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Research Article

A STUDY OF MULTIPLE HEAVY METAL TOLERANCE IN ROOT NODULATING BACTERIA

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ABSTRACT

In this study overall 22 root nodules strains of leguminous plants were used and their maximum resistance level (MRL) was determined by using iron and lead. Those strains were Tolerant,they were used for further study of cell surface properties. Surface elements like expolysaccharide, neutral beta glucans, lipopolysaccharides as well as those components that are responsible for motility of rhizobial cells. Further the strains which shows maximum tolerances were selected for co resistance to nickel, cadmium, copper, cobalt, chromium, zinc and aluminium till MIC on the basis of MRL and cell surface propertiesand it was observed that there was a decline in the growth of Rhizobium,with respect tothe increase in the heavy metals concentrations. Maximum selected isolates showed multiple tolerances to heavy metals with minimum inhibitory concentration (MIC) for heavy metals ranging from 25 mg/ml – 350 mg/ml. whereas maximum resistance level was observed in isolates RT4d for nickel and zinc (300 mg/ml). the present study concluded that seven selected isolates maintains cell surface components as well as having multiple tolerances to other heavy metals could lead to a successful approach for bioremediation of heavy metal contaminated regions.

Keywords: bioremediation, cell surface properties, heavy metal, symbiotic nitrogen fixation.

INTRODUCTION

Heavy metal contamination is worldwide (Nriagr, 1990) Since beginning of industrial revolution,pollution of biosphere by toxic metals has more widely increased. number of human activities directly or indirectly polluting the environment.by variety of anthropogenic sources like smelters,mining,power station and by application of metal containing pesticides, fertilizers and sewage sludge soil become contaminated with metals (Gilleret al., 2001).the main environmental pollution is the heavy metal pollution of soil and its negative impact on agriculture which affects the animal as well as human health. most common heavy metals contamination are Cd, Cr, Cu, Hg, Pb and Ni (Zhon and Qiu, 2005). From industrial, agricultural and domestic activities the amount of heavy metal into water bodies is increasing and is of great concern to human beings because of their toxic effects on food chain (Matakaetal., 2006).for ecological evolutionary and environmental reason the Toxicity of heavy metals is a big problem(Nagajyotiet al., 2008).the soil fertility looses when the heavy metals accumulate in the soil to an abnormal level and causes dramatic changes in the microbial composition and in their activities . (Krujatzet al., 2011),

For toxic heavy metal contamination of soil environment, an effective and inexpensive attention is necessary (Wani and Khan, 2012).heavy metals cannot be degraded biologically to more less toxic products. therefore to neutralize the effect of metal stress, microorganisms have evolved varieties of mechanisms, by which they tolerate the uptake of heavy metal ions. Previous studies reported that the microbial resistance to heavy metals occurs,due to the presence of cell surface components, (Gauriet al., 2012) These surface components include acidic expolysaccharide, neutral beta-

glucans, lip polysaccharides and the elements responsible for motility of rhizobial cells (Gray and Reuber, 1991) As the cell surface components of Rhizobium are signs for maintaining nodulation in leguminous plants, the isolates were examined for cell surface production under lead and iron stress The present study is aimed to determine how much the isolates of Rhizobium are tolerant and sensitive to increasing concentrations of lead and iron ,cell surface production under these stress and impact of multiple tolerances with other heavy metals in tolerant isolates.

MATERIALS AND METHODS

Collection of samples

Total five modulated plants in replica i.e. barseem from different parts of Dehradun, Uttarakhand were taken. Healthy plants were uprooted carefully and those plants possessing healthy nodules with pink color were selected and brought to the lab in polythene bags. (Gauriet al., 2011)

Biochemical tests for differentiation of Rhizobium and Agrobacterium:

Congo red Test

Rhizobia appears as white, translucent, glistening elevated and comparatively small colonies with entire margins in contrast to stained colonies of Agrobacterium in this test whencongo reddye isincorporated in YEMA medium .

Hoffer's alkaline broth Test (Hofer, 1935)

Agrobacterium grows at higher pH level whereas Rhizobium unable to do so. A medium i.e. Hoffer's alkaline having high pH of 11.0 was used to screen isolated nodulated bacteria.

Lactose agar Test

Agrobacteria utilize lactose by the action of enzyme ketolactosewhere as Rhizobia cannot utilize this sugar. Culture of nodulated bacteria were streaked on lactose agar plate and incubated for 4-10 days.

