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BIOLOGICAL TREATMENT OF SEWAGE WATER BY HEAVY METAL RESISTANT BACTERIA

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ABSTRACT

Industrial effluents should be treated for their high BOD before they are discharged into the environment. Most of the inoculated bacteria used for the removal of high BOD are not efficient as they cannot withstand the high concentrations of heavy metals present in the effluents. In the present investigation 20 cellulose degrading bacteria having high resistance to heavy metals were isolated from soil collected from four different sites of industrial area of Faridabad. One heavy metal sensitive bacteria (HMS-1) were also checked for enzymatic activities. Among the 20 isolates, 12 isolates showed higher cellulose reduction activity when compared to other strains varying from 16 to 83%. Further they also exhibited the starch hydrolysis activity varying from 16 to 66%. Finally these isolates were also checked for bioremediation of sewage water and it has been observed that they have the great potential to reduce various parameters such as BOD and COD as compared to HMS-1 strain. The bacterial isolates were screened for metal resistance against Cd, Ni, Pb and Hg. A particular high value of MIC was observed for cadmium ranging from 200ppm to 250ppm. Data indicated that the isolated bacteria have a great potential in organic matter degradation of industrial effluents having high concentration of heavy metals as compared to heavy metal sensitive bacteria.

KEYWORDS: Cellulose, sewage water, heavy metal resistant bacteria, Starch, soil sample, Heavy metals.

INTRODUCTION

With the rapid industrialization due to increasing population, the water pollution has become a global concern. The effects of water pollution are not only devastating to human but also to other forms of life as animals, fish, and birds. Most of the industrial effluents are rich in organic matter, thus increasing the BOD and rendering them unfit for disposal into water bodies. The Environmental Protection Agency (EPA) uses BOD levels to measure effluent strength and to establish effluent guidelines as required by the Federal Water Pollution Control Act. Soil organisms play a vital role in the sustainable functioning of ecosystems. They not only act as the primary driving agents of nutrient cycling but are also involved in regulating the dynamics of soil organic matter. Microorganisms such as bacteria are responsible for decomposing organic waste. When organic matter such as a dead plants, leaves, grass clippings, manure, and sewage or food waste is present in water supply, the bacteria brings out the process of breaking down this waste, thus reducing the BOD of the waste water. Decomposition of organic carbonaceous materials in soils is undertaken by groups of soil organisms acting in concert.[1, 2] Carbonaceous materials subjected to microbial decomposition include cellulose, hemicelluloses, starch and lignin. Bacteria play is very significant role for the enzymatic hydrolysis of the complex organic polymer. The use of bacterial consortia for the waste water treatment has been in practice since long. Therefore the impacts on these microbial processes of environmental contaminants, including heavy metals, are of concern. The industrial effluents contain large amounts of heavy metal contaminants which are difficult to be tolerated by these microorganisms and therefore, it affects the degradation of organic compounds indirectly. Hence there is an urgent need for microbial population which is highly resistant to heavy metals as well as have higher organic matter decomposition ability. The objectives of this study were to isolate potential bacterial strains that can decompose the organic matter in sewage water having heavy metals.

MATERIAL AND METHODS

Sample collection

The soil samples were collected from the four different locations of industrial areas, Faridabad, at the depth of 0-15cm below surface. All samples were kept in clean sterile bags, labelled accordingly and stored at 4^0C.

Isolation of bacteria resistant to cadmium

Enumeration of cadmium resistant bacteria was conducted using plate dilution techniques.[3-4] Soil samples were serially diluted up to 10^5 and aliquots of 0.1ml from each dilution was spread on Luria Bertani media plates supplemented with and without 5mM of cadmium as $Cd(NO_3)$.[2] The plates were incubated at 30^0C for 24 hours. The colonies that appeared on the medium supplemented with 5mM cadmium nitrate were considered as cadmium resistant bacteria. Morphologically distinct colonies were picked, purified and stored at 4^0C. The isolates were purified by re-streaking on Luria Bertani medium plates and single colonies were transferred on slants. The isolates were stored on LB agar slants in a refrigerator and maintained by regular transfers.

