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Research Article

Efficiency of immobilized microbial combination for the bioremediation of tannery effluents in Vellore District, Tamil Nadu, India

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Abstract

Tannery effluents one of the most polluting industrial wastes. The effluent contains chromium, arsenic, zinc, cadmium, copper and mercury it's accumulating into water bodies and agriculture field causes serious problems. In this study the sample was collected from Vellore District, Tamil Nadu and ten heavy metal resistant bacterial isolates were identified. The bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its potential application in industry. Three categories were used to remediate the tannery effluents followed by Bioaccumulation (Living Cell), Biosorption (Dead cell) and Immobilization. Immobilized bacterial cell has great value to remediate the heavy metals compare to others.

Keywords: Tannery effluent, Bacteria, Bioaccumulation, Biosorption and Immobilization.

Introduction

Environmental pollution has become a major concern of developing countries in the last few decades. There is a growing sense of global urgency regarding the pollution of our environment by an array of chemicals used in various activities (Palaniappan *et al*., 2009; Saranraj *et al*., 2010; Sadeeshkumar *et al*., 2012; Saranraj and Stella, 2014). Pollution of water and soils by heavy metals is an emerging problem in urbo industrialized countries. Since, the advent of development through mining and smelting, tanning, sewage, warfare and metallurgical industries the survival of plants and animals are much affected (Xi *et al*., 2009; Sriram *et al*., 2013).

Tanning industry is recognized as a serious environmental threat all over the world. In India, leather industry contributes 15% of the world total leather production (Alam *et al*., 2009; Saranraj and Stella, 2014) and it is the fourth exchange earner with a share of around 7% in the country's total exports.

Tanning industry contributes significantly towards exports, employment generation and occupies an important role in Indian economy on the other hand; tannery wastes are ranked as the highest pollutants among all the industrial wastes (Soyalsan and Karaguzel, 2007). The tannery industries released most commonly occurring metals at the discharge sites are lead, chromium, arsenic, zinc, cadmium, copper and mercury. The presence of these metals in the water and soil may cause serious threat to human health and ecological systems (Sundar *et al*., 2010).

Materials and Methods

Collection of tannery effluent samples

The tannery effluent to be bioremediated was collected from Vaaniyambadi, Vellore district of Tamil Nadu, India. Before sampling the effluent, the polythene container was cleaned thoroughly using distilled

water. Immediately after the effluent sampling, the effluent sample was taken to the laboratory and stored at room temperature in the laboratory for further analysis using standard methods.

Estimation of heavy metals in tannery effluent

The estimation of trace heavy metals such as for Cr, Zn, Cu, Pb, Ni in the industrial effluent and soil was performed as per Malik *et al*. (1984).

Estimation of Chromium, Zinc, Iron, Copper, Lead, Cadmium, Manganese and Nickel by Atomic Absorption Spectrophotometric (AAS) method

Three concentrations of each standard metal solution were selected to find out the expected metal concentration of a sample. Then, each standard was aspirated into flame and the absorbance was recorded. A calibration curve was prepared by plotting the absorbance of standards versus their concentrations. The estimations of chromium, copper, lead, zinc and nickel were done at the wavelengths of 357.9 nm (chromium, iron, copper and manganese), 324.7 nm (lead), 228.8 nm (cadmium) 248.3 nm (zinc) and 232.1 nm (nickel). The concentration of each metal ion was calculated in milligrams per litre, by referring to the appropriate calibration curve.

Isolation and Identification of Bacterial Isolates from Tannery Effluent

Pour plate technique was used for the isolation of bacteria from the tannery effluent collected from Vaaniyambadi, Vellore district, Tamil Nadu, India. Ten different bacterial strains were isolated and identified.

Screening of Bacterial Isolates for its Heavy Metal Resistance

Disc diffusion method

The isolated bacterial strains (*Pseudomonas fluorescens*, *Proteus* sp., *Bacillus* sp., *Escherichia coli, Serratia* sp., *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter asburiae*, *Alcaligenes* sp. and *Micrococcus* sp.) were tested for their resistance to heavy metals (Cr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+}) by Disc diffusion method. Freshly prepared Muller Hinton agar (MHA) plates were seeded with respective cultures individually. The disc impregnated (20 µl) with respective metal solution (100 mg/L metal solution of Cr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+}) were placed on the four corners of each petridishes and suitable control disc was also placed. The plates were then incubated at 28 ± 2° C for 24 hrs. After incubation, the presence of inhibition zone was visualized. A zone size less than 1 mm was considered as resistance strain (Cervantes *et al*., 1986). The bacterial isolates resistant to all the metals used were taken for further study.