Catalase test (Graham and Parker, 1964)

The 48 hours culture of the isolated strains on YEMA was flooded with hydrogen peroxide and observed for the bubble formation.

Oxidase test (Kovaks, 1956)

Few drops of p-aminodimethylaniline oxalate were added on the surface of 48 hours cultures of the rhizobial strains on YEMA and observed for the production of colour.

Determination of maximum resistance level (MRL)

Agar plates of YEM medium incorporated with individual concentrations of iron and lead were prepared.Iron was added as iron sulphateat concentrations ranging from 25mg/ml up to 200 mg/ml and lead as lead acetate at same concentration.Rhizobial suspensions were streaked onto the surface of the metal-amended agar, the plates were kept at 28°C for 2 days for growth and determined the maximum resistance level (MRL).The MRL is defined as the highest metal concentration that permits visible growth.

Cell surface properties

Test for the production of β-glucan

Strains of Rhizobium were streaked on YEM agar having aniline blue at a rate of 1mg/ml. and were given different iron and lead stress (0-200mg/l).Plates were incubated at 28°C for 2 days.colony having blue color were considered to be β Glucan positive and white colored colony were negative.

Test for Production of Lipopolysaccharide

Strains of Rhizobium were streaked on TY medium having Sodium deoxycholate (SDC) at a rate of 1mg/ml and were given different iron and lead stress (0-200mg/ml) and incubated at 28°C for 2 days. LPS producing strains of Rhizobiumshows growth.

Test for Motility (Swamynathan and Singh, 1995)

Rhizobiumstrains were spotted on swarm plates having tryptone yeast Extract medium with 0.3% agar. They are given different iron and lead stress and incubated at 28°C for 48 hours. The motility of bacterial strains was determined by spreading the colonies in the swarm plates. The diameter was measured in centimeter Units.

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Test for EPSII (Sudan Black B)

Rhizobium were streaked on the agar surface of YEM incorporated with Sudan black B with heavy metal (iron and lead) for 2 days.colonies which were unable to incorporate the Sudan Black B stain appeared white; while colonies which

able to incorporate the dye appeared bluish black.

Determination of co-resistance to other heavy metal (Rajbanshi, 2008)

Isolates which were tolerant to lead and iron were tested for their resistance to other heavy metals chosen i.e. Nickel, Cadmium, Copper, Cobalt, Chromium, Zinc and Aluminum incorporated in YEMA media.The starting concentration of heavy metals in this test was 25mg/ml, which was gradually increased to 350 mg/ml.

RESULTS

Total 22 strains of Rhizobium were isolated from root nodule of barseem collected from different agriculture fields of Dehradun (Uttarakhand).

Cultural and Biochemical characteristics

colonies having sticky appearance shows the production of mucous by analysis of colony morphology indicated round colonies having diameter of 5-7mm.All the isolates were gram negative rods.All the strains were catalase and oxidase positive.as well as all isolates showed growth on lactose peptone agar and unable to grow on Hoffers alkaline broth. Congo red test indicated white colonies on the medium.(Table 1.0)

Effect of iron and lead on growth of isolates

Rhizobium strains were cultivated on different iron and lead concentrations supplemented in YEMA medium for MRL.At 25mg/ml concentration of iron out of 22 strains 17 showed growth and 5 were negative,same were observed at 50 mg/ml, at 75mg/ml concentration 16, at 100mg/ml 13, at 125mg/ml and 150mg/ml 12, at 175 mg/ml 11 and at 200mg/ml iron concentration only 7 strains showed growth as shown in fig 1.0a. On addition of lead acetate at 25 mg/ml, 50mg/ml, 75mg/ml 15, at 100mg/ml 12, at125mg/ml 10, at 150 mg/ml 13, at 175 mg/ml 11 and at 200mg/ml only 7 strains showed growth (fig10b).

No growth was observed above 200 mg/ml of iron and lead concentrations therefore 200 mg/ml concentration was considered as MRL.