Determination of minimal inhibitory concentration (MIC)

Minimum inhibitory concentration of the different heavy metals was determined by the plate dilution method as described by Malik *et al*.[5] The metals Cd, Hg, Ni and Pb were used as $Cd(NO_3)_2$, $HgCl_2$, $NiSO_4$, $Pb(NO_3)_2$ respectively. Stocks of metal salts were prepared in distilled water and sterilized by filter membrane (0.22µm) and stored at 4^0C. Luria Bertani medium was prepared and amended with various amounts of heavy metals Cd, Pb, Ni and Hg to achieve the desired concentration of 10, 20, 30, 40, 60, 80, 100, 150, 200, 250 and 300ppm. Inoculums of all strains were spread in the metal amended and control plates (without metal). The plates were incubated at 30^0C for 72 hours. The concentration of the metal which permitted growth and beyond which there was no growth was considered as the MIC of the metal against the strain tested.

Screening of Cellulose Degrading Bacteria and amylase activity

The cellulose degrading potential of the isolated colonies was checked on cellulose agar media composed of $MgSO_4$ (0.025g), cellulose (2.5g), peptone (0.5g), NaCl (0.02g), $CaCl_2.6H_2O$ (0.02g), K_2HPO4 (0.2g) in distilled water (l/liter) pH 6.8-7.2. Agar was added 2%.Confirmation of cellulose degrading ability of bacterial isolates was performed by streaking on the carboxymethyl cellulose(CMC) agar media with the following composition peptone (1.0%), K_2HPO_4(0.2%), $MgSO_4.7H_2O$ (0.03%), $(NH_4)_2$ SO_4(0.25%), Gelatin (0.02), CMC (1.0%), agar (1.0%), distilled water/liter and pH6.8-7.2. After incubation for48hours, CMC agar plates were flooded with 1% Congo red and allowed to stand for 15min at room temperature. Then one molar NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The diameter of the clear zones was measured and % cellulolytic activity was measured using the formula.

Z-C/C x 100 where, Z = halozone diameter, C = Colony diameter.

Further these selected isolates were streaked on starch agar plates with starch as the only carbon source. After incubation at 37^0C for 24-48 hrs, individual plates were flooded with gram's iodine to produce a deep blue colour, which is the basis of the detection and screening of an amylolytic strain. Starch hydrolysing potential of the bacterial colonies showing zone of clearance in starch agar plates was calculated. All experiments were carried out in triplets.

Utilization of starch in the presence of cadmium

The utilization of starch by the selected isolates in broth culture was studied in starch broth supplemented with cadmium under shake culture conditions at 30°C for 48h. The inoculums of each bacterial isolates were prepared by suspending a loopful of bacterial culture from 24 h old slant culture into 9ml of sterile distilled water. The absorbance of the suspension was adjusted to obtain 1 x 10^6 cells/ml by dilution with sterile distilled water. One percent of inoculate of each selected isolate were added to 250ml of sterilized nutrients medium broth supplemented with cadmium. Further these selected isolates were streaked on starch agar plates supplemented with cadmium and incubated at 37^0C for 24-48 h, and starch solubilisation efficiency in the presence of cadmium was calculated.

Bioremediation of sewage water by selected isolates

Twelve bacterial isolates were selected for bioremediation of sewage water. All were heavy metal resistant bacteria except HMS-1 which was heavy metal sensitive. This bacterial strain was previously isolated by Biotechnology department, MRIU, Faridabad. Effluent was collected from sewage water (Faridabad) and autoclaved at 120^0C for 15 min to make them free from other microorganisms before inoculating them with selected bacterial isolates. The inoculums was taken from the pure culture grown on Luria Bertani agar slants and selected bacterial isolates inoculated into each flask labelled containing 250ml of sterilized sewage water . To study, compare and evaluate the biodegradation efficiency, experiment was carried out with control. Experiment was carried over for a period of 5 days under laboratory condition .Immediately after the collection of sewage water and inoculation of bacterial strains including control, the biological parameters such as, BOD and COD were measured. Then on the 5th day the same parameters were measured to evaluate and compare their individual bioremediation efficiency. Dissolved Oxygen (DO) was measured using the modified Winkler's method and biochemical oxygen demand (BOD) with the five day incubation method. Chemical oxygen demand (COD) was carried out using the potassium permanganate method. The effect of the twelve different bacterial isolates on sewage water for the bioremediation process was determined and the best bio remediating bacteria was concluded based on higher percentage reduction potential of physico-chemical and biological parameters.