Determination of Minimum Inhibitory concentration (MIC) (Cervantes *et al*., 1986)

The Minimum inhibitory concentration (MIC) of heavy metal resistant bacterial isolates (*Pseudomonas fluorescens*, *Proteus* sp., *Bacillus* sp., *Escherichia coli, Serratia* sp., *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter asburiae*, *Alcaligenes* sp. and *Micrococcus* sp.) grown on heavy metals (Cr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+}) incorporated media was determined by gradually increasing the concentration of the heavy metal by 10 µg/ml each time in the specific media until the strains failed to give colonies on the plate. The starting concentration used was 50 µg/ml. The culture growing on the last concentration was transferred to the higher concentration by streaking on the plate. The MIC was noted when the isolates failed to grow on plates.

Bioremediation of heavy metals in tannery effluent using bacterial isolates

Preparation of Heavy metal solution

The stock solutions of the heavy metals were prepared by mixing 100 mg of respective heavy metal *viz*., Cr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+} in one litre of deionized water (Semra Ilhan *et al*., 2004).

Heavy metal adsorption by living microbial cells (Bioaccumulation) (Vargas *et al*., 2009)

About 1% living bacterial biomass (*Pseudomonas fluorescens*, *Proteus* sp., *Bacillus* sp., *Escherichia coli, Serratia* sp., *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter asburiae*, *Alcaligenes* sp. and *Micrococcus* sp.) were suspended individually in a solution (100 ml) supplemented with heavy metals. After incubation, cells were harvested

by centrifugation. The supernatants of the samples were analysed and the quantity of each metal removed was measured using AAS and expressed as mg/L.

Heavy metal adsorption by dead microbial cells (Biosorption) (Vargas *et al*., 2009)

Biomass from the bacterial isolates (*Pseudomonas fluorescens*, *Proteus* sp., *Bacillus* sp., *Escherichia coli*, *Serratia* sp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter asburiae*, *Alcaligenes* sp. and *Micrococcus* sp.) grown in Nutrient broth were harvested by centrifugation and washed with distilled water three times. The pellet was dried and milled. Aliquots of dried microbial cells (200 mg/L) were prepared in distilled water and homogenized in a mixer to destroy aggregated cells. About 1 ml of cell suspensions were added to the metal solution (100 ml) prepared and incubated. After incubation, the suspensions were centrifuged and filtered for biomass removal. Heavy metal concentration in the supernatant was measured as previously described.

Heavy metal adsorption by immobilized microbial cells (Johncy Rani *et al*., 2010)

The bacterial cells (*Pseudomonas fluorescens*, *Proteus* sp., *Bacillus* sp., *Escherichia coli*, *Serratia* sp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter asburiae*, *Alcaligenes* sp. and *Micrococcus* sp.) were immobilized as beads according to the procedure of Leung *et al*. (2000). Two percent sodium alginate solution is prepared in sterile distilled water by heating it to 60°C and mixing it thoroughly on a magnetic stirrer. Later, 100 ml of the sodium alginate was cooled to room temperature and 10% (10 ml culture in 100 ml sodium alginate solution) of the cell culture was added, the optimum condition was also studied as described above. The contents were mixed well by vigorous shaking to get a homogenized mixture. In a separate beaker, 100 ml of 0.1 M calcium chloride solution was taken. The sodium alginate containing cell culture suspension was extruded drop wise through a syringe and allowed to fall in the beaker containing calcium chloride solution. The beads of sodium alginate gel formed are left in the beaker overnight for hardening. Then beads were washed and stored in distilled water at 28 ± 2°C. One gram of material contained 16 to 17 beads, each bead approximately weighing 60 mg. The beads (1 g) containing $>10^5$ cfu/ml biomass were added to the conical flask containing 50 ml of samples and incubated at room temperature for 72 hrs. After which, the samples were withdrawn for heavy metal analysis using AAS.

Results and Discussion

The resistance of bacterial isolates *Pseudomonas fluorescens*, *Proteus* sp., *Bacillus subtilis*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter asburiae*, *Alcaligenes* sp. and *Micrococcus* sp. which were isolated from the tannery effluent was tested against toxic heavy metals (Cr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+}) by Disc diffusion method and the results were showed in Table – 1.

