The present study deals with isolation and characterization of iron reducing bacteria that could potentially degrade xenobiotic compounds.

Materials and Methods

Sample collection

Soil samples were collected from Kolar Gold Fields, Bangalore, India from 1000

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feet deep pit. The samples were collected in sterile containers and stored in dark conditions. The sample was processed by serial dilution within 24 hours of sample procurement.

Sample processing and Isolation

To isolate iron reducing bacteria, anaerobic conditions are pre-requisite, use of complex growth substrate is also necessary for isolation. The most common source of carbon and energy for these organisms will be hydrogen and short chain organic acids. Iron reducing bacteria can be most commonly isolated from anaerobic enrichments. In the present study, the ferric ion is used in the form of Iron oxy hydroxide (Geothite) which is available in soil or can be artificially prepared in laboratory.

The selective media (Sodium lactate; yeast extract; sodium chloride; magnesium sulphate; Ascorbic acid; potassium hydrogen phosphate; vitamins and trace elements) was prepared under low oxygen tension by constant heating and incorporation of appropriate reducing agents (sodium dithionate was added in low concentration to keep the undesirable effect of SO_4^{2-} acting as a terminal electron acceptor and as a competitor to iron oxy hydroxide) and Geothite solution was added to it and autoclaved. The serially diluted samples were inoculated into selective media by pour plate method.

Roll tube technique

The above serially diluted slurry samples were used for the isolation of anaerobic organisms by Roll tube technique. About 0.5 ml of the diluted sample was taken in a sterile roll tube and appropriate molten agar medium (at 45°C) was added with continuous nitrogen flushing. Then the tubes were sealed quickly and rolled on a wet ice cold sponge for quick solidification of the medium. The tubes were then incubated at 30°C in an inverted position to prevent disturbance of colonies by condensed of water lets during sub-culturing. The technique helps in better observation of isolates on the inner wall of the tubes, study of colony characteristics and their identification and further isolation by using syringes.

Nitrogen flushing is essential to maintain the anaerobic condition and prevention of oxygen contamination that could be detrimental to obligate anaerobes.

Characterization of the isolates

A series of biochemical tests were performed on the potential isolates which included the Oxidase test, Catalase test. Lactose fermentation test, Indole test, starch hydrolysis, methyl red and VP test and nitrate test.

Dye reduction test

The isolate was tested for dye reduction test where by the selective media was prepared and supplemented with 1% of dye solution. The dyes including Methylene blue, Methyl Orange, Congo red, Safranin and Crystal violet were tested for degradation against the potential isolate.

Hydrocarbon degradation test

Hydrocarbon degradation depends on various factors and the most important aspect is the availability of hydrocarbon to bacterial cell which depends on their concentration in soil. In this experiment minimal medium was prepared with benzene as the carbon source. Growth of bacteria on minimal medium could be considered as positive for benzene utilization and degradation.

Results and Discussion

Soil samples were serially diluted and later inoculated into enrichment broth and colonies isolated from the highest dilutions was considered for further screening as they could represent the highest number in the soil sample. The isolates were tested for their ability to grow on goethite medium, the colonies exhibited irregular, undulate and smooth characteristics on agar medium and were gram positive rods on staining (**Fig: 1**). Three isolates were selected for further characterization based on their cultural and microscopic characteristics and labelled as IRB-1, IRB-2 and IRB -3.