RESULTS AND DISCUSSION

The present investigation was focused on the isolation of cadmium resistant bacteria displaying multiple resistances to various heavy metals, besides having the capacity to decompose organic matter and consequently could be used for the simultaneous removal of more than one heavy metal from the samples having high biological oxygen demand. Soil samples were collected from the different sites of Faridabad industrial area, Haryana, India. Twenty different bacterial strains were isolated from the contaminated soil samples on LB media supplemented with 5 ppm of $Cd(NO_3)_2$. It is very well understood that the sampling environment exposed consistently to heavy metals favours the adaptation of heavy metal resistant bacteria. This study resulted in the isolation and purification of 20 bacterial isolates from heavy metal contaminated soil on agar media containing 5 ppm of Cd. Out of these 20strains, 12strains were selected for further studies depending on their colony morphology.

Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration of the bacterial isolates was investigated using plate assay to select bacterial isolates capable to grow and resist high level of metal toxicity. The isolates showed very high degree of resistance to all heavy metals. Minimum Inhibitory Concentration of Cd for the isolate A1 was 200 ppm, while for the other isolates it was only 150ppm. MIC of Pb was 100 ppm for the all strains. MIC of Ni was 200 ppm for A1and A3, while for the other strains it was 100ppm. MIC of Hg was 80ppm for all isolates. Among the heavy metals nickel was less toxic, whereas mercury was highly toxic to all strains (Table. 1).

Table 1. Minimal inhibitory concentrations (MIC) of various heavy metals to the isolates.

Heavy metals	Bacterial isolates										
mg/l(ppm)	A1	A2	A3	B1	B2	B3	B4	C1	C2	C3	C4
Cadmium	200	150	150	150	150	150	150	100	150	100	150
lead	100	100	100	100	100	80	100	100	100	100	100
Nickel	200	150	200	100	100	100	100	150	100	150	150
Mercury	80	80	80	80	80	80	80	80	80	80	80

Determination of organic matter decomposition

The decomposition of cellulose and starch by the bacterial isolates was studied and it was observed that all the isolates were capable to decompose cellulose and starch activity .The isolate A2 showed maximum decomposition of cellulose (83.3 %) followed by A1 while A4 and B1 showed minimum cellulose degradation of 16%. HMS-1 reduced cellulose and starch with 53% and 45% respectively. The two isolates A4 and B2 showed maximum decomposition of starch while B1 showed minimum starch decomposition of 16% (Figure-1).

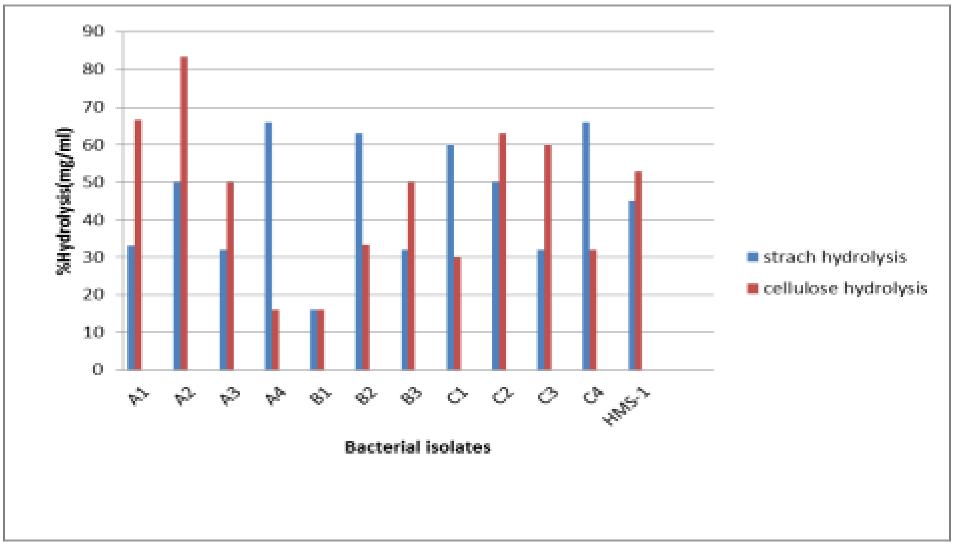

Figure: 1 Percentage of cellulose and starch hydrolysis by bacterial strains.

