



Original Research Article

High metal resistance and metal removal properties of antibiotics producing Actinobacteria isolated from rhizosphere region of *Casuarina equisetifolia*

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A B S T R A C T

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Actinobacteria are ubiquitous and known for its heavy metal resistance and antibiotic production. Antibiotic activity and metal resistance property of *Casuarina equisetifolia* associated actinobacteria were studied. The actinobacteria were screened for antagonistic activity against bacterial and fungal pathogens. Four strains identified as *S.roseisederoticus* (V5), *S.flavochromogenes* (V6), *S.vastus* (V7) and *S.praguaeneses* (V8) showed both antimicrobial properties and high tolerance to heavy metals. Heavy metal removal property was also observed towards Chromium (Cr^{6+}), Cadmium (Cd^{2+}), Zinc (Zn^{2+}) and Lead (Pb^{2+}), which are known to be potent environmental polluting agent. High metal resistance and removal characteristic of these strains, up to 8000 mg/L resistance 73% removal capacity of Cr by V5, 8000mg/L and 60 % for Pb by V6 were observed respectively than for Zn and Cd were the resistance was significantly less. An FTIR result confirms the involvement of proteins in metal binding.

Introduction

Actinobacteria are group of gram positive filamentous bacteria with high G+C content. It is the most widely distributed group of microorganism in the nature belonging to the phylum Actinobacteria and they primarily inhabit soil (Oskay M., Tamer, et al. 2004). They are characterized by their ability to produce various bioactive compounds (Imda, Masazumi et al. 2007) which includes, almost 80% of the world antibiotics mostly from the genera streptomycetes (Kuski, Barns, et al. 1997).

Streptomycetes have provided about two third (more than 4000) of naturally occurring antibiotics discovered including many of those important medicines, such as Aminoglycosids, Anthracyclines, Chloramphenicol, Beta lactum, Macrolids and Tetracyclins (Anansiriwattana, Tanasupawat, et al. 2006, Manjula, Rajguru, et al. 2009). Other products of secondary metabolism may enable the bacteria to cope with stress factors including toxic levels of heavy metals. While many heavy metals (Fe,

Zn, Mn, Co, Cu, Ni, V, Mo) are essential for maintenance are still toxic in high concentrations (Lopez-Maury, Garcia-Dominguez, et al. 2002). Many microorganisms show adaptation to the toxic materials constantly released in the environment, they have developed strategies to resist tolerate, metabolize and detoxify these toxic metals (Pasti, Pometto A. L., et al. 1990) these metal resistance ability of the microbe can be capitalized to bioremediation which has stimulated great interest in the research community.

The stress condition of toxic environment can result in syntheses of some novel metabolites with medicinal value by the microorganisms. From the past two decade the rate of antibiotic research are being increased steadily and every possible source are being screened due to emergence of new type microbial infection (Perez and Patterson, 2002), increasing prevalence of antibiotic resistance pathogens and pharmacological limitations to deal with them (Namasivayam and Palaniappan, 2005).

So there exist a demand for the discovery of newer drugs and antibiotics. It is suggested that the exploration of specimens from new area and habitats have a vital role to play in the possibility of isolating newer strains which are resistance to environmental contaminants. At same time produce newer active compounds that may help in fighting the emerging phenomenon of antibiotic resistance and environmental hazards (Gharaibeh and Mahasneh, 2003). The aim of study was to investigate heavy metals resistance and antibiotic production of actinomycetes spp. isolated from rhizosphere region of heavy metal treated tree species *Casuarina equisetifolia* to and their antibiotic properties *In-vitro*.

Materials and Methods

Sample collection and isolation

Rhizosphere soil samples were collected from five different one year old *Casuarina equisetifolia* tree species which was previously treated with salts of Chromium(Cr^{6+}), Lead(Pb^{2+}), Cadmium(Cd^{2+}) and Zinc(Zn^{2+}) at concentration of 100mg/l, 100mg/l, 50mg/l and 50mg/l respectively. The samples collected were about 250 g surrounding the root area at a depth of 10-15cms in separate sterile 1kg polyethylene bags.

The five rhizosphere samples were uniformly mixed to get 1.25 kg of composite sample. From this 500 g of the soil was dried sieved and stored in a sterile polyethylene bag at 4 °C for further studies (Bhat and Kaveriappa, 2009). For the study soil samples were treated with CaCO_3 (10:1 w/w) and then suspended in ringers solution (1/4strength). About 1 gm of composite sample was serially diluted using 0.1% peptone water and diluted aliquots was spread plated on sterile KenKnight and Munaier's agar medium (Himedia, 693) supplemented with metal salts of Chromium, Cadmium, Lead and Zinc at a concentration of 100mg/l, 50mg/l, 100mg/l and 50mg/l respectively. Inoculated plates were incubated at 28 C for 14 days and isolated colonies were subcultured.

