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RESEARCH ARTICLE

Bioremediation of organic pollutants by using free and immobilised cells of *Pseudomonas putida* and *Pseudomonas aeruginosa*

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SUMMARY : Immobilisation of cells on a suitable support simplify the treatment of liquid waste as the entrapment of living cell increasing the retention time of cells on contaminated water. The present study aimed at free and immobilised cells of *Pseudomonas putida* and *Pseudomonas aeruginosa* were used as remediating material for the removal of organic pollutants from tannery effluent. Removal of organic pollutants is drastic under immobilised conditions compared to free cells of *Pseudomonas putida* and *Pseudomonas aeruginosa*. Compared to free cell, immobilised cells of *Pseudomonas putida* and *Pseudomonas aeruginosa* are more efficient in the removal of organic pollutants from tannery effluent. Immobilised cells of *Pseudomonas putida* and *Pseudomonas aeruginosa* exhibited maximum percentage bioremediation level of organic pollutants from tannery effluent.

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Biotechnology has been investigated as an alternative method for treating the metal-containing wastewater of low concentrations. In response to heavy metals, micro-organisms have evolved various measures *via* processes such as transport across the cell membrane, bioabsorption to cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions. It has been proved that they are capable of adsorbing heavy metals from aqueous solutions, especially for the metal concentration below 50 mg/L (Lu and Wilkins, 1995). The metal-binding capacities of several biological materials have been identified to be very high, including marine algae, fungi and yeasts. It was reported that these micro-organisms could accumulate a wide range of metal species.

Bioremediation, a biodegradation process in which sites contaminated with xenobiotics are cleaned up by means of bacterial bio-geochemical processes, preferably *in situ*, exploits the ability of micro-organisms to reduce the concentration and/or toxicity of a large number of pollutants. It is an economical, versatile, environment-friendly and efficient treatment strategy, and a rapidly developing field of environmental restoration. Bioremediation utilizes the microbial ability to degrade and/or detoxify chemical substances such as petroleum products, aliphatic and aromatic hydrocarbons (including polycyclic aromatic hydrocarbons and polychlorinated biphenyls), industrial solvents, pesticides and their metabolites, and metals. The presence of a large number of diverse bacterial species in nature expands the variety of chemical pollutants that can be degraded and the extent to which pollutant sites can be decontaminated.

EXPERIMENTAL METHODOLOGY

Initial analysis of the effluent was carried out by analysing the physico – chemical parameter of the effluent. The physico – chemical parameters such as total dissolved solids, total phosphate, inorganic phosphate, total hardness, BOD, COD, calcium, magnesium are carried out (APHA, 1991). Bacteria were identified based on

colony characteristics, Gram staining methods and various biochemical studies as given by Bergey (1984). Immobilization of Bacteria in Alginate beads (Chibata *et al.*, 1996).

Experimental condition for bioremediation :

For the present study the following treatments were employed in order to study the interaction of the bacteria with the tannery effluent.

- Effluent sample (100 ml) inoculated with *Pseudomonas putida* (100 mg).
- Effluent sample (100 ml) inoculated with *Pseudomonas aeroginosa* (100 mg).
- Effluent sample (100 ml) inoculated with immobilized *Pseudomonas putida.*
- Effluent sample (100 ml) inoculated with immobilized *Pseudomonas aeroginosa.*
- Raw effluent sample (100 ml) - Control

The experiment was conducted in batch cultures in duplicates for a total period of 20 days (once in 5 days) in 250ml Erlenmeyer flasks under the same conditions as described for the culture maintenance. Effluent samples (control and bacteria treated) were periodically (every five days) analysed for various physico-chemical parameters and recorded.

EXPERIMENTAL FINDINGS AND DISCUSSION

Inoculated bacteria are known to grow fairly efficiently in different types of effluent and generally this growth is measured with the biomass component (Sallal, 1988). In the present study both *Pseudomonas putida* and *Pseudomonas aeruginosa* were found to grow in tannery effluent. However, *Pseudomonas putida* recorded marginally better growth than *Pseudomonas aeruginosa*. The slow growth of *Pseudomonas putida* and *Pseudomonas aeruginosa* in tannery effluent might be due to high COD, Mg and Chloride. Moreover, the colour of the effluent was yellowish brown initially which could prevent easy light penetration into the effluent and it probably contained some toxic substances also from fungi which are abundant in this effluent. Rajannan and Oblisami (1979) noticed similar observations in paper mill effluent. Such slow growth in different effluents such as domestic sewage (Subramanian and Shunmugasundaram, 1986; Manoharan and Subramanian, 1992 b), Ossein (Manoharan and Subramanian, 1993) and dye (Vijaykumar *et al*., 2005) has already been reported.

