JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2008. Vol. **2**(2), p. 507-512

Study of *Azospirillum* sp. on the Heavy Metal Tolerance, Phytostimulation and Bioprotection

Savanta V. Raut* and Kanaka K. Ghosalkar

Department of Microbiology, Bhavan's college, Andheri (W.), Mumbai - 400 058, India.

(Received: 15 June 2008; accepted: 02 August 2008)

A heavy metal tolerant *Azospirillum* sp. was isolated from different sources. These isolates were characterized and identified as *Azospirillum lipoferum*. These isolates tolerate more concentration of heavy metals and studied for production of IAA. These isolates were used to study efficiency in soil with the heavy metal as biofertilizer with plant *Triticum aestivum* var LOK-1. The impact of these isolates as a biofertilizer, clearly show significant results for growth of plant and shows increase number of *Azospirillum lipoferum*.

Key words: *Azospirillum lipoferum,* IAA, heavy metals tolerance, *Triticum aestivum* var LOK-1.

Nitrogen is the most abundant element, its efficient utilization and harnessing requires the presence of the process of nitrogen fixation, a domain of very few organisms including those belonging to the genera *Azotobacter, Klebsiella, Rhizobium, Azospirillum, Anabaena, Nostoc,* etc. However one important fact that cannot be denied is that the efficiency of these organisms is limited by the increasing pollution of soil by virtue of the use of industrial effluent and sewage sludge in agricultural fields. The effluents contain a variety of organic and inorganic chemical like pesticides and heavy metals which affect the microbial population in the soil, as well as the processes mediated by these organisms. Considerable study has been carried out about organisms in the rhizosphere like *Azotobacter, Rhizobium,* etc. in detail.

*To whom all correspondence should be addressed.

However comparatively lesser details are available about the metal resistant/ tolerant organisms belonging to the genera *Azospirillum.* In the present study, tolerance of heavy metals, IAA production and phytoprotection studies of *Azospirillum* isolates were carried out. These studies can be useful for efficient biofertilizer in metal polluted soil.

MATERIAL AND METHODS

Sampling, enrichment, isolation and identification

Soil samples and pieces of roots of rice were collected from Ambernath, of grass were collected from Bhavan's college Chemistry Dept. backyard and from NNP locality Goregaon (E.) and maize root were collected from Dighanchi (Sangli). Serial enrichment of *Azospirillum* sps. from these samples was carried out using semisolid Nfb medium in microaerphilic conditions. Maintenance of the isolates was done on sterile BMS agar slants followed by their

identification with reference to "The Bergey's Manual of Systematic Bacteriology." with standard culture of *Azospirillum lipoferum* (MTCC 2694) .

Determination of MIC

Minimal Inhibitory Concentration of the metals Nickel (nickel chloride), Zinc (zinc sulphate), Cadmium (cadmium chloride), Copper (copper sulphate), Chromium (chromium trioxide) and Lead (lead sulphate) for the standard strain and the isolates was determined by the plate method by spotting the isolates wherein 5 isolates were spotted per plate, retaining one slot in the plate empty as control. Media used for the study was BMS agar into which the filter sterilized respective metal solutions were added post-autoclaving to attain the specific concentration in " ppm" depending on the range of MIC selected and the results were recorded after an incubation period of 24 hrs. at R.T.(19)

Quantitatively analysis of the production of indole acetic acid (IAA)

Azospirillum spps. are known to produce growth hormones; one of them being (IAA). For the production of same, NFB broth supplemented with 4mM tryptophan was used in which the standard strain and the 14 isolates were grown for a period of 13 days at R.T. following which the quantification was carried out using the IAA assay by the "Salper reagent method" of colorimetric estimation which is sensitive within the range of 2-25 mcg/ml of IAA.