Antibiotic activity

Gram -ve bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa*(ATCC 27856). Gram +ve bacteria: *Bacillus subtilis*(ATCC 2063), *Staphylococcus aureus*(ATCC 25923), *Klebsiella pneumoniae*(ATCC 13883).

Fungal pathogens: *Fusarium oxysporium*, *Fusarium solani*, *Aspergillus niger* and

Aspergillus flavus were used as test organisms to determine the antibiotic activity of the actinomycetes isolates in primary screening by cross streaking method.

Isolation of antibacterial metabolites

The respective actinomycetes colonies were inoculated into 100ml sterile modified nutrient broth (MNB-Glucose 500mg, Peptone 500mg, Beef extract 300mg, NaCl 500mg, Agar 1.5 gms and 100 ml dst. water, pH 7 ± 0.1) in a conical flask. It was then incubated at 28°C for 8 days in an incubator shaker at 120 rpm. After incubation the supernatant obtained by centrifuging the culture at 8000 rpm for 15 minutes. The supernatant was subjected to solvent extraction using butyl alcohol in a ratio 1:1 and the concentrated extract was used for further studies.

Well diffusion assay

Fresh bacterial and fungal cultures were lawn swabbed on Muller-Hinton agar and Sabouraud's dextrose agar respectively. 6mm wells were made using surface sterilized cork borer. 100µl of respective actinomycetes supernatant extract were added to the wells. Bacterial and fungal plates were incubated at 37 °C, (24 hrs) and at room temperature (72 hrs) respectively.

Heavy metal: Maximum Tolerance

Concentration (MTC)

To each plate of MNA medium containing 4 different wells of 6-7mm diameter, 100µl of the appropriate concentration of metal salt solutions were added and allowed to diffuse at 28 °C for 24 hrs. This would have created a concentration gradient of the metals in the medium surrounding the wells.

To each plate of respective metals, the

freshly cultured isolates V5, V6, V7 and V8 were swabbed and incubated at 28 °C for five days. After incubation the plates were observed for zone of inhibition to determine the tolerance (Hassen, Saidi et al. 1998).

Metal uptake

100ml of sterile kusters media was prepared with 10mg/l of Cr^{6+} in four different 250ml conical flasks. Actinomycetes cultures V5, V6, V7 and V8 were inoculated respectively. This process was repeated for the other metals namely Cd^{2+} , Zn^{2+} and Pb^{2+} respectively. The culture were incubated at 28 °C in a incubator shaker at 120 rpm, and then aliquots were taken at regular intervals of 1,2,3,4,5,6 and 7 days and it was centrifuged at 8000 rpm for 15 minutes. The residual heavy metal in the supernatant was estimated using Atomic Absorption Spectrophotometer (AAS Varian AA240).

FT-IR spectroscopic studies

FT-IR spectroscopy was performed in order to study the conformational changes on metal tolerant Actinomycetes cultures V5, V6 and V8 owing to the interaction with the Cr^{6+} metal. 100ml of sterile kusters media was prepared with 10mg/l of Cr^{6+} in four different 250ml conical flasks. Actinomycetes cultures V5, V6 and V8 were inoculated respectively. Similarly cultures were inoculated in in Kusters media without any addition of metals for control. The culture was incubated at 28 °C in an incubator shaker at 120 rpm, after 7 days cultures were centrifuged at 8000 rpm for 15 minutes. The resulting pellets were dried in hot air oven at 45° C for 24 hrs. The spectra were recorded on a FT-IR spectrometer (Perkin Elmer, India) in a range of about 400–4000 cm^{-1} .

Taxonomic Investigation of the Potential Strain

International *Streptomyces* Project mediums (ISP 1 to ISP 7) were used for the study morphological characteristics of actinobacterial isolates. The colour, aerial mycelium, size, nature of colony and reverse side colour taken in to consideration. Gram staining and spore chain morphology were studied. Carbon sources utilization of Arabinose, Xylose, Inositol, Mannitol, Fructose, Rhamnose, Sucrose and Raffinose as sources of energy was also studied (Nonomura 1974). The amino acid and the sugar present in the cell wall components were determined by standard TLC method (Harper and Davis 1979).

