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Invitro Screening of Trichoderma in Rockphosphate Solubilization And Mycoremediation of Heavy Metal Contaminated Water

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ABSTRACT

Trichoderma ,echoed as a potential biofertilizer and in mycoremediation of Iron and lead of polluted water .The fungi was able to solubilize different sources of insoluble rock phosphates,Raj phos, Mussorie rock phosphate, Gafsa phos-60, Gafsa phos-100,North Carolina rock phosphate as free and immobilized cultures.The concentration of available phosphorus (P) under invitro condition was high for immobilized cells compared to free cell cultures in liquid broth. Drastic reduction of pH was also seen in the liquid broth supplemented with different sources of unavailable P.Further,mycoremediation studies using Trichoderma, the concentration of heavy metals reduced in bore well and car wash water with immobilized cells compared to free cells. The positive response of immobilized culture for removal of heavy metal, iron and lead makes the process of mycoremediation of the polluted water more feasible as there is no growth of the fungus and therefore less of pollution and the removal of toxic pollutant.

Keywords : Phosphate solubilising microbes,immobilized cells,mycoremediation

Trichoderma is a fungus that exists in almost all soils and a wide range of habitats. In soil, they are the most widespread, culturable fungi. They prefer locations with a large supply of plant roots, which they promptly colonize. Intricate chemical reactions in the soil convert applied phosphate fertilizers into highly insoluble forms. However, the soil also has fungi that convert these insoluble forms into soluble forms. Such fungi are said to possess a mineral phosphate solubilizing ability that is an alternative to chemical fertilizers.Tirchoderma harzianum is known to be a potential phosphate solubilizer (Padmavathi and Madhumathi).In addition ,immobilization of microbial cells is a well known method having potential application in industries for their stability and recovery. But in agriculture the immobilized system used should facilitate the release of microbial inoculants to elicit the benefit of plant microbe interactions. Immobilized cells retain high catabolic activity, are protected from predators, parasites and inhibitors and efficiently mineralize substrates(Brodelius and Vandamme,1987).The world has a tremendous supply of water, but most of it is too polluted for human consumption or for agriculture. Polluted water is increasing day by day as consequences of anthropogenic activities. Industrial growth,urbanisation and due to other man-made problems water bodies receive a heavy flux of sewage, domestic waste, industrial effluents, agricultural wastes etc.,These chemicals added to the water bodies range from simple nutrients to highly toxic heavy metals. These chemicals are considered as pollutants, as their addition has decreased the legitimate use of water for any purpose. Considering the possible threat of pollutants in water, an in situ mycoremediation was taken up under laboratory conditions, a cleanup approach was designed, where in immobilized cells of Trichoderma spp. was used in the treatment of heavy metals dissolved in the water and its possibility in controlling the microbial density and the removal of heavy metals. Immobilization is done to see that secondary metabolites are involved in the biotransformation of the pollutants without the growth of the microbe.Efficient biodegradation by immobilized fungal cells by sodium alginate method (Zhou et al.,2007).The ability of lead(Pb) and Iron(Fe) bisorption by immobilized Trichoderma spp was studied in the present work. We have zeroed in on Lead and Iron, the toxic heavy metals found in drinking water, able to cause serious ailments in humans, animals as well as aquatic flora and fauna.Iron being one the most heavy metal contaminants in water supplies. Rainwater as it infiltrates the soil and underlying geologic formations dissolves iron, causing it to seep into aquifers that serve as sources of groundwater for wells. Household plumbing materials and water service lines even legally “lead-free” plumbing may contain up to 8 percent lead. . Lead in drinking water can also cause a variety of adverse health effects. In this study car wash water and bore well water near to the vehicle service station were chosen.

Material and Methods

Trichoderma harzianum culture maintanence

Trichoderma harzianum cultures were obtained from PDBC. These cultures were maintained on Trichoderma specific media ($MgSO_4$ 0.20g ,K_2HP0_4 0.90g,Kcl 0.15g,NH_4Cl 3.00g,PCNB 0.20g, Rose Bengal 0.15g,Agar 20.0g,chloramphenicol 0.25g,Captan 0.20g ,Propamocarb 1.60g,water 1000ml,pH 6.0)slants at 4ºC.

Screening of Trichoderma harzianum for phosphate solubilization.