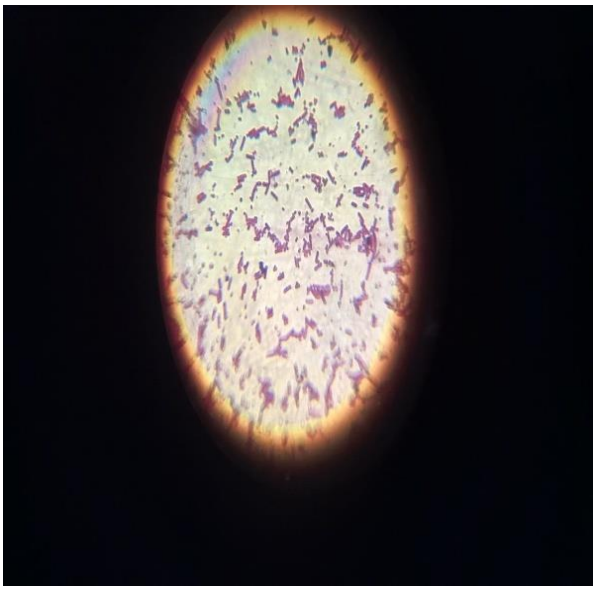

Figure 1: Microscopic view of the isolate

Figure 2: Roll tube cultivation

Cultural characteristics

The isolates were grown anaerobically by roll tube method (**Fig: 2**); cultural and morphological tests revealed that all the three isolates could be *Bacillus* sp. the isolates showed spore formation and growth under aerobic and anaerobic medium and preferably better growth was observed in medium supplemented with sodium lactate and yeast extract.

Earlier workers have reported an iron reducing isolate of using lactate, acetate, and H_2 as electron donors for dissimilatory metal reduction with Hydrogen consistently yielded the most rapid growth coupled to metal reduction. Media formulation can result in higher growth and better utilization of iron as a reducing agent, in the present study maximum iron reduction was observed with sodium lactate rather than sodium acetate or sodium formate.

Acetate and H_2 have been suggested to be the two major extracellular intermediates in the oxidation of fermentable organics in Fe(III)-reducing sediments [10,8,3].

Biochemical characteristics

All the three isolates were subjected to biochemical tests and the isolates were confirmed to be *Bacillus* sp. (**Table 1**). Although these organisms are facultative anaerobes, known to grow anaerobically by fermentation yet it had not been studied widely under anaerobic conditions.

Table 1: biochemical test results of potential isolates IRB-1, IRB-2 AND IRB-3

Sl. No.	Biochemical Tests	Results
1	Oxidase test	-
2	Catalase test	+
3	Glucose fermentation test	+
4	Lactose fermentation test	+
5	Indole test	+
6	Methyl red	+
7	Voges Proskauer test	+
8	Citrate test	+
9	Starch hydrolysis test	+
10	Nitrate test	+
11	H_2S production	-

Dye reduction test

The iron reducing isolate having capability to degrade xenobiotic substances in nature can carve a niche that helps in bioremediation in natural environments. Reports of many Fe (III)-reducing organisms capable of reduction of humic substances and humic substance analog 2,6-anthraquinone disulfonate have been reported [(9)]. The isolate showed degradation of only Methylene blue dye while for all the remaining dye the isolate absorbed the dye but not degradation (Table 2).

Table 2: Dye reduction results

Sl. No.	Type of Dye	IRB - 1	IRB-2	IRB-3
1	Crystal violet	-	-	--
2	Methy Orange	-	-	-
3	Safranin	-	-	-
4	Methylene blue	+	+	+
5	Malachite Green	-	-	-

Hydrocarbon degradation test

Out of the three isolates the IRB-2 was able to grow on benzene minimal medium while the IRB-2 and IRB-3 showed no growth. The IRB-2 based benzene degradation was tested for optimization at various pH (6.5, 7.0, 7.5 and 8.0) and temperatures (28°C, 37°C and 45°C). The IRB-2 showed maximum benzene degradation at pH 7.5 and at 45°C. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature [(5)]. Microorganisms take time to degrade the xenobiotic compounds, in few cases degradative percentage was enhanced after longer incubation (Bharathi *et al.*, 2013).

Conclusion

Among the three isolates the IRB-2 was able to degrade both hydrocarbon (Benzene) and dye methylene blue in comparison to IRB-1 and IRB-3 which could degrade Methylene Blue dye but was not able to degrade hydrocarbons. But absorption of the dye was observed for Crystal Violet, Methyl Orange, Safranin and Malachite Green but was later released by the biomass on suspension in hypotonic solution. Hence the isolate IRB-2 could have better application in bioremediation process (The isolates can be used in consortium for treatment of urban waste polluted with hydrocarbons and dyes, [(12)] which has to be further characterized at molecular level.

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