Statistical analysis

The amount of heavy metal removed by the cell was calculated by the formulae:

$$\% \text{ Removal} = (C_i - C_f / C_i) \times 100$$

Where, C_i = initial metal concentration (mg/l), C_f = final metal concentration (mg/l)

Results and Discussion

Antimicrobial activity

A total of 23 isolates of actinobacteria were isolated from rhizosphere region of *Casuarina equisetifolia* tree species. In cross streak method out of 23 actinobacterial isolates subjected to primary screening process, 4 isolates namely V5, V6, V7, and V8 successfully inhibited the growth of test organism showing antimicrobial activity. In Secondary Screening using well diffusion assay, V8 was found to be having broad spectrum activity against both bacterial and fungal cultures (against 4 bacterial strains

and 1 fungal strain). V7 inhibited the growth of both Gram negative and Gram positive bacteria, V6 was found to be inhibiting only Gram negative bacteria and V5 was active against gram positive bacteria (Table. 1).

Heavy metals: Maximum tolerance concentration (MTC)

The tolerance concentration was very high for the isolates V5 and V8 with maximum tolerance around 8000 mg/l towards Cr and Pb. The MTC of the isolates towards each metal is given in fig. 1.

Heavy Metal % Removal

The % removal Cr^{6+} , Pb^{2+} , Cd^{2+} and Zn^{2+} were calculated and derived in the form of graph. (Fig.2). whereas maximum % removal was shown by V5 and V6 towards Cr (78.5 and 64.32), V6 and V7 for zinc (67.9 and 60.59) and V5 and 8 towards Pb (71.73 and 66.7)

Characterization of the potential strain

Based on morphological study of aerial mycelium, colour and growth of the colony the Potential strains were identified as *S.roseisederoticus*, *S.flavochromogenes*, *S.vastus* and *S.praguaeneses* using nonomura key. Results are listed (Table 2) in comparison with the existing data. The cell wall was found to be type 1 (Table 3).

FTIR Spectroscopy

The % transmittance vs. wave number (cm-1) gave the peaks between 3500-500 (Fig. 3) these peaks suggested that the metal bands mainly to the proteins. 2926.16, 2923.95 and 2925.24 of V5, V6 and V8 suggest alphabet CH stretch. (1677.19, 1636.14 and 1633.57; C=O stretch -1439.45, 1365.00 and 1439.20.

Changes in peak length after Cr⁶⁺ metal uptake suggest bending of metal in this region.

It is observed that the colonization of actinobacteria are higher in rhizosphere soil than normal soil (Travis 2003) due to root exudates which attracts most microbes. The antibiotic activity of the V5, V6, V7 and V8 can be explained on the basis that actinobacteria from rhizosphere exhibits extensive actinobacteria and antifungal activity actinobacteria of many plants produce a large spectrum of antifungal activity. Actinobacteria of many plants produces a large spectrum of antifungal substrate (Anthony, et al. 2004.). The high resistance of actinobacteria towards Cr, Pb, Cd, and Zn due to the adaptation of microbes to the toxicity of high concentration of these metals in the rhizosphere of the plant (Gadd and Griffiths 1977) by strategies like oxidative stress and multiple efflux pump. The metal uptake

properties of the isolates explained by (Bae, et al. 2001) as microbes synthesize compounds which bind to metals, reduce toxicity, compounds which accumulate heavy metal by metal binding peptides (Valls and Lorenzov 2002). FT-IR results suggests that metals binds to protein motifs like C=O,-NH bond and -CH₃ bond. This concludes that metals bind majorly to proteins on the actinobacteria. The production of antibiotics, high metal tolerance and removal properties suggests that the production of antibiotic metabolites may be influenced by the chemical nature (heavy metals) as described earlier (Srivibool 1981).

The actinobacterial isolates can be further studied for the relationship between antibiotic property and high metal resistance. Their metal removal properties can be exploited in the field of bioremediation by finding suitable application.