Preliminary screening for phosphate solubilization was done by a plate assay method using Sperber’s Agar Media.Trichoderma harzianum cultures were point inoculated in Sperber’s media and incubated at 37 °C . Sperber’s agar medium supplemented with tricalcium phosphate (TCP). The medium contained: glucose 10 g; Ca $(PO)_4$ 5 g; $(NH)_4 S0_4$ 0.5 g; NaCl 0.2 g; $MgSO_4 .7H_2 O$ 0.1 g; KCl 0.2 g;Yeast extract 0.5 g; $MnSO_4 .7H_2 O$ 0.002 g; $FeSO_4.7H_2 O$ 0.002g and agar 15 g. The initial pH of the medium was adjusted to 7 [8]. The plates were incubated at room temperature for four days and the ability of isolates to solubilize the phosphate was assessed by observing halo zone around the growing colonies indicating phosphate solubilization. The results were expressed as solubilization efficiency (E):

$$E = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

Immobilization of Trichoderma harzianum

Immobilization of Trichoderma harzianum culture was done by Sodium alginate method.50ml suspension of the fungal mycelia (4g wet mycelia) was thoroughly mixed in sodium alginate solution that was sterilized.The suspension was added drop by drop to 2% calcium chloride solution.The beads were then collected and kept in 20% glucose solution for half an hour. They were washed thoroughly in sterile distilled water and 15 beads were used for Phosphate solubilization and 5g of beads for treatment of waste water.For waste water treatment ,a control and sample with 5g of beads was introduced into conical flask.Control car wash and car wash+immobilized Trichoderma harzianum, control borewell water and borewell+ immobilized Trichoderma harzianum were the treatments maintained.The conical flasks were incubated on a rotary shaker at 24^0C at 125rpm.The pH of the samples were checked daily .The percent removal of iron and lead was determined from the difference in the concentrations of the heavy metals in the water sample before and after addition of the beads.

Capacity to solubilize different sources of insoluble Phosphate in liquid cultures

Solubilization activity was carried out in 100 ml Sperber's broth medium supplied with hydroxyl apatite and modified Sperber's broth medium consisiting of different insoluble sources of phosphate(Mussorrie rock phosphate,Gaphsa phos-100 and 60,Raj phos and North Carolina Rock Phosphate) Then,after sterilization 1.0 ml of harvested fungal spore suspension was inoculated to each of the broth.A control without any inoculation was maintained. The cultures were incubated on a rotary shaker at 25ºC for 7 days. Available Phosphorus in broth cultures was estimated by Ascorbic acid method. In addition pH change of the medium was monitored for production of organic acids.

Determination of metal concentration in the waste water

The car wash and the borewell water samples were analysed for Iron and Lead concentration by mass spectroscopy.

Results & Discussion:

Trichoderma harzianum was tested for Mineral Phosphate Solubilizing (MPS) activity on Hydroxy apatite medium (HAP), it showed zone of solubilization and grew profusely after 168 hrs of incubation (68.75mm) as evident from Plate 1.In broth cultures after ten days of incubation, maximum cell dry weight (1990mg/100ml) was observed on HAP .Though it is evident from the Table 1& Plate 2 that the growth was seen on modified Sperber's medium supplemented with Rock Phosphates. Solubilization of insoluble phosphorus was followed by drastic reduction of pH in the medium, though the initial pH of the medium was 7.Available P was estimated by ascorbic acid method.It was found that the fungus could solubilize maximum P in HAP medium (1.82ppm), followed by MRP (1.57ppm), GP-100(1.28ppm), 0.46ppm of available P was estimated in RP and GP-60 medium. The pH reduction in all the cultures ranged from 4.30-4.00. From Table 2& Plate 3 ,immobilized culture of Trichoderma harzianum after seven days of incubation showed maximum solubilization of insoluble phosphate in HAP(1.98ppm)with a drastic reduction in pH(3.16) followed by MRP(1.88)and pH reduction is 3.79,GP-100(1.62ppm,pH 3.10),NCRP(0.97ppm,pH 5.36),GP-60(0.91ppm,pH 5.70) and RP (0.46ppm,pH 4.30) was found to be the least. As it was seen that Trichoderma harzianum could solubilize insoluble phosphate and a drastic reduction in pH, the possible mechanism would be the production of organic acids in the medium. Henceforth the immobilized culture was used for treatment of toxic metals in waste water .From Table 3,Initial Chemical analysis for the heavy metal showed presence of lead only in carwash(CW-0.058ppm) but not in bore well(BW) water. Iron was present in both the water samples (BW-0.017ppm,CW-0.33ppm).After seven days of treatment with immobilized Trichoderma harzianum,it was found that the heavy meal content decreased for both (BW-Iron:0.011ppm,CW-Iron:0.16ppm and lead-0.043ppm) water samples. The table also shows the percent removal of the heavy metals after treating with the immobilized Trichoderma harzianum, 33.3% and 51.5% of iron in BW and CW, respectively and 25.8% of lead in CW. Phosphorus is one of the major plant nutrients. It is next to nitrogen in its requirement as an essential nutrient plant. Plant obtains this element from soil solution. Therefore its availability to plants depends on the form in which it is present in the rhizosphere.Phosphorus is present in the soil in mineral form as iron, aluminium, calcium or magnesium phosphates, or as organic phosphates as iron or calcium phytates. Intricate chemical reactions in the soil convert applied phosphate fertilizers into highly insoluble forms. However, the soil also has fungi that convert these insoluble forms into soluble forms. Such fungi are said to possess a mineral phosphate solubilizing ability that is an alternative to chemical fertilizers. It is evident from the current study, that Trichoderma is an efficient fungus in rock phosphate solubilization, bicontrol and in treatment of waste water. However the liquid environment offered encouraging results where the fungus showed good activity in broth cultures. Morris and Allen (1994) while studying the oxalate metabolism by microorganisms echoed a confirming trend in liquid broth, attributed to simple cation dissociation. The data also shows that the ability of the fungus to solubilize phosphorus depends upon the source of phosphorus. Low solubilization of phosphorus could be to the resistance of these phosphatic compound to the organic acids released by these PSM.The pH of the medium was reduced because of the production of organic acids. The solubilization of insoluble phosphorus sources from the immobilized cell cultures with the simultaneous reduction of pH have shown that these immobilized cultures were able to produce enzymes that could act as biocatylst in the conversion of compounds present in the media to organic acids or respective metabolites which in turn acts on insoluble P sources to release available P. Moreover the amount of available P produced in immobilized cultures were more when compared to free cultures, because the available P is not mobilized by the entrapped cultures for their growth or metabolic activity. Though most of the work on immobilized cultures of microorganisms is done for the commercial production of enzymes. immobilized PSM is of the first kind in the study. Being a potential biofertilizer Trichoderma harzianum immobilized cultures were further used in the study for treatment of heavy metal contaminated water. The obtained results are in good agreement with our initial assumption that the immobilized fungal beads of Trichoderma harzianum can be used as an effective method of bioremediation. The positive response of immobilized culture for removal of heavy metal, iron and lead makes the process of bioremediation of the polluted water more feasible as there is no growth of the fungus and therefore less of pollution and the removal of toxic pollutant. Here the secondary metabolites produced by the immobilized culture would have transformed the metal content to a less toxic form.

Table 1: Growth , solubilization of phosphorus and change in pH by Trichoderma harzianum on Sperber'medium and modified Sperber's medium after 10 days of incubation

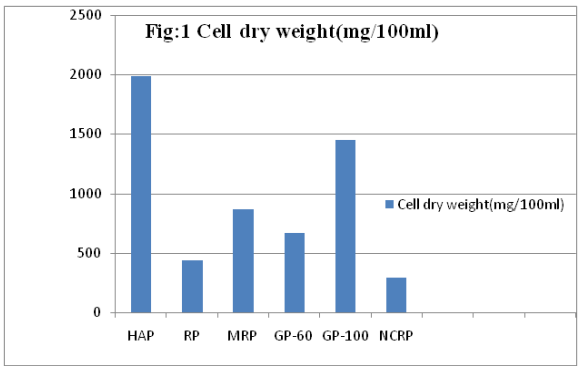
P source	Celldry weight (mg/100ml	Soluble P (ppm)		pH	
Hydroxy apetite	1990	C=0.05(2.00)	1.82(53.00)	C=6.72	4.00
Raj Phos	440	C=0.01(0.30)	0.46(13.00)	C=7.00	4.00
Mussoorie RP	870	C=0.04(1.00)	1.57(46.00)	C=6.81	4.30
Gafsa Phos-60	670	C=0.05(2.00)	0.57(17.00)	C=7.10	4.10
Gafsa Phos-100	1450	C=0.05(2.00)	1.28(37.000	C=6.76	4.10
North Carolina RP	290	C=0.08(2.00)	0.46(13.00)	C=6.88	4.30