Initially the effluent was turbid and yellowish brown in colour. This turbidity and yellowish brown colour was due to total dissolved solids of the effluent (Sharma *et al*., 2003). Turbidity of the effluent showed continuous decrease and by 10^{th} day of the effluent became almost clear when treated with bacteria in alginate immobilised condition. Whereas, with free cells it took 15 days to make clear the turbidity of the effluent. Organic substances are being broken down into simpler inorganic forms for absorption by growing bacteria as pointed by Sharma *et al*. (2003). Decrease in BOD, COD and dissolved organic matter is observed.

The initial pH of the effluent was 8.1 and it was increased to 8.7 and 8.6 in the effluent with immobilised *Pseudomonas putida* and *Pseudomonas aeruginosa* and with free cells it was around 8.4. Alkaline nature of the effluent showed gradual increase as a result of break down of bicarbonates due to cellular respiration Manoharan and Subramanian (1992a and b, 1993a) found a rise in pH value up to 10^{th} day in various effluents inoculated with free blue green algae.

There was a fairly good correlation between the initial

Table 1 : Initial level of physico-chemical parameters of the effluent

Sr. No	Parameters	Initial
1.	pH	8.1
2.	Temperature	20.10
3.	Total dissolved solids	2860
4.	Carbonate	2.60
5.	Bicarbonate	212
6.	Nitrate	229
7.	Nitrite	166
8.	Ammonia	89
9.	Total phosphate	176
10.	Inorganic phosphate	93
11.	Total hardness	419
12.	Magnesium	133
13.	Calcium	286
14.	Chloride	1080
15.	Free carbon dioxide	24
16.	Biological oxygen demand	230
17.	Chemical oxygen demand	595

Note: All values are expressed in mg / L, except pH and temperature

levels of bicarbonate and free CO_2 in the effluent. There was a very low level of carbonate in the effluent during the experimental period, but fairly high levels of bicarbonate and free CO_2 were present. A gradual reduction in bicarbonate from 5th day onwards was noticed in the effluent treated with *Pseudomonas putida* and *Pseudomonas aeruginosa* in both free and immobilised conditions. However, the reduction was maximum in effluent with immobilised conditions than with free cells. On the other hand CO_2 was not completely removed in the effluent treated with *Pseudomonas putida* and *Pseudomonas aeruginosa* in both free and immobilised conditions. Thus, bacteria may have a complete advantage of assimilating HCO_3^- as a source of inorganic carbon for cellular respiration and they have high CO_2 affinity and low CO_2 compensation point (Colman, 1989). In the present investigation, though HCO_3^- was not completely removed from the effluent, their initial level was very high when compared to CO_2 level (Table 1). This could be the reason for not complete removal of HCO_3^- from the effluent. However, the removal of these carbon sources effectively by bacteria both in free and immobilised conditions as could be expected was observed in the present investigation.

BOD and COD are the parameters, which will determine the strength of waste. COD is generally considered as a major indicator of organic pollution in water (Dash and Mishra, 1999 a). Industrial waste water analysis in India have been reported to show higher BOD and COD that Bureau of Indian standards (BIS) permissible limits reported by Panesar *et al*. (1999) and in their study, on the effluents arising from tannery in Tamil Nadu state, has reported BOD and COD ranging from 105 to 200 mg/L and 300 to 500 mg/L, respectively. In the present study, also the initial levels of BOD and COD were 230 and 595mg/L, respectively (Table 1). Inoculation of bacteria both in free and immobilised conditions brought down the BOD and COD levels considerably when compared to control (Table 2). The reduction percentage of BOD ranged from 74 to 83 with free cells and 80 to 87 with immobilised bacteria. Similarly COD percentage ranged from 63 to 67 with free cells and 67 to 72 with immobilised bacteria (Table 2). The BOD reduction of 40 per cent of dairy effluent by using lactose fermenting yeast has been reported (Chand and Srinivasan,1984). The metabolization of whey lactose for vitamin B_{12} production using *Propioniobacterium* sp. which consequently resulted in BOD reduction up to 74 percentage in dairy effluent has also been reported by Capoor and Singh (1985).