Table.1 Secondary Screening for Antimicrobial activity

Test	Antimicrobial activity			
	V5	V6	V7	V8
Gram +ve				
<i>B. subtilis</i>	11.3±1.5	25.3±0.5	16.0±1.7	26.6±1.5
<i>S. aureus.</i>	9.0±1.7	0.0±0.0	6.3±1.5	0.0±0.0
Gram -ve				
<i>E. coli.</i>	0.0±0.0	11.0±1.0	9.6±1.5	15.3±1.5
<i>P. aeruginosa</i>	0.0±0.0	0.0±0.0	11.0±1.0	11.3±1.5
<i>K. pneumoniae</i>	0.0±0.0	11.0±1.7	0.0±0.0	8.3±0.5
Fungal strains				
<i>A. niger</i>	0.0±0.0	0.0±0.0	0.0±0.0	10.0±0.0
<i>A. flavus</i>	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
<i>F. oxysporum</i>	0.0±0.0	0.0±0.0	0.0±0.0	15.3±0.5
<i>F. solani</i>	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Here data is represented as Mean ± Standard deviation (SD)

Table.2 Taxonomical investigation

Char-acteristics	V5	<i>S.roseise-</i> <i>-roticus</i>	V6	<i>S.flavochro-</i> <i>-mogenes</i>	V7	<i>S.vastus</i>	V8	<i>S.praguaen-</i> <i>es-es</i>
Morphological characteristics								
Colour of aerial mycelium	White	White	White (grey)	White (grey)	White (grey)	White (grey)	White	White
Melanoid pigment	-	-	-	-	-	-	-	-
Reverse side pigment	+	+	+	+	+	+	+	+
Soluble pigment	-	-	-	-	-	-	-	-
Spore chain morphology	Spiral	Spiral	Rectiflexible	Rectiflexible	Spiral	Spiral	Spiral	Spiral
Spore surface morphology	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Utilization of sole carbon sources								
Arabinose	+	+	+	+	+	+	-	-
Xylose	+	+	+	+	+	±	-	±
Inositol	+	±	-	-	+	+	+	±
Manitol	+	+	-	-	+	+	+	±
Fructose	+	+	-	±	+	+	+	+
Rhaminose	+	+	+	+	+	+	-	-
Sucrose	+	±	-	-	+	+	+	±
Raffinose	+	±	-	-	-	+	+	±

Here, +: positive, -: negative, ±: intermediate

Table.3 Cell wall amino acids and whole cell sugars of isolates

Str.ain No	LL-DAP	Meso-DAP	Glycine	Whole cell Sugars	Wall type
V5	+	-	+	-	I
V6	+	-	+	-	I
V7	+	-	+	-	I
V8	+	-	+	-	I

Here: +: present, -: not present

Figure.1 Maximum Tolerance Concentration of V5,V6, V7,V8 against Cr⁶⁺, Cd²⁺, Zn²⁺, Pb²⁺ (*P* value< 0.005)

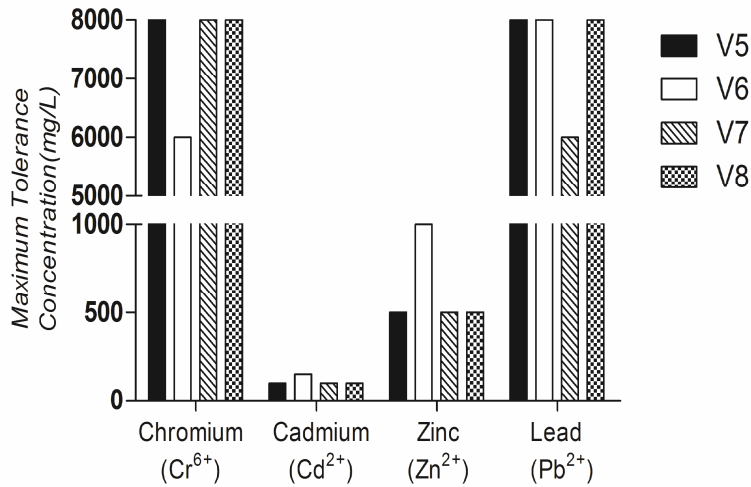


Figure.2. Heavy metal removal by actinobacterial strains: % removal of Cr⁶⁺, Cd²⁺, Zn²⁺ and Pb²⁺ by (a) V5 (b) V6 (c) V7 (d) V8.