Study of the antimicrobial activity of the std. strain and the isolates

Antibacterial activity of the standard strain and the isolates was studied against *Agrobacterium tumefaciens* (MTCC 431), *Erwinia carotovora* (MTCC 1428), *Pseudomonas syringae* (MTCC 1604) and antifungal activity was studied against *Fusarium oxysporum (*MTCC 3075) by the **"**paper disc method**"** using acidic extract of the isolates and the std. culture that were absorbed on sterile 6 mm.Whatman filter paper discs against the above mentioned test cultures.

Plant studies of the isolates and the std. strain for phytostimulation

Plant study was carried out using Garden soil from Dahisar (E.) residential area. The soil that was selected was not supplemented with any organic manure and free from any heavy metal pollution .The soil was sieved slightly to remove large stones. 100 gms of soil was taken for the study in thermocol cups.The 14 isolates and the std. strain from BMS agar slants were used at cell density of 10^{5}/ml for inoculation.The plant / seed used for the study was wheat i.e. *Triticum aestivum var* LOK-1 wherein 10 seeds were added to each cup. The inoculation of the culture into the soil was "carrier based" using jaggery solution as the carrier . soil was mixed with solutions of the different heavy metal salts to attain the final MIC concentration in ppm which was decided from the MIC studies that were performed for the isolates. The pots without the metal solution served as the control.

Viability studies carried out for the isolates to ensure their efficacy as a biofertilizer

Viable count of the soil was performed immediately after sowing the seeds in the soil to know the "0 day" count and it was also performed for the soil as well as the roots after removing the plants from the soil which was done after attainment of complete growth that was obtained after 12 days to give the " final count".

Table 1. Results for the MIC value of heavy of the 4 isolates of *Azospirillum lipoferum* which are used for plant studies and the std. strain

Isolates	Zinc ppm.	Copper ppm	Nickel ppm	Lead ppm	Cadmium ppm	Chromium ppm.
STD.	300	300	300	<2000	<100	<200
GH_{2a}	300	300	300	2500	<100	<200
$GC1_2$	300	300	300	2000	<100	250
RH	300	300	300	<2000	125	200
$M_1 C_1$	300	300	300	2000	100	200

Table 2. Statistical analysis of the macroscopic characters recorded after the plant study as performed by the Student's t test. Pot :- Soil + 10 seeds of *Triticum aestivum var* LOK-1

Parameter	Pot-1	Pot-2	Pot-3	Pot-4	Pot-5	Pot-6	Pot-7	Pot-8	Pot-9	Pot-10	Pot-11	Pot-12	Pot-13	Pot-14
% germination	90	50	100	70	80	90	100	90	90	90	80	70	90	60
Shoot length [cm.]	20.5	25.85	24	26.75	25.25	31.5	26.5	30.35	27.25	*28.5*	24.1	26	24.35	26.75
CD[p=0.05]	10.91	N.S.	2.69	S	3.32	S	5.83	N.S.	1.138	*S*	1.733	S	1.33	S
Leaf length cm.	20.5	24	19	23.55	26.75	29.55	21.85	23.80	19.75	21.6	21.5	23.95	20.75	23.25
CD[p=0.05]	3.837	NS	3.657	S	5.7	NS	1.907	S	0.669	S	0.796	S	0.894	S
No.of leaf	2.66	3.66	2.33	3	2	3	2	2	2.33	3	2.66	3	2.33	3
CD[p=0.05]	2.133	NS	2.02	S	0.740	S	0.595	S	0.01	S	1.025	NS	0.401	S
No.of roots	3	5	4.33	5.33	3.33	4.66	2	5.66	4.66	5.33	4.33	5	6	6.66
CD[p=0.05]	1.463	S	1.133	S	0.783	S	2.747	S	0.512	S	1.16	NS	0.052	S

Pot-1:- All metals
Pot-2 :- All metals+ all isolates
Pot-3 :- Zinc
Pot-4 :- Zinc + M_1C_1
Pot-5:- copper
Pot-6:- copper + M_1C_1
Pot-7:- Nickel
Pot-8 :- Nickel+ M_1C_1
Pot-9:- Lead
Pot-10:- Lead + GH_{2a}
Pot-11:- cadmium
Pot-12:- Cadmium + RH
Pot-13:- Chromium
Pot-14:- Chromium + $GC1_2$