Use of acclimatized algal cultures in considerably reducing BOD and COD with different effluents including dye industry has been reported (Saxena *et al*., 1990; Govindan, 1983; 1984 and 1985; Manoharan and Subramanian, 1992a and b, 1993 a; Dash and Mishra, 1999; Sharma *et al*., 2003; Vijaykumar *et al*., 2005). Effectively of immobilized bacteria in removing BOD and COD over free cells has already been reported. Considering the above observations, the result obtained in the present investigation is quite conceivable.

Common inorganic nutrients present in waste water include nitrite, nitrate, ammonia and phosphate. All these compounds are essential requirement for the growth of bacteria. They have high nutrient uptake capabilities as they can accumulate inorganic phosphate and nitrogen and store them as polyphosphate (Fay, 1983). Suspended cultivation of micro organisms is one of the biological processes which have been employed to eliminate the residual inorganic nutrients as a tertiary treatment step from secondary treated effluents (Oswald *et al*., 1978; Tam and Wong, 1989; Prakasham and Ramakrishna, 1998).

Table 2: Percentage removal of physico-chemical parameters of effluent on 20th day

Sr. No	Parameters	*Pseudomonas putida*		*Pseudomonas aeruginosa*	
		Free cells	Immobilised	Free cells	Immobilised
1.	Colour	Decolourized	Decolourized	Slightly	Decolourized
2.	Total dissolved solids	69	74	52	69
3.	Carbonate	100	100	100	100
4.	Bicarbonate	80	90	72	81
5.	Nitrate	78	87	61	78
6.	Nitrite	88	100	70	82
7.	Ammonia	83	94	89	100
8.	Total phosphate	97	100	81	100
9.	Inorganic phosphate	91	100	82	100
10.	Total hardness	53	64	47	58
11.	Magnesium	86	100	68	81
12.	Calcium	37	48	37	48
13.	Chloride	42	46	37	40
14.	Free carbon dioxide	71	79	63	75
15.	Biological oxygen demand	83	87	74	80
16.	Chemical oxygen demand	67	72	63	67

Note: pH increase in their level in all the treatments with both free and immobilised cells of *Pseudomonas putida* and *Pseudomonas aeruginosa*

In the present investigation, in tannery effluent, all forms of inorganic nitrogen were observed in appreciable quantities (Table 1). A complete removal of ammonia by both bacteria in immobilised *Pseudomonas aeruginosa* and 94 per cent by immobilised *Pseudomonas putida* and above 80 per cent removal by free cells of bacteria were observed on 20th day (Table 2). On the other hand, free cells of bacteria could reduce nitrite around 70 per cent and above 80 per cent by immobilised bacteria, respectively, while nitrate removal was above 60 and 70 per cent with free and immobilised bacteria, respectively (Table 2). Among inorganic nitrogen compounds, ammonia is the preferred source in bacteria, and removal of these compounds is always related with metabolism dependent process (Prakasham and Rai, 1992) and utilized for the mass transfer application. In most of the effluents ammonia, nitrate and nitrite remain together. Under such conditions, bacteria utilize first ammonia, then nitrite and nitrate. This is mainly because, ammonia is required to be least processed to incorporate into cell constituents, whereas nitrates require a special enzyme for transport of nitrate into cell and then converted to nitrite and then ammonia by the sequential action of nitrite and nitrate reductase (Guerrero and Lara, 1987). This could be the reason for the observed variations in the inorganic nitrogen removal in the present investigation. The specific use of bacteria, both free and immobilised forms, in the efficient removal of different forms of combined nitrogen has also been reported (Proulx and de la Noue, 1988). Manoharan and Subramanian (1992a and b, 1993a) found that there was 100 per cent removal of nitrite and ammonia and 50-100 per cent removal of nitrite from different effluents by *Oscillatoria* sp. alone and in combination with natural population of microbes. Similarly, Vijayakumar *et al.* (2005) reported that *Oscillatoria brevis* could remove more than 90 per cent of all forms of inorganic nitrogen from the dye effluent.