Production of β -glucan under iron and lead stress

Rhizobiumisolates were tested for the presence of cyclic ß-Glucans on the basis of their staining with aniline blue at different iron and lead concentrations.The percentage of ß-Glucan production was 81%, 72%, 68%, 63%, 68%, 59%, 59%,50%,at25,50,75,100,125,150,175,200 mg/ml of iron respectively. Eleven isolates showed blue colour colony at 200 mg/ml of iron as fig 2.0a The percentage of ß-Glucan production was 86%, 81%, 72%, 72%,54%,68%,54% and50% at 25, 50, 75 ,100,125,150,175and 200mg/ml of lead acetate (Fig2.0b)

Production of lipopolysaccharide in recovered isolates under iron and lead stress:

Rhizobiumisolates were streaked on TY plates containing 1mg/ml sodiumdeoxycholate (SDC) at different concentrations of iron and lead .The percentage of lipopolysaccharide production in iron tolerant isolates were 90%,86%,63%,63%,72%,59%,31%,13% at 25, 50, 75, 100, 125, 150, 175, 200mg/mliron. At 25, 50, 75, 100, 125, 150, 175, 200mg/ml percentage of lipopolysaccharide production in lead tolerant isolates were 90%, 82%, 59%, 54%, 45%, 50%27, 9%. Highest lipopolysaccharide production was seen at 25 mg/ml and lowest was recorded at 200 mg/ml of iron and lead as observed in fig 3.0.a and b.

Swarming behavior of isolates under iron and lead stress

Most of the tolerant strains maintained the swarming behavior upto 200mg/ml of iron and lead with a reduction of the diameter from 3cm to 0.1cm.(table2.0and3.0) Percentage of motility was 86%, 95%, 72%, 72%, 63%, 63%, 59%, 45%, 50% at 0, 25, 50, 100, 125, 150, 175, 200 mg/ml of iron respectively. Percentage of motility in lead tolerant strains were 86%, 95%, 68%, 72%, 68%, 77%, 63%, 63%, 63% at 0, 25, 50, 100, 125, 150, 175, 200 mg/ml of lead. Maximum diameter was observed in lead tolerant strain RT4d (3.0 cm) at 50 mg/ml lead.

Production of EPS II in recovered isolates under iron and lead stress

Rhizobium isolates were streaked on YEMA plates containing 1mg/ml Sudan black B at different concentration of iron and lead. The percentage of EPS II production in iron tolerant isolates were95%, 72%, 54%, 50%, 45%, 40%, 40%, 40% at 25, 50, 75 ,100 ,125, 150, 175, 200mg/ml of iron. (Fig4.0.a) The percentage of EPS II production in lead tolerant isolates were 88%, 64%, 47%, 47%, 47%, 35%, 70%, 76% 82% at 25, 50, 75, 100, 125, 150, 175, 200mg/ml of lead (Fig4.0 b).

Table-1 Biochemical tests for differentiation of Rhizobium and Agrobacterium

Sl. No.	ISOLATES	CONGO RED	HOFFER'S ALKALINE BROTH	LACTOSE AGAR	CATALASE	OXIDASE
1	RT1a	-	-	-	+	+
2	RT1b	-	-	-	+	+
3	RT1c	-	-	-	+	+
4	RT1d	-	-	-	+	+
5	RT1e	-	-	-	+	+
6	RT2a	-	-	-	+	+
7	RT2b	-	-	-	+	+
8	RT2c	-	-	-	+	+
9	RT2d	-	-	-	+	+
10	RT2e	-	-	-	+	+
11	RT3a	-	-	-	+	+
12	RT3b	-	-	-	+	+
13	RT4a	-	-	-	+	+
14	RT4b	-	-	-	+	+
15	RT4c	-	-	-	+	+
16	RT4d	-	-	-	+	+
17	RT4e	-	-	-	+	+
18	RT5a	-	-	-	+	+
19	RT5b	-	-	-	+	+
20	RT5c	-	-	-	+	+
21	RT5d	-	-	-	+	+
22	RT5e	-	-	-	+	+

- Negative; + Positive

Table 2 Swarming behavior of isolates under ironstress.