RESULTS AND DISCUSSION

Enrichment and isolation: After successful enrichment of the root and soil samples and their subsequent isolation 23 isolates were obtained. These isolates were identify by biochemical characters . 17 such isolates were identified as *Azospirillum spp.* 14 of the 17 isolates were found to be *Azospirillum lipoferum.*

MIC of heavy metals

The isolates that were resistant to the different heavy metals under consideration were screened using the PLATE METHOD for performing MIC amongst the 14 isolates and the std. culture the details of which are given in Table 1.

IAA assay by the SALPER reagent method

The results for the same are indicated by a graphical representation indicated in graph 1 .

Antimicrobial activity

None of the isolates show antimicrobial activity and thus are not effective against plant pathogens.

Plant studies: 12 days after the set up of the plant study, complete growth of the plants ie wheat was obtained which were studied for the different macroscopic characters the detailed information along with the statistical analysis for which is provided in tables 2.

Viability studies

Viable count study was performed on the day of setting up the plant study and on day 12 i.e. after the plants had attained complete growth and were removed from the soil. The results for the same with the % increase or decrease in the viability are indicated in table 3.

The project aimed at isolating and screening *Azospirillum spps*. that are mainly tolerant to heavy metals and then these isolates were checked for various other attributes that are typical of any *Azospirillum spps.* Biochemical characterization was carried out for the 17 isolates and the std. strain of *Azospirillum lipoferum* (MTCC 2694). 14 isolates were found to be *Azospirillum lipoferum* whereas 3 were not belonging to the genera of *Azospirillum.*

MIC of the 14 isolates and the std . strain were determined by the "PLATE METHOD", wherein the determination was carried out in "TRIPLICATES" the consolidated results of which suggest that isolate M_1C_1 could tolerate

Table 3. Results of the viability studies performed on "0" and "12" day of the plant study setup for the *Azospirillum* count in the soil and the roots using NFB medium. Period of incubation is 48 hrs. at R.T

Particulars of the soil sample	Avg cfu/gm of soil / Avg cfu/gm of root pieces	% Change in the inoculum size
Zero Day Count:		
Control: Soil + Wheat	5×10^{7}	Not applicable
Test: Soil + Wheat + Std.	3×10^{10}	Not applicable
Twelve Day Count		
Control: Soil + Wheat	$(1.52 + 2.2) \times 10^{8} = 3.72 \times 10^{8}$	86 %(Increase)
Test: Soil + Wheat + Zinc	$(4.89 + 1.78) \times 10^{7} = 6.67 \times 10^{7}$	25 % (Increase)
Test: Soil + Wheat + Zinc + M_1C_1	$(2.97 + 2.4) \times 10^{10} = 5.37 \times 10^{10}$	44 % (Increase)
Test: Soil + Wheat + Copper	$(3.08 + 2.97) \times 10^{7} = 6.05 \times 10^{7}$	17.34% (Increase)
Test: Soil + Wheat + Copper + M_1C_1	$(2.18 + 2.03) \times 10^{10} = 3.21 \times 10^{10}$	37.7 % (Increase)
Test: Soil + Wheat + Nickel	$(4.72 + 1.11) \times 10^{7} = 5.83 \times 10^{7}$	14.23 % (Increase)
Test: Soil + Wheat + Nickel + M_1C_1	$(2.89 + 1.09) \times 10^{10} = 3.98 \times 10^{10}$	24.62 % (Increase)
Test: Soil + Wheat + Lead	$(3.015 + 2.73) \times 10^{7} = 5.74 \times 10^{7}$	12.96 % (Increase)
Test: Soil + Wheat + Lead + GH_{2a}	$(1.98 + 2.0) \times 10^{10} = 3.98 \times 10^{10}$	24 % (Increase)
Test: Soil + Wheat + Cadmium	$(2.07 + 3.12) \times 10^{7} = 5.19 \times 10^{7}$	3.6 (Increase)
Test: Soil + Wheat + Cadmium+ RH	$(1.4 + 1.97) \times 10^{10} = 3.37 \times 10^{10}$	10.9 (Increase)
Test: Soil + Wheat + Chromium	$(2.56 + 3.03) \times 10^{7} = 5.59 \times 10^{7}$	10.6 (Increase)
Test: Soil + Wheat + Chromium + $GC1_2$	$(1.6 + 2.23) \times 10^{10} = 3.83 \times 10^{10}$	21.6 (Increase)
Test: Soil + Wheat + All metals	$(1.72 + 1.03) \times 10^{7} = 2.75 \times 10^{7}$	45 (Decrease)
Test: Soil + Wheat + All metals + consortium.	$(2.7 \times 1.01) \times 10^{10} = 3.71 \times 10^{10}$	19 (Increase)