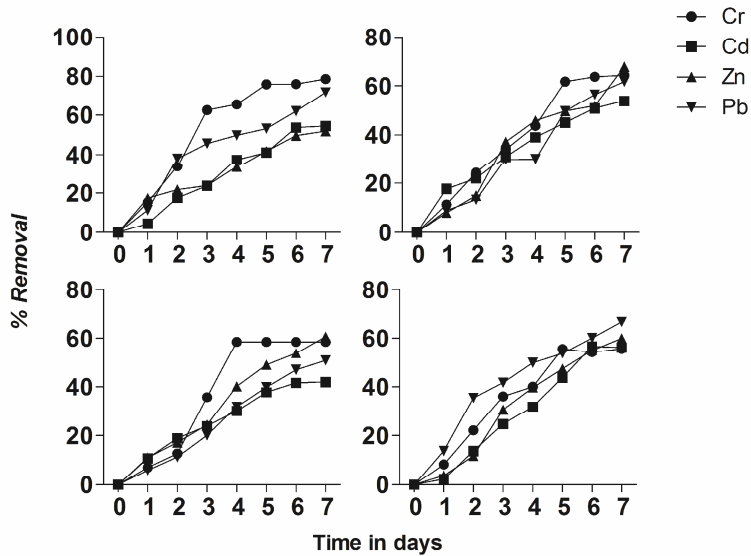
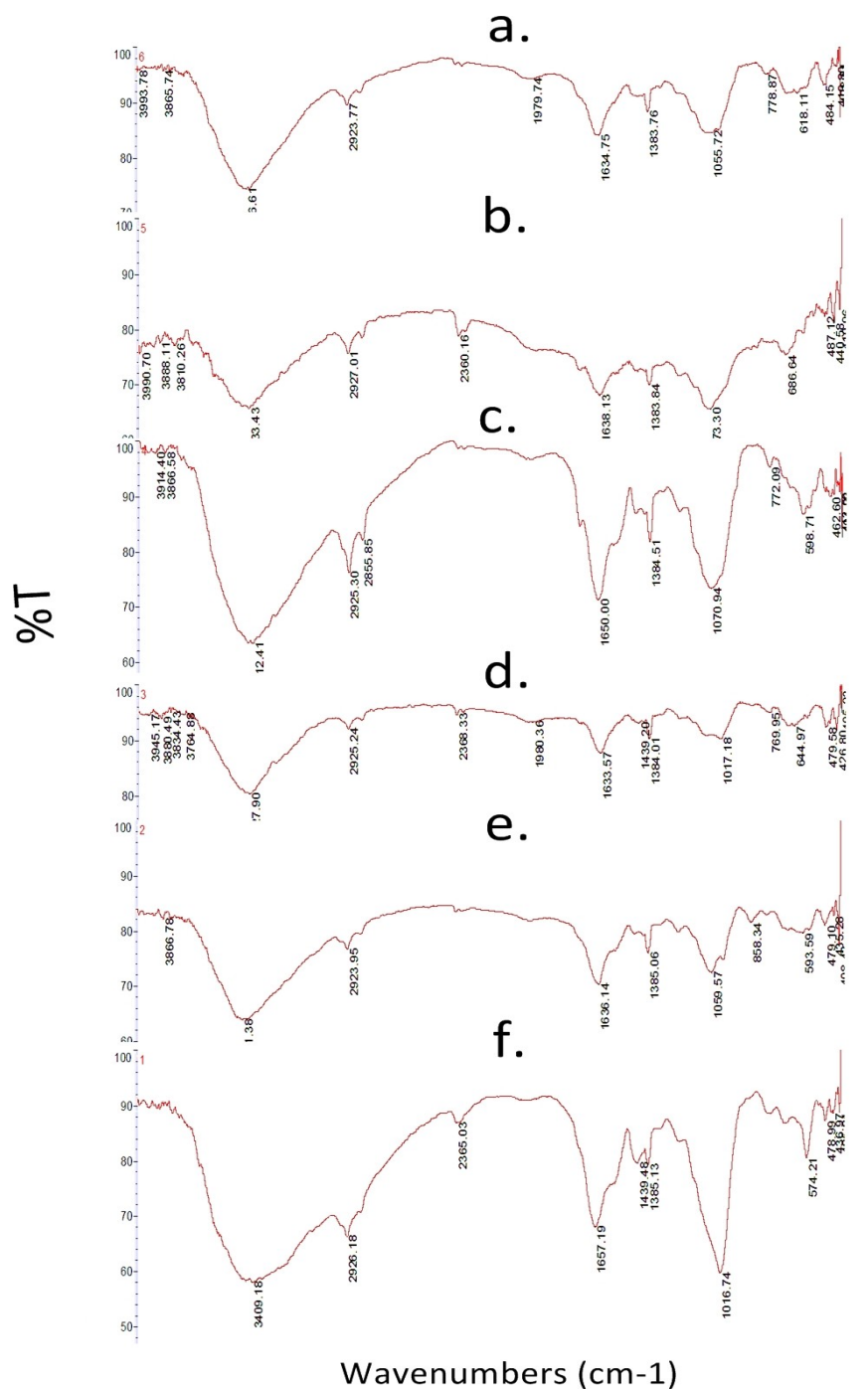


Figure.3 FTIR analysis of actinobacterial strains: (a) V5 without Cr⁶⁺ (b) V5 with Cr⁶⁺ (c) V6 without Cr⁶⁺ (d) V6 with Cr⁶⁺ (e) V8 without Cr⁶⁺ (f) V8 with Cr⁶⁺.