Sl. No.	ISOLATES	control	25	50	75	100	125	150	175	200
1	RT1a	0.8	2.0	2.1	0.8	1.0	1.0	0.6	-	0.2
2	RT1b	-	0.6	-	0.9	-	0.5	-	-	-
3	RT1c	2.0	2.0	2.5	0.6	1.0	0.5	-	0.6	0.4
4	RT1d	1.5	0.5	0.6	0.9	0.9	-	0.8	0.8	-
5	RT1e	1.0	1.0	-	-	-	1.0	0.7	-	0.2
6	RT2a	-	0.6	-	0.8	2.1	0.6	0.3	0.2	-
7	RT2b	2.2	1.0	3.0	1.0	1.4	0.5	-	-	0.5
8	RT2c	0.6	0.2	0.1	0.5	-	-	-	-	-
9	RT2d	1.0	0.2	1.0	0.8	0.7	1.0	0.5	-	1.0
10	RT2e	2.3	1.1	1.0	0.9	0.9	0.4	-	-	-
11	RT3a	0.9	0.9	-	-	0.6	0.1	0.1	-	0.5
12	RT3b	1.0	1.0	0.6	-	-	0.4	0.4	0.6	0.1
13	RT4a	1.2	0.7	-	0.9	-	-	1.0	-	-
14	RT4b	-	1.6	1.5	2.4	2.4	-	-	1.6	1.2
15	RT4c	1.4	-	-	0.7	-	-	0.1	0.1	-
16	RT4d	2.5	2.1	2.1	2.0	1.3	1.0	1.0	0.5	0.4
17	RT4e	2.1	1.5	1.4	1.0	-	-	1.0	1.0	0.5
18	RT5a	1.0	0.8	0.6	-	-	-	-	-	-
19	RT5b	2.0	1.6	0.5	-	2.0	0.4	-	0.1	-
20	RT5c	1.0	0.5	0.5	1.0	1.0	1.7	1.6	-	-
21	RT5d	3.0	1.8	1.8	1.5	1.0	0.8	0.3	0.1	0.2
22	RT5e	0.5	0.9	1.1	0.8	0.7	-	-	-	-

Table 3 Swarming behavior of isolates under lead stress.

Sl. No.	ISOLATES	control	25	50	75	100	125	150	175	200
1	RT1a	0.8	2.0	2.1	0.8	1.0	1.0	-	1.0	-
2	RT1b	-	0.6	-	0.9	-	0.8	2.0	-	0.8
3	RT1c	2.0	2.0	2.5	0.6	0.6	1.0	2.5	0.7	0.5
4	RT1d	1.5	0.5	1.6	0.9	0.8	0.7	0.5	1.5	0.5
5	RT1e	1.0	1.0	-	-	1.2	-	-	-	1.3
6	RT2a	-	0.6	-	0.8	0.5	0.6	2.0	1.8	1.8
7	RT2b	1.9	1.9	2.0	1.6	-	1.8	1.4	1.2	1.2
8	RT2c	1.0	1.2	1.9	1.2	-	-	-	-	-
9	RT2d	1.0	0.2	1.1	0.8	0.7	0.9	-	0.5	-
10	RT2e	0.5	0.2	-	-	-	-	-	-	-
11	RT3a	0.9	0.9	-	-	-				
12	RT3b	1.0	1.0	1.6	-	-	-	0.5	-	-
13	RT4a	1.2	0.7	-	0.9	1.2	1.0	0.9	-	1.2
14	RT4b	-	1.6	2.4	1.5	1.4	0.9	0.8	0.8	-
15	RT4c	1.4	1.8	1.8	0.7	-	0.8	1.0	0.8	1.1
16	RT4d	2.2	1.0	3.0	1.0	1.0	0.8	-	-	0.7
17	RT4e	2.6	2.3	2.0	1.5	1.2	1.1	0.5	0.1	0.1
18	RT5a	1.2	1.5	2.0	1.0	0.9	0.6	0.2	0.1	0.1
19	RT5b	2.0	1.6	1.8	-	0.9	0.7	0.7	0.7	0.6
20	RT5c	1.0	0.5	0.9	1.0	1.0	0.9	0.9	0.8	0.7
21	RT5d	1.5	1.9	2.1	1.8	1.7	1.5	-	1.1	-
22	RT5e	0.5	-	-	1.5	0.9	0.6	0.6	-	0.5

Table 4 Co-resistance (MRL) in Fe tolerant isolates to other heavy metals

S. No.	Isolate / Heavy Metal (mg/ml)	Nickel	Cadmium	Copper	Cobalt	Chromium	Zinc	Aluminium
1.	RT1d	200	200	200	200	200	200	200
2.	RT2b	200	250	200	150	150	150	150
3.	RT4a	150	250	200	250	250	250	250
4.	RT4b	200	250	200	250	200	250	200
5.	RT4d	300	200	200	150	150	300	250
6.	RT4e	200	200	200	200	200	200	200
7.	RT5d	200	200	150	200	200	200	200