Zinc, Nickel and Copper upto a maximum concentration of 300 ppm. With respect to Lead , MIC value as high as 2500 ppm was obtained for the isolate GH_{2a}. For Cadmium and Chromium the MIC values were 125 ppm and 250 ppm for isolates RH and $GC1_2$ respectively. Therefore these cultures that were then used for plant studies. Thus the tolerance levels that have been obtained for the six metals under consideration with the *Azospirillum* isolates are certainly significant when compared to a similar type of study that was carried out for *Azotobacter chroococcum* wherein the maximum MIC obtained for Zinc. Nickel, Copper, Lead, Cadmium, Chromium were 158, 150, 182, 1500, 75, 1750 ppm respectively. Except for Chromium where the MIC value for *Azospirillum* is much lower than that for *Azotobacter,* the tolerance levels for all other metals are much higher in *Azospirillum* isolates.

It was found that IAA production was maximum in GC22a (0.16M/L) followed by RL1 and RL2 (0.075M/L) and thus these isolates can be considered for phytostimulation of crop plants or alternatively can be considered for commercial IAA production after subsequent optimization and standardization studies. Most surprisingly it was observed that the production of IAA was the least in case of the std. culture that was procured from MTCC.

In order to establish the efficacy of the heavy metal tolerant isolates of *Azospirillum* to be used as a biofertilizer, plant study was carried out. In pots where a single metal was tested with a single culture, the recovery in the germination was not very significant indicating that rather than using these cultures as single candidates, it is better to carry out consortium studies for them and accordingly optimize the different parameters for biofertilizer formulation.

Also when the statistical t test is applied with 5 % confidence limits it is observed that with respect to shoot length, the difference between treated and untreated soil in case of the "all metal" and "nickel" pots is not significant but in the rest of the sets of other metals, the results are significant. For leaf length, similar type of results are observed with respect to " all metals" pot. With respect to the no. of roots, the difference in the treated and untreated for the "cadmium metal" and the "all metal" pot are not significant. Except for these other test pots have shown statistically significant and valid results.

and this has an effect on the soil micro flora. However in the pot having all metals and the consortium of the cultures, there is an increase by 19 % which is certainly achieved by first nullifying the 45 % decrease and then gaining the increase.

CONCLUSIONS

The study has been currently terminated with the isolation of 14 potential isolates of *Azospirillum lipoferum* with respect to heavy metal tolerance. These isolates also have other special attributes like IAA production with the maximum concentration obtained being 0.16 M/ L for GC22a. According to MIC studies M1C1, GH2a, RH and GC12 were found to be potential isolates for heavy metal tolerance which has been further confirmed by plant studies using wheat as the test crop. Plant studies and viability studies also indicate that when the isolates are used as single candidates for the remediation of soil, the results are unequivocal, but when used as consortium, these isolates prove to be very efficient prospective candidates for biofertilizer formulation. The only drawback of these isolates is that they do not have antimicrobial activity.