Entrapment matrices play a major role in change of rate of removal of inorganic nitrogen by bacteria. It was observed that, Chitosan immobilised *Anabena dioliolum* is more efficient in removing nitrate and nitrite over ammonia (Mallick and Rai, 1994). In the present study, alginate immobilised bacteria removed ammonia efficiently from the effluent when compared to other inorganic nitrogen compounds. Moreover the removal efficiency was more with immobilised bacteria than with free cells (Table 2).Contrary to this, Grabisu *et al*.(1991) reported that polyvinyl and polyurethane immobilised *Phormidium laminosum* cells showed more than 90 and 60 per cent reduction of nitrate uptake to that of free cells. However, in the present investigation, it was observed that the immobilised bacteria were more efficient in removing all forms of inorganic nitrogen when compared to free cells.

Pseudomonas putida and *Pseudomonas aeruginosa* in both free and immobilised conditions could bring down the level of all forms of phosphate in the effluent (Table 2). The capacity of bacteria to remove large amount of phosphorus from industrial waste water has been demonstrated by several workers (Witt, 1963; Neos and Verma, 1996; Chan *et al.*, 1979; Tam and Wong, 1989). Further, the bacteria are known to absorb and store large amount of phosphorus as polyphosphate granules (Fay, 1983). Manoharan and Subramanian (1992a and b, 1993a) and Vijaykumar *et al.* (2005) found a total or near total removal of all types of phosphate by *Oscillatoria* from different effluents. Tang *et al.* (1997) also reported a higher phosphate uptake, despite low biomass of cyanobacteria. Dash and Mishra (1999) also observed 100 per cent removal of phosphate from the paper mill effluent while treating with *Westiellopsis prolifica*. In the present investigation, immobilised bacteria, efficiently removed all forms of phosphate from the effluent when compared to free cells (Table 2). Alginate immobilised bacteria were better in removing all forms of phosphate (Table 2). Contrary to the present findings, Robertson and Kuenen (1995) and Mallick and Rai(1994) earlier reported that inorganic phosphate removal was found to be more effective in free cells rather than in immobilised conditions. However, Proulx and de la Noue (1988) found chitosan immobilised *Phormidium* cells were able to remove phosphate up to 90 per cent with the retention time of 24 hours from urban waste water. Phosphate uptake is more pronounced in growing cells to that of stationary phase cells. Robertson and Bhatt (1973) have found that exponentially growing cells eliminate phosphate from the medium five times more rapidly than the cells of older age is an important factor in phosphate removal from waste water.

Calcium and magnesium contribute to the hardness of effluent. A general decrease in their concentration with respect to zero days was observed in the present investigation. The percentage removal of calcium was 37-48 and that of magnesium was 68-100 in all treatments with both bacteria (Table 2). However, the immobilised bacteria efficiently removed calcium and magnesium from the effluent over the free cells. Among the immobilised forms, *Pseudomonas putida* was effective in removing calcium and magnesium (Table 2). Reports are available on the removal of calcium and magnesium from different effluents with bacteria [Manoharan and Subramanian (1992 a and b, 1993 a); Dash and Mishra (1999); Sharma *et al.* (2003); Vijayakumar *et al.* (2005)]. Substantial reduction in calcium and magnesium levels cannot be explained by uptake. Divalent cations such as calcium and magnesium are known to be essential for flocculation and would co flocculate, which could explain the observed reduction.

Chlorides are generally considered to be one of the major pollutants in effluents which are difficult to remove by conventional biological treatments. In the present study, 37-46 per cent removal of chloride from the effluent by both

bacteria under different conditions (free and immobilised) was observed (Table 2). Maximum level of chloride removal was noticed in effluent with immobilised *Pseudomonas putida* (46 %) and minimum (37 %) with free living cells of *Pseudomonas aeruginosa*. When compared to *Pseudomonas aeruginosa, Pseudomonas putida* responded well in the removal of chloride. Manoharan and Subramanian (1992a and b, 1993a) also reported the removal of chloride from various effluents including Ossein effluent by *Oscillatoria pseudogeminata*. A similar observation attributing 50 per cent chloride reduction under laboratory conditions by *Oscillatoria brevis* was also reported in dye effluent (Vijayakumar *et al.,* 2005).

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