Table 5. Co-resistance (MRL) in Pb tolerant isolates to other heavy metals

S. No.	Isolate / Heavy Metal(mg/ml)	Nickel	Cadmium	Copper	Cobalt	Chromium	Zinc	Aluminium
1.	RT1d	200	200	200	200	200	200	200
2.	RT2b	150	150	100	150	150	250	150
3.	RT4a	150	250	200	250	250	150	150
4.	RT4b	200	300	200	250	200	250	200
5.	**RT4d**	200	200	200	250	150	200	250
6.	RT4e	200	200	200	200	200	250	200
7.	RT5d	200	200	150	200	200	200	150

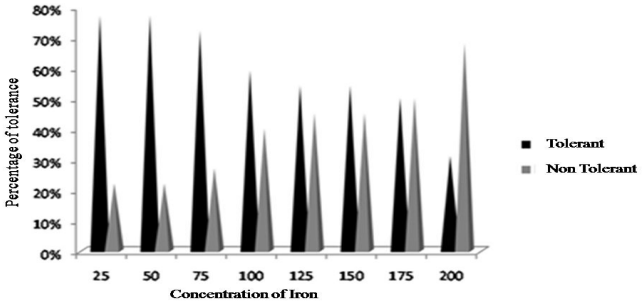

Figure 1a Heavy metal tolerance to iron

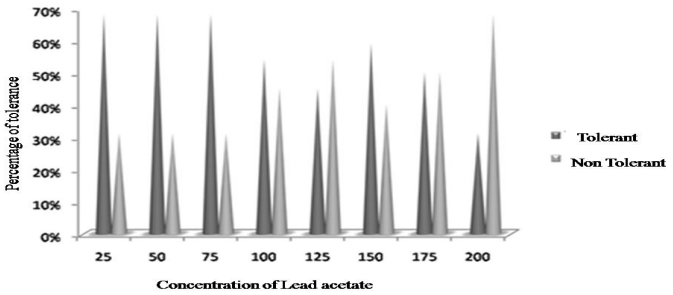

Figure 1b Heavy metal tolerance to lead acetate

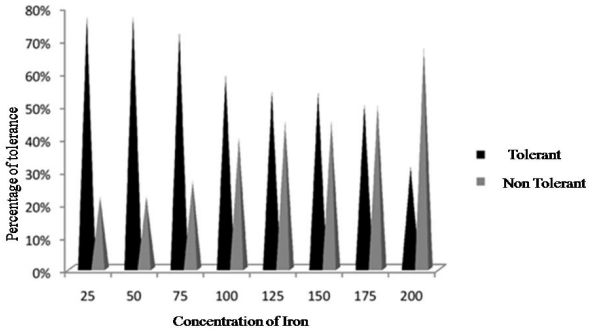

Figure 2a Production of beta Glucan under Iron stress

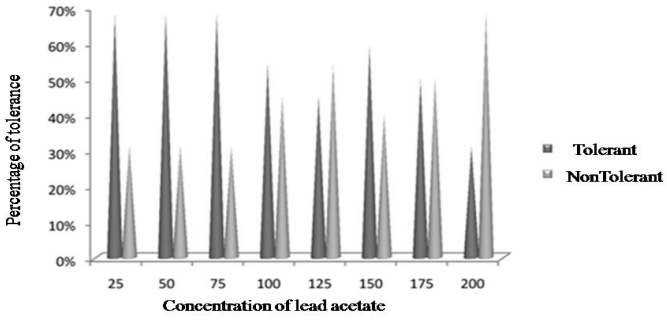

Figure 2b Production of beta -Glucan under Lead stress

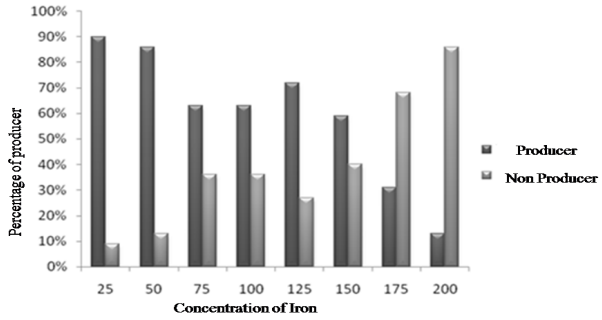

Figure 3a LPS production under iron stress

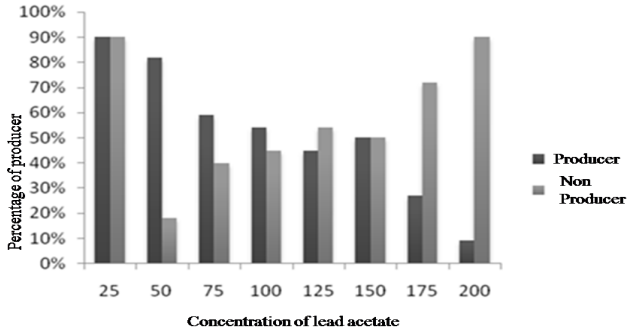

Figure 3b LPS production under Lead stress

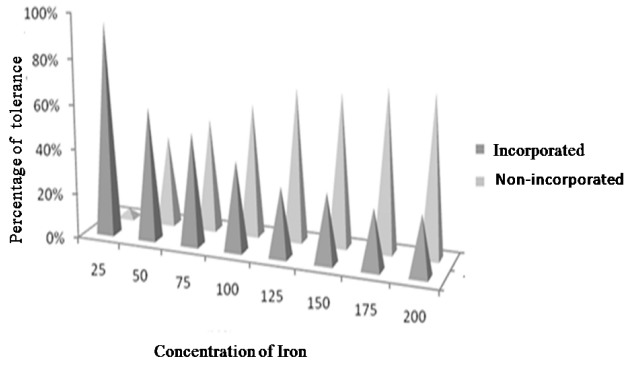

Figure 4 aEPSII (Galactoglycan) under Iron stress

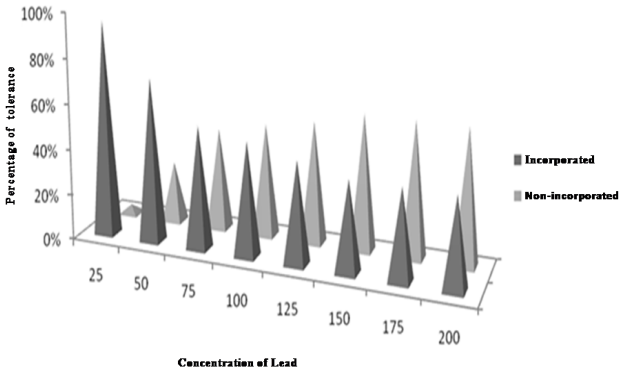

Figure 4b EPSII (Galactglycan) under Lead stress

Determination of co-résistance (iron) and (lead) of isolates with other heavy metals