ACKNOWLEDGEMENTS

The auther is grateful to University of Mumbai for the financial support for this project.

REFERENCES

1. Alexander G., R. Bally, B.L. Taylor, I.B. Zhulin, Loss of cytochrome c oxidase activity and acquisition of resistance to quinone analogs in a laccase positive variant of *Azospirillum lipoferum*, *Bacteriol,* 1999; **181**(21): 6730-6738.
2. Alexander G., S.E. Greer, I.B. Zhulin, Energy taxis is the dominant behaviour in *Azospirillum brasilense*, *J. Bacteriol.,* 2000; **182**(21): 6042-6048.
3. Bekri M. A., J. Desair, V. Keijers, P. Proost, M. Leeuwen, J. Vanderleyden, A. Broek, *Azospirillum irakense* produces a novel type of pectate lyase, *J. Bacteriol,* 1999; **181**(8): 2440-2447.
4. Boergen W., C.Genetello, M.V. Montagu, D, Inze, A new bioassay for auxins and cytokinins, *Plant Physiol,* 1992; **99**: 1090-1098.
5. Broek A.V., M. Lambrecht, K. Eggermont, J. Vanderleyden, Auxins upregulate the expression of indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*, *J Bacteriol.,* 1999; **181**(4): 1338-1342.
6. Gerk L.P., K.Gilchrist, I.R. Kennedy, Mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense*-wheat associations, *Appl. Environ Microbiol,* 2000; **66**(5): 2175-2148.
7. Gleba D., N.V. Borisjuk, L. G. Borisjuk, R. Kneer, A.Poulav, M.Skarhinskaya, S. Dushenkov, S. Logendra, Y.Y. Greba, I. Raskin, 1999, Use of plant roots for phytoremediation and molecular farming, *Proc. Natl. Acad. Sci.* USA, **96**(11): 5973-5977.
8. Hirschi K.D.,V.D.Korenkov, N.L.Wilganowski and G.J.Wagner, Expression of Arabidopsis CAX2 in tobacco altered metal accumulation and increased manganese tolerance, *Plant Physiol.,* 2000; **124**(1): 125-134.
9. Kadouri D., S. Burdman, E.Jurkevitch, Y. Okon, Identification and isolation of genes involved in PHB biosynthesis in *Azospirillum Brasilense* and characterization of a phb C mutant, *Appl. Environ Microbiol,* 2002; **68**(6): 2943 -2949.
10. Meda A.R., E.B. Scheuermann, U.E. Prechsl, B.Erenoglu, G.Schaaf, Iron acquisition by phytosiderophores contributes to Cadmium tolerance, *Plant Physiol.,* 2007; **143**(4): 1761-3.
11. Myers M.L., D.H. Hubell, Plant cell wall carbohydrates as substrates for *Azospirillum brasilense*. *Applied and Environmental Microbiology*, 1987; 2745-2748.
12. Pawlowska T.E. and J.Charvat, Heavy metal stress and developmental patterns of Arbuscular Mycorrihizal Fungi, *Appl. Environ. Microbiol.,* 2004; **70**(11): 6643-6649.
13. Rajapaksha R.M.C.P., M.A.Tobor-Kaplan, E. Baath, Metal toxicity affects fungal and bacterial activities in soil differently, *Appl. Environ Microbiol*, 2004; **70**(5): 2966-2973.
14. Athar R., S. T. Khan and Dr. M. Ahmad, Isolation of Pesticides and Heavy Metal Tolerant Strains of *Azotobacter chrococcum* from the Rhizospheric Region of Wheat Crop, Handbook of Biofertilizers and Biopesticides.
15. Somers E., D.Ptacek, P.Gysegom, M.Srinivasan, J.Vanderleyden, 2005, *Azospirillum basilense* produces the Auxin like Phenylacetic acid by using the key enzyme for Indole acetic acid biosynthesis, *Appl Environ Microbiol.*, **71**(4): 1803-1810.