Utilization of starch in the presence of cadmium

Starch activity was checked in the presence and absence of cadmium .The presence of Cd showed a little effect on starch hydrolysis .This showed that the presence of Cd did not interfere in the starch hydrolysis. With the reference of figure.2, isolate A4 showed 66% hydrolysis in the absence of Cd as compared to 60% hydrolysis in the presence of Cd.

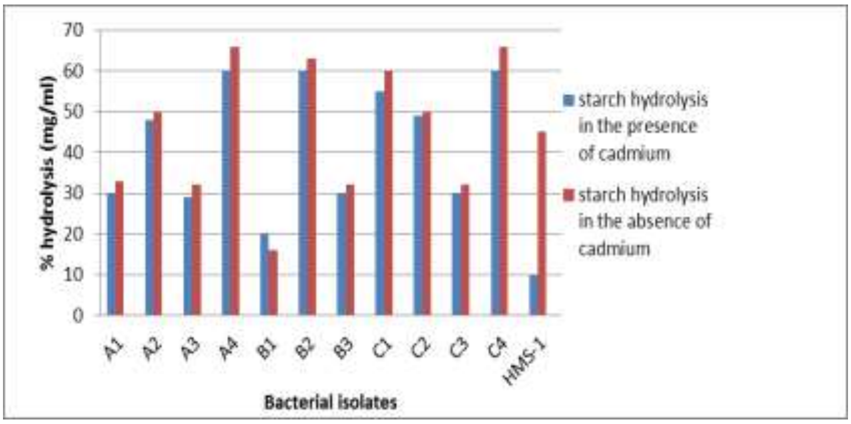

Figure 2. Percentage of starch hydrolysis in the presence and absence of cadmium by bacterial isolates.

Bioremediation of sewage water by selected isolates

The twelve bacterial isolates were checked to their capability of bioremediation of sewage water. All the bacterial isolates were tested for parameters such as BOD and COD up to 5 days of incubation with sewage water. The percentage reduction in all the parameters measured for control and the samples treated with twelve different bacteria is shown in Table-2. In case of BOD, isolate A4 showed maximum % reduction (19.24[a]). The reduction of BOD for all isolates varied from 10.78[a] to17.7[a]. Percentage reduction of COD for all isolates varied from 12.41[a] to 19.71[a], maximum reduction being showed by isolate A3 (19.7[a]). Strain HMS-1 showed poor bioremediation potential. Heavy metal resistant bacteria showed higher reduction capacity than HMS-1 strain.

Table 2. Variation in biological oxygen demand (BOD) and chemical oxygen demand (COD) in the sugar mill effluent treated with various bacteria.

Bacterial isolates treated with sugar mill effluent(mg/l)	Percentage Reduction of BOD and COD removal by bacterial isolates	
	BOD	COD
Control	8.54[d]	11.86[d]
A1	13.56[abcd]	15.30[abcd]
A2	11.97[cd]	14.25[cd]
A3	13.47[abcd]	19.60[a]
A4	17.76[a]	18.49[abc]
B1	11.53[cd]	16[abcd]
B2	12.08[bcd]	16.97[abc]
B3	17.28[ab]	14.68[bcd]
C1	10.79[d]	17.78[abc]
C2	11.21[cd]	15.96[abcd]
C3	11.84[cd]	19.21[ab]
C4	16.41[abc]	18.88[ab]
HMS-1	9.74[d]	14[cd]

These experimental data were analyzed by analysis of variance (ANOVA) and Duncan's New Multiple Range (DMRT) at significant 5%