Seven (RT1d, RT2b, RT4a, RT4b, RT4d, RT4e, RT5d) bacterial isolates resistant to one heavy metal were tested for their resistance to Nickel, Cadmium, Copper, Cobalt, Chromium, Zinc and Aluminum chosen in this study. The starting concentration of heavy metals in this test was 25 mg/ml, which was gradually increased to 350mg/ml All resistant isolates showed multiple tolerances to heavy metals as represented in table 4.0 and 5.0. Maximum resistance level in iron tolerant was observed in isolate RT4d for Nickel and Zinc (300mg/ml). Lead tolerant isolate RT4d showed maximum resistance level (300mg/ml).

DISCUSSION

Metal deposition into soil for long-termresults in high metal concentrations, which directly affects negatively to soil micro flora (Smith and Giller, 1992; Matsuda et al., 2002).in the above study the Rhizobium strains were cultivated on YEMA incorporated with heavy metals (lead and iron), for detection of MR, cell surface properties and co resistance with other metals.

It has been observed that growth of strains were decrease with the increase in heavy metal concentration in medium and at higher concentrations (above 200mg/ml) none of the organism shows growth .Bacteria exposed to high levels of heavy metalsin their environment have adapted to this stress by developing various resistance mechanism.These mechanisms could be utilized for detoxification and removal of heavy metals frompolluted environment (Ahmed et al., 2005 and Singh et al., 2013). Same conclusion was made by Ausilet al., in 2002 he took 20 Rhizobium isolates for detecting MRL of four different heavy metals (Cu, Cd, Zn and Mn) using 5 levels of concentrations.