Table – 1: Determination of heavy metal resistant bacterial isolates

S. No	Bacterial Isolates	Heavy Metals (100 mg/L)				
		Cr (VI)	Zn (II)	Ni (II)	Cu (II)	Pb (II)
1.	*Pseudomonas fluorescens*	R	R	R	R	R
2.	*Proteus* sp.	R	R	R	R	R
3.	*Bacillus subtilis*	R	R	R	R	R
4.	*Escherichia coli*	R	R	R	R	R
5.	*Serratia marcescens*	R	R	R	R	R
6.	*Pseudomonas aeruginosa*	R	R	R	R	R
7.	*Staphylococcus aureus*	R	R	R	R	R
8.	*Enterobacter asburiae*	R	R	R	R	R
9.	*Alcaligenes* sp.	R	R	R	R	R
10.	*Micrococcus* sp.	R	R	R	R	R

All the ten bacterial isolates *viz*., *Pseudomonas fluorescens, Proteus* sp., *Bacillus subtilis, Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter asburiae, Alcaligenes* sp. and *Micrococcus* sp. were resistant to all the heavy metals. Amalesh Samanta *et al*. (2012) explained that the Microorganism was able to interact with a range of toxic metals, including copper, iron, magnesium, gold and lead. This ability was attributed to differences between the net negative charge of bacteria and the cationic charge of many metals. The theory stated that nucleation sites on the cell surface had the ability to bind metals of opposite charge. Once bound to the cell wall, this resulted in a nucleation site where a large concentration of metals could bind and precipitate on the cell wall.

The minimum inhibitory concentration (MIC) of bacterial isolates (*Pseudomonas fluorescens, Proteus* sp., *Bacillus subtilis, Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa, Staphylococcus aureus, Pseudomonas putida, Alcaligenes* sp., *Micrococcus* sp.) to heavy metals (Cr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+}) was determined and the results were showed in Table – 2. The bacterial isolates showed high tolerance to Chromium as compared with other heavy metals. Among the bacterial isolates, *Bacillus subtilis* showed maximum heavy metal tolerance (280 µg/ml for Cr^{2+} and 260 µg/ml for Zn^{2+} and Ni^{2+} 250 µg/ml, 240 µg/ml for Cu^{2+} and 210 µg/ml for Pb^{2+}) followed by *Serratia* marcescens, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Enterobacter asburiae, Escherichia coli, Alcaligenes* sp., *Micrococcus* sp. and *Proteus* sp. The bacterial isolate *Staphylococcus aureus* showed minimum heavy metal tolerance (130 µg/ml for Cr^{2+} and 100 µg/ml for Zn^{2+}, 120 µg/ml for Ni^{2+}, 100 µg/ml for Cu^{2+} and 100 µg/ml for Pb^{2+}) for all heavy metals.

Table - 2: Determination of MIC of bacterial isolates to heavy metals

S. No.	Microbial Isolates	MIC (µg/ml)				
		Cr (VI)	Zn (II)	Ni (II)	Cu (II)	Pb (II)
1.	*Serratia marcescens*	270	250	250	210	220
2.	*Proteus* sp.	160	140	120	100	110
3.	*Bacillus subtilis*	280	260	250	240	210
4.	*Escherichia coli*	180	170	160	160	130
5.	*Staphylococcus aureus*	130	100	120	100	100
6.	*Pseudomonas aeruginosa*	200	210	190	180	190
7.	*Pseudomonas fluorescens*	230	230	210	200	220
8.	*Enterobacter asburiae*	190	180	170	140	150
9.	*Alcaligenes* sp.	180	170	160	140	160
10.	*Micrococcus* sp.	180	150	170	140	110

Bioremediation (Bioaccumulation, Biosorption and Immobilization) of heavy metals (Cr^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+} and Pb^{2+}) was studied by using live bacterial cultures, inactivated or dead bacterial cells and immobilized beads. Ten different bacterial isolates showed resistance against toxic heavy metals were used for the bioremediation studies. The results revealed that all the organisms were found effective in remedying heavy metals. The heavy metal adsorption by living bacterial cells was studied and the results were showed in Table – 3. The bioaccumulation studies revealed that the highest heavy metals adsorption was showed by the bacteria *Bacillus subtilis* (58.5 mg/L for Cr^{2+}, 58.1 mg/L for Zn^{2+}, 57.3 mg/L for Ni^{2+}, 57.8 mg/L for Cu^{2+} and 54.2 mg/L for Pb^{2+}) followed by *Serratia* marcescens, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Enterobacter asburiae, Escherichia coli, Alcaligenes* sp., *Micrococcus* sp. and *Proteus* sp., whereas *Staphylococcus aureus* showed the lowest activity of heavy metal adsorption (33.2 mg/L for Cr^{2+}, 33.0 mg/L for Zn^{2+}, 32.6 mg/L for Ni^{2+}, 32.6 mg/L for Cu^{2+} and 30.4 mg/L for Pb^{2+}). Microbes deals with poisonous chemicals by applying enzymes to convert one chemical into another form and taking energy or utilizable matter from this process. The chemical transformations generally involve breaking of large molecules into several small molecules in simpler form. (Gupta *et al*., 2003; Anitha *et al*., 2010; Saranraj *et al*., 2010; Jayanthi *et al*., 2013; Saranraj and Sujitha, 2013; Jayanthi *et al*., 2014)