The BOD standards laid down by the Government agencies for the discharge of wastewater into the environment are 30 mg/L for discharge on inland surface and 100 mg/L for disposal. Moreover, many industries use chemicals for treatment, which is very expensive and poses a threat to aquatic life. This necessitates the implementation of an eco-friendly method for treatment of waste water. The inhibitory effect of heavy metals is a common phenomenon that occurs in the biological treatment of waste water and sewage having high BOD. It has been reported that BOD_5 is suppressed significantly by even small concentrations (12mg/L) of copper or chromium.In order to have efficient treatment system, it is imperative to reduce BOD of the effluent having a range of heavy metals. In the present investigation, efforts were diverted to isolate specific novel bacteria which can survive under such extreme environment and can effectively degrade organic matter present in the industry effluent. It was found that most of the strains were capable of cellulose and starch degradation. Moreover these isolates were also found to be resistant to a variety of heavy metals like Cd, Pb, Ni and Hg. The starch solubilising efficiency was not affected in the presence of heavy metals. Bacteria exposed to high levels of heavy metals in their environment have adapted to this stress by developing various resistance mechanisms. Microorganisms possess a variety of mechanisms to deal with high concentration of heavy metals and often are specific to one or a few metals. Microbes have developed mechanism to tolerate the metals either by presence of heavy metals through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in aerobic respiration. Efflux mechanism export toxic metal ions to the outside of the cell.[6] These may be non-ATPase or ATPase-linked and are generally highly specific for a particular heavy metal ion.[7] Accumulation serve to prevent the exposure of essential cellular components to the contaminant and may be a result of the presence of metallothionins or cysteine-rich protein.[7] In the mechanism of reduction, contaminated ions are enzymatically reduced upon entry to the cell and the less toxic, reduced ions are exported from the cell into the environment.[8] Some bacteria may adapt to the presence of heavy metals by altering the sensitivity of cellular components, which may be achieved through mutations that decrease the sensitivity but do not alter basic function or by increasing the production by the sensitive cellular component.[9] A number of genes, located on bacterial plasmids and chromosomes, have been identified that encode specific resistance to a number of heavy metal ions, including Ag^+, AsO_2, AsO_4^+, Cd(ll), Co(ll), CrO_4^{2-}, Cu(ll), Hg(ll), Ni(ll), Sb(ll), TeO_3^{2-} and Zn^{2+}.[10] Different biomass types, such as bacteria, fungi and algae have been screened and studied extensively by many authors over past decades with the aim of identifying highly efficient metal removal biological systems.[11-13] The ability of microbial strains to grow in the presence of heavy metals would be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of organic matter.[14] In the present study all bacterial isolates were resistant to mercury at the concentration of 80 ppm except B2 that showed MIC of 100ppm. Among the heavy metals investigated, nickel was less toxic; whereas mercury was highly toxic to all strains. Table -1 showed the initial high level of BOD and COD, as they were greatly reduced after treatment with each bacterial strain. HMS-1 which was sensitive for heavy metals appeared to be less effective than other bacterial strains which were highly resistant to heavy metals. Several studies on application of growing microbial cells for metal scavenging have been reported. However, in toxic metal removal applications, it is important to ensure that the growing cells can maintain a constant removal capacity even in the presences of heavy metals. Recent studies show that the strains isolated from contaminated soils and electroplating effluent-contaminated sludge have excellent capability of removing significant amounts of metals from bioaccumulation-desorption cycles and a suitable method is required to optimize the essential operating conditions.[15] The reduction of BOD, COD and heavy metals by *Pseudomonas aeruginosa* and *Saccharromyces* in pharmaceutical effluent was studied by G.B Adebayo *et al.*[16] Mixed culture of *Pseudomonas* and *Saccharromyces spp.* reduced COD and BOD to well below 250mg/l and 30mg/l respectively. Results revealed that the concentrations of the metals which were relatively high were reduced to the nearest minimum most especially by the combination of the bacteria and fungi (94.3-100% reduction). Some of these metals (Zinc and Mn) were even completely removed from the effluent within the period of the experiment.

CONCLUSION

Present study exploits the heavy metal tolerance potential of bacteria to treat the BOD and COD of sewage collected from Faridabad (Haryana). COD is the amount of chemical oxidant required for the oxidation of organic matter present in the polluted samples while BOD is the amounts of oxygen required by the aerobic bacteria to biochemically oxidize the organic matter present in the polluted samples. The high values of BOD in all the samples suggest that the aerobic bacteria are oxidizing the oxygen present in the polluted samples. This may be one of the causes for the reduction of population of aquatic organisms. The normal range of BOD for good water quality is 5-6mg/l and COD is 6-10 mg/l.[17] Higher BOD and COD values obtained in this study indicate that the water is considerably polluted with organic and chemical pollutants. The experiments conducted in the present study conclude that all bacterial strains exhibit the ability of organic matter decomposition. Besides, they show adaptive response against various heavy metals and thus could have potential application for bioremediation of metal contaminated soils and waste water having high BOD and COD.

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