References

- Anansiriwattana, W., S. Tanasupawat, S. Amnuoyopol and Suwanborirux, K. 2006. Identification and antimicrobial activities of actinomycetes from soils in Samed Island and geldanamycin from strains PC4-3. Thai J Pharm Sci. 30: 49-56.
- Anthony, J., J. Strap and D. Crawford, L. 2004. Production and chemical composition of antifungal metabolites isolated from mangrove rhizosphere soil. J Industrial Microbiol Biotechnol. 12: 27-34.
- Bae, W., R. K. Mehra, R. Mulchandani and Chen, W. 2001. Genetic engineering of *Escherichia coli* for enhanced uptake and bioaccumulation of mercury. Appl Environ Microbiol. 67: 5335-5338.
- Bhat, R. and Kaveriappa, K. M. 2009. Rhizosphere mycoflora of some species of myristicaceae of the Western Ghats. India Asian J Microbial Biotech Env. 11: 543-557.
- Gadd, G. M. and Griffiths A. J. 1977. Microorganisms and heavy metal toxicity. Microb Ecol 4: 303-17.
- Gharaibeh, R. S. and Mahasneh, A. 2003. Genotypic and phenotypic characteristic of antibiotic producing soil *Streptomyces* investigated by RAPD PCR. J Basic Microbio. 43: 18-27.
- Harper, J. J. and Davis, G. H. G. 1979. Two-Dimensional Thin-Layer Chromatography for Amino Acid Analysis of Bacterial Cell Walls. Int J Syst Bacteriol. 29: 56-58.
- Hassen, A., N. Saidi, M. Cherifh and Boudabous, A. 1998. Effects of heavy metals on *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. Bioresour Technol. 65: 73-82.
- Imda, C. N., K. Masazumi, K. Takeshi and Nosko, H. S. 2007. Isolation and characterization of antimicrobial substance produced by marine actinomycetes in the presence of sea water. Actinomycetologica. 21: 27-31.
- Kuski, C. R., S. M. Barns and Busch, J. D. 1997. Diverse uncultivated bacterial groups from soils the arid south western United States that is present in many geographic region. Applied Environ Microbiol. 63: 3614-3621.
- Lopez-Maury, L., M. Garcia-Dominguez, F. J. Florencio and Reyes, J. C. 2002. A two-component signal transduction system involved in nickel sensing in the *Cyanobacterium synechocystis* sp. PCC 6803. Mol Microbiol. 43: 247-256.
- Manjula, C., P. Rajguru and Muthuselvam, M. 2009. Screening for antibiotic sensitivity of free and immobilized actinomycetes isolated from India. Advan Biologic Res. 3(3-4): 84-85.
- Namasivayam, K. R. S. and Palaniappan, R. 2005. Antibacterial activity of *Annona squamosa* (L) seed extract against human pathogenic bacteria." National Academy Science Letters. 13:76-78.
- Nonomura, H. 1974. Key for classification and identification of 458 species of the Streptomyces included in ISP. J ferment Tech 52: 78-92.
- Oskay M., A. U. Tamer and Azeri C. 2004. Antibacterial activity of some actinomycetes isolated from farming soil of Turkey. African J Biotechnol. 3(9):441-446
- Pasti, M. B., Pometto A. L., M. P. Nuti and Crawford D. I. 1990. Lignin solubilizing ability of actinomycetes isolated from termite (termitidae) gut. Appl Environ Microbiol. 56: 2213-18.
- Perez, S. and Patterson, T. F. 2002. Antifungal resistant in pathogenic fungi. Clin Infect Dis. 35: 1073-1080.
- Srivibool, R. 1981. A collection of actinomycetes from mangrove soils and screening for antimicrobial producing strains. Biosep Science Journal. 6: 23-33.
- Travis, D. 2003. Root exudation and rhizosphere biology. Plant physiology. 132: 44-53.
- Valls, M. and Lorenzov V. 2002. Exploiting the Genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbial Res. 26: 327-338.