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Table – 3: Heavy metal adsorption by living bacterial cells (Bioaccumulation)

S. No	Living bacterial cells	Heavy metals absorbed (Initial concentration – 100 mg/L)				
		Cr(VI)adsorbed (mg/L)	Zn(II) adsorbed (mg/L)	Ni(II) adsorbed (mg/L)	Cu(II) adsorbed (mg/L)	Pb(II) adsorbed (mg/L)
1.	*Bacillus subtilis*	58.5	58.1	57.3	57.8	54.2
2.	*Serratia marcescens*	56.6	55.3	54.6	54.2	52.8
3.	*Pseudomonas fluorescens*	53.2	51.8	51.3	51.6	50.2
4.	*Pseudomonas aeruginosa*	51.3	50.8	50.6	49.8	47.4
5.	*Enterobacter asburiae*	46.4	47.4	47.2	46.8	44.8
6.	*Alcaligenes* sp.	43.2	43.0	42.4	41.6	40.8
7.	*Escherichia coli*	42.6	41.5	40.6	40.0	38.7
8.	*Micrococcus* sp.	38.6	38.4	36.3	36.6	34.5
9.	*Proteus* sp.	36.4	36.9	35.8	35.2	33.0
10.	*Staphylococcus aureus*	33.2	33.0	32.6	32.8	30.4
SEd		2.74	2.64	2.70	2.72	2.68
CD (P = 0.05)		5.49	5.29	5.6	5.46	5.38

The heavy metal adsorption by dead bacterial cells was tested and the results were showed in Table – 4. Among the ten bacterial isolates, *Bacillus subtilis* showed the maximum heavy metal adsorption (70.4 mg/L for Cr^{2+}, 69.6 mg/L for Zn^{2+}, 70.3 mg/L for Ni^{2+}, 68.9 mg/L for Cu^{2+} and 64.6 mg/L for Pb^{2+}) followed by *Serratia* marcescens, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Enterobacter asburiae, Escherichia coli, Alcaligenes* sp., *Micrococcus* sp. and *Proteus* sp. The bacterial isolate *Staphylococcus aureus* showed the minimum adsorption of the heavy metals (42.4 mg/L for Cr^{2+}, 43.5 mg/L for Zn^{2+}, 42.2 mg/L for Ni^{2+}, 40.8 mg/L for Cu^{2+} and 39.8 mg/L for Pb^{2+}). Biosorption studies carried out on some promising natural biosorbents (algae, fungi, bacteria and yeast) and some waste materials which could serve as an economical means of treating effluents charged with toxic metallic ions. The major advantages of biosorption over conventional treatment methods include low cost, high efficiency, minimization of chemical and biological sludge and regeneration of biosorbent and possibility of metal recovery (Nilajana *et al*., 2007; Saranraj and Stella, 2012; Saranraj *et al*., 2014).

The heavy metal adsorption by immobilized bacterial isolates was analyzed and the results were showed in Table – 5. Among the ten bacterial isolates, *Bacillus subtilis* showed maximum heavy metal adsorption (79.2 mg/L for Cr^{2+}, 78.4 mg/L for Zn^{2+}, 77.4 mg/L for Ni^{2+}, 76.8 mg/L for Cu^{2+} and 74.2 mg/L for Pb^{2+}) followed by *Serratia marcescens, Pseudomonas fluorescens, Pseudomonas aeruginosa, Enterobacter asburiae, Escherichia coli, Alcaligenes* sp., *Micrococcus* sp., *and Proteus* sp., while *Staphylococcus aureus* showed the least heavy metal adsorption (50.4 mg/L for Cr^{2+}, 50.9 mg/L for Zn^{2+}, 50.0 mg/L for Ni^{2+}, 49.8 mg/L for Cu^{2+} and 47.4 mg/L for Pb^{2+}). Immobilized cells have been reported to be very effective in heavy metal removal. Heavy metal toxicity and other extreme properties of waste effluents that may limit the use of living cell systems Immobilized cells appear to be of greater potential in controlling particle size, better capability of regeneration, easy separation of biomass and effluent and recirculation, high biomass loading, minimal clogging and reduced depletion of nutrient source (Katiyar and Katiyar, 1997; Sureshkumar *et al*., 2011; Saranraj and Stella, 2012).