Figure in the parenthesis indicates percent of added P solubilized

C=Uninoculated control

pH of the medium at the time of inoculation was 7.00

RP=rock phosphate



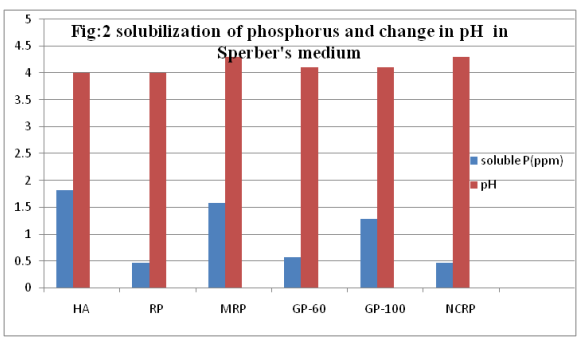


Table 2: Phosphate solubilized and pH changes by immobilized Trichoderma harzianum on Sperber'medium and modified Sperber's medium after 07 days of incubation

P source	Soluble P (ppm)		pH	
Hydroxy apetite	C=0.05(2.00)	1.98(57.00)	C=6.89	3.16
Raj Phos	C=0.02(0.30)	0.46(23.00)	C=7.00	4.30
Mussoorie RP	C=0.05(1.40)	1.88(55.00)	C=6.80	3.79
Gafsa Phos-60	C=0.06(2.00)	0.91(26.00)	C=6.99	5.70
Gafsa Phos-100	C=0.06(2.00)	1.62(47.00)	C=6.78	3.10
North Carolina RP	C=0.08(2.00)	0.97(28.00)	C=6.99	5.36

Figure:3 Phosphate solubilized and pH changes by immobilized

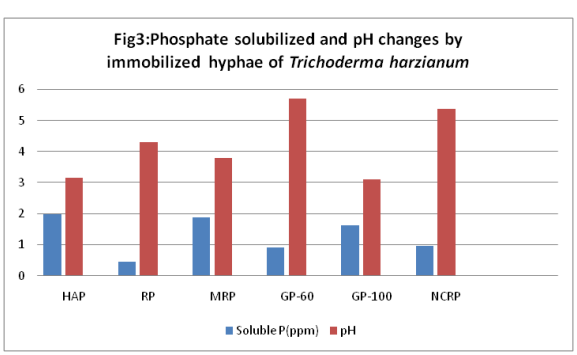


Table 3: Analysis of lead and Iron in waste water samples

Borewell(BW)	Iron(Fe)ppm	Lead(Pb)ppm
Initial	0.017	Nil
Final(BW+IMF)	0.011	Nil
Percent removal(%)	33.3	Nil
Carwash(CW)		
Initial	0.33	0.058
Final(CW+IMF)	0.16	0.043
Percent removal(%)	51.5	25.8

IMF:Immobilized fungal cells of Trichoderma harzianum

Final analysis was done after 7 days of incubation

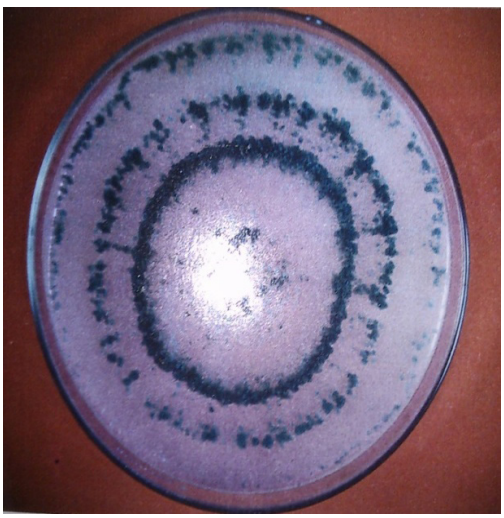


Plate 1:Trichoderma grown on Sperbers agar medium



Plate 2:Solubilization of P by Trichderma in Sperbers broth

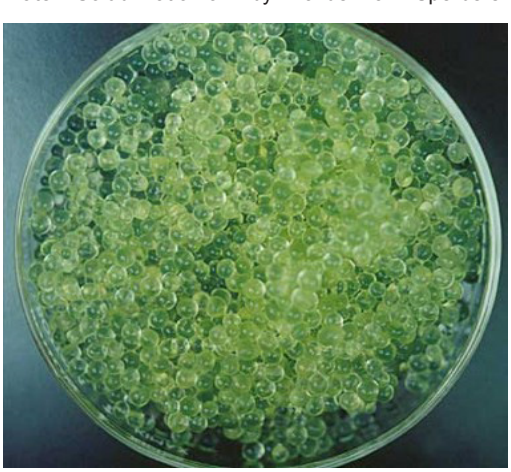


Fig:3 Immobilized fungal spores of Trichoderma harzianum

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