This also has been observed that growth of strains were decreased with the increased in heavy metal concentration in medium and at higher concentrations (above 200mg/ml) and none of the organism shows growth .Bacteria those were exposed to high levels of heavy metals in their environment thave adapted to this stress by producing various resistance mechanism. these resistance mechanisms could be used detoxification and removal of heavy metals from polluted environment (Ahmed et al., 2005 and Singh et al., 2013).Same conclusion was made by Ausilet al., in 2002 he took 20 Rhizobium isolates for detecting MRL of four different heavy metals (Cu, Cd, Zn and Mn) using 5 levels of concentrations.

In Rhizobium-legume symbiosis, the regulation of cell surface polysaccharides is highly relevant. Polysaccharides which are closely associated with the bacterial cell surface have been shown to be vital in early infection events for rhizobium (Brink et al.,1990).furtherRhizobium strains were selectedinorder to study their cell surface properties under iron and lead stress conditions. by results it has been noticed that β-glucan production in both iron and lead tolerant strains decreased with increase in metal concentration and at higher concentrations,it was absent in several strains. Geremia etal., 1987 have linked the absence of β -glucans to a defective flagellum that results in the absence of chemotactic response which in turn leads to the formation of ineffective nodules.

It was observed that in iron and lead tolerant strains LPS production was decreasing up to 200 mg/ml of iron and lead Kannenberg and Brewin (1989)reported that alteration in LPS occurred in response to environmental changes. Carlson (1995) stated that the major component of the bacterial outer membrane and for Rhizobialspecies, is Lipopolysaccharide, having a role in the establishment of an effective nitrogen fixing symbiosis with a legume host.

Swarming behavior is marker of motility and chemotaxis:

As per our results that swarming activity of both heavy metal tolerant strains were increasing up to 50 mg/ ml thereafter with the increase in iron and lead concentration in the medium growth rate decreases on the contrary in few strains swarming activity were maintained up to 200 mg/ml of heavy metals. Gulashet al., (1984) showed that the better nodules were from strains with higher swarming behavior. according to him Chemotaxis and motility might have provided increased chemical or physical contact with the root, enhanced occupation of sites potentially suitable for infection and rapid or efficient infection development.Ames and Bergman(1981)reported that, chemotactic wild type strains which are motile was capable of forming between 65% and 98% of the nodules when competing against equal numbers of non-flagellated or non motile mutant isolates.The similar contest found by Mellor et al.,(1987) they also concluded that motile strains of Rhizobium trifolii formed approximately five times more nodule than non motile strains

as well as suggesting that the motility is a factor in competition for nodule formation of iron on nodulation may perhaps be as much due to inhibition of motility as to direct toxicity especially at low concentrations.Microbespopulations are known to affect heavy metals mobility and availability to the plant by the release of chelating agents, acidification, phosphate solubilization, and redox changes.

Synthesis of Exo-polysaccharide appears to be a common feature associated with numerous microorganisms. these may be involved in invasion and nodule development, bacterial release from infection threads, bacterial development, suppression of plant defense response and protection against plant antimicrobial compounds (Anna etal., 2006).in present study, EPSII was checked by streaking the isolates on the plates of YEMA having 1mg/ml Sudan black B at different concentration of lead and iron.Similar method was followed by Reuber and Walker (1993) and Liuet al, (1998) .In our experiments EPSII production were different with Fe and Pb. EPSII production were decrease with increasing concentrations of Fe while in lead tolerant isolates it varied with increasing concentrations,in the case of iron tolerant isolates.

The cell surface molecules of rhizobium play vital role for maintaining factors which are essential for nodulation under stress conditions(Singh et al ,2015).Seven tolerant strain which showed all cell surface components were further tested with co resistance with other heavy metals.Multiple tolerances occur only to those toxic compounds that have same mechanisms underlying their toxicity. multiple tolerances are common phenomena among heavy metal resistant bacteria because the heavy metals are all same in their toxic mechanism. Rajbanshi (2008) also selected bacterial isolates which were resistant to one heavy metal for their co resistance with other heavy metals.

CONCLUSION

The present study concludes that under lead and iron stress, the tolerant organisms are better to adapt the stressful environmental conditions Therefore supplementation of soil with multiple heavy metal tolerant strains could be successful agent for bioremediation of heavy metal contaminated land and will be beneficial so as to enhance the process of symbiotic nitrogen fixation.

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