Table - 4: Heavy metal adsorption by dead bacterial cells (Biosorption)

S.No	Dead bacterial cells	Heavy metals absorbed (Initial concentration – 100 mg/L)				
		Cr(VI)adsorbed (mg/L)	Zn(II) adsorbed (mg/L)	Ni(II) adsorbed (mg/L)	Cu(II) adsorbed (mg/L)	Pb(II) adsorbed (mg/L)
1.	*Bacillus subtilis*	70.4	69.6	70.3	68.9	64.6
2.	*Serratia marcescens*	67.6	67.3	65.8	65.2	63.8
3.	*Pseudomonas fluorescens*	64.8	63.6	64.4	62.6	60.4
4.	*Pseudomonas aeruginosa*	63.0	61.7	61.8	60.4	58.6
5.	*Enterobacter asburiae*	58.8	57.5	56.3	57.4	56.8
6.	*Alcaligenes* sp.	53.4	53.8	52.5	51.4	51.8
7.	*Escherichia coli*	52.8	50.4	50.6	50.2	48.8
8.	*Micrococcus* sp.	48.9	48.4	46.2	47.8	46.4
9.	*Proteus* sp.	45.2	46.2	44.0	45.4	40.0
10.	*Staphylococcus aureus*	42.4	43.5	42.2	40.8	39.8
SEd		3.05	2.88	3.11	2.92	2.90
CD (P = 0.05)		6.3	5.78	6.24	5.86	5.82

Table - 5: Heavy metal adsorption by immobilized bacterial cells (Immobilization)

S. No	Immobilized bacterial cells	Heavy metals absorbed (Initial concentration – 100 mg/L)				
		Cr(VI)adsorbed (mg/L)	Zn(II) adsorbed (mg/L)	Ni(II) adsorbed (mg/L)	Cu(II) adsorbed (mg/L)	Pb(II) adsorbed (mg/L)
1.	*Bacillus subtilis*	79.2	78.4	77.4	76.8	74.2
2.	*Serratia marcescens*	75.6	74.8	72.6	72.3	71.4
3.	*Pseudomonas fluorescens*	73.2	70.3	71.0	69.8	68.4
4.	*Pseudomonas aeruginosa*	70.3	68.8	69.4	67.5	66.2
5.	*Enterobacter asburiae*	67.8	65.6	64.8	65.2	64.2
6.	*Alcaligenes* sp.	60.4	62.2	61.0	58.2	58.2
7.	*Escherichia coli*	59.6	59.8	58.2	57.4	54.2
8.	*Micrococcus* sp.	55.4	56.4	55.2	54.0	52.3
9.	*Proteus* sp.	53.8	54.2	53.8	53.2	51.8
10.	*Staphylococcus aureus*	50.4	50.9	50.0	49.8	47.4
SEd		3.15	2.85	2.88	2.88	2.93
CD (P = 0.05)		6.40	5.80	5.78	5.78	5.88

Conclusion

From the present study it was concluded the immobilized bacterial cell *Bacillus Subtilis* showed maximum heavy metal adsorption (79.2 mg/L for Cr^{2+}, 78.4 mg/L for Zn^{2+}, 77.4 mg/L for Ni^{2+}, 76.8 mg/L for Cu^{2+} and 74.2 mg/L for Pb^{2+}) followed by Immobilized bacterial cell *Serratia marcescens* (75.6 mg/L for Cr^{2+}, 74.8 mg/L for Zn^{2+}, 72.6 mg/L for Ni^{2+}, 72.3 mg/L for Cu^{2+} and 71.4 mg/L for Pb^{2+}), and immobilized bacterial cell *Pseudomonas fluorescens* (73.2 mg/L for Cr^{2+}, 70.3 mg/L for Zn^{2+}, 71.0 mg/L for Ni^{2+}, 69.8 mg/L for Cu^{2+} and 68.4 mg/L for Pb^{2+}).

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