

## Isolation and Characterization of Indole Acetic Acid (IAA) Produced by a Halo Tolerant Marine Bacterium Isolated from Coastal Sand Dune Plants

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Halotolerant bacteria are capable of tolerating the presence of saline stress and can even survive in the absence of saline stress. This study was done to enhance Indole Acetic Acid production by media optimization and then to isolate the same. The work starts with sub culturing of the 46 halotolerant rhizobacterial (HTRB) strains from the culture collection of previously isolated bacteria. These HTRB strains were screened for the production of Indole Acetic Acid and further quantified with UV-VIS spectrophotometer. It has been found that strain AMET7041 has produced higher amount of IAA and hence was selected for further studies. Media parameters such as Carbon, Nitrogen, Tryptone and Salinity were altered for enhanced IAA production. The optimized parameters were used to mass culture strain AMET7041. Further, the IAA responsible for the plant growth promotion was also isolated, separated and authenticated via standard methodologies. The culture filtrate was concentrated and then components separated using silica gel column chromatography. The IAA was separated using various concentrations of solvents Hexane and Chloroform in silica gel column chromatography and its identity was confirmed in TLC as well as UV Visible spectroscopy.

**Keywords:** Halotolerant Rhizobacteria, IAA, plant growth, purification, Column Chromatography.

Soil salinity is one of the major agricultural problems limiting crop productivity in most of the arid and the semi-arid regions of the world. Soil Salinity is caused by excess accumulation of salts, typically most pronounced at the soil surface. As soil salinity increases, salt effects can result in degradation of soils and vegetation. Nowadays soil salinity has become an important issue in agriculture, which is also one of the most urgent global problems to provide enough water and land for agriculture for meeting the world's food

consumption (Pfiser, 1999). Sodium in the soil adversely affects the growth and yield of the most crops, which are highly salt-sensitive (glycophytes) (Bashan *et al.*, 2000). None of the top cultivable plants are salt tolerant. Salinity is a natural factor of the ecosystem in arid and semi-arid regions of the world and can also be induced by irrigation (Abrol, I. P *et al.*, 1988). Nearly 20% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity (Zhu, J. K. 2001).

Biochemical signals transmitted by the roots are said to be the major criteria in conferring the salt tolerance in plants (Shen, 2001; Munns, 2002). However, significance and role of the roots of plants in improving fertility and crop

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productivity of the salt-affected soils have mostly been remained unexplored (Munns, 2002). Statistical studies established that inoculation with *Azospirillum halopraeferens*, a mixture of two *Azospirillum brasilense* strains, a mixture of *Vibrio aestuarianus* and *Vibrio proteolyticus*, or a mixture of *Bacilluslicheniformis* and *Phyllobacterium sp.* has increased the height, dry weight of the plant and even the number of branches (Bashan Y. *et al.*, 2000). Enhancement of plant growth promotion under saline stress happens by the reduction of ethylene production via ACC deaminase activity (Siddikee *et al.*, 2010).

In this context, the study was done to check the plant growth promotion activities of Halotolerant Rhizobacteria obtained from culture collection of previously isolated bacteria on crop plants such as Finger Millet (*Elcusinacoracocna*), Pearl Millet (*Pennisetumglaucum*), Green Gram (*Vigna radiate*), Black Eyed Peas (*Vignaunguiculata*). Plant Growth promoter, Indole Acetic Acid was isolated and characterized. Then the effects of HTBR strain on the above plants with respect to their Protein, Chlorophyll and Carotenoid content was performed.

#### **Collection of halotolerant rhizobacteria**

A total of 46 halotolerant rhizobacteria (HTRB) which were originally isolated from the halophytes were obtained from the Department of Biotechnology, AMET University, Chennai. These samples had their origin from the rhizospheres of *Sueveda sp.* found along the Kelambakkam salterns, Tamil Nadu. These HTRB were previously characterized for their salt tolerance (Jayaprakashvel *et al.*, 2011). Nutrient agar medium supplemented with 1% of tryptone and prepared with sea water was used for subculturing of the samples.

#### **Screening the halotolerant bacteria for IAA production**

The halo tolerant strains were tested for IAA production by the method, described by Brick *et al.* (1991). Bacterial cultures were grown for 48 h on their respective media at  $36 \pm 2$  °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M  $\text{FeCl}_3$  solution). Development of pink colour indicates IAA production.

#### **Quantitative assay of IAA production**

HTRB broth cultures grown for 3 days were centrifuged at 10,000 rpm for 10 minutes and supernatant was removed. To 2 ml of supernatant, 3 ml of Salkowski reagent was added and incubated for 30 minutes. A Blank containing Salkowski reagent and water (3+2 ml) was used for calibration in ELICO UV Vis Spectrophotometer. The test samples were read at 530nm for absorbance (OD). OD values were compared with a standard graph made from authentic IAA to calculate the actual quantity of IAA and were expressed as  $\mu\text{g/mL}$ .

#### **Optimization of media parameters for enhanced IAA production**

Optimization of carbon and nitrogen source, tryptophan concentration and process parameters such as pH, temperature salinity were considered for enhanced IAA production from the selected strain. Methods by Prashanth and Mathivanan (2010) were followed in this experiment with slight modifications.

#### **IAA isolation and characterization**

Culture filtrate was taken by centrifuging the mass culture content at 10,000rpm for 10 minutes. The culture filtrate was mixed with equal volume of ethyl alcohol, and then solvent separation was done. The solvent layer was concentrated. The concentrated solvent (Crude Sample) was used to run Thin Layer Chromatography (TLC). After the movement of the solvent up to the solvent front, the bands in the TLC were identified after soaking in Iodine.

Retention factor was calculated according to the standard formulae as follows,

Retention factor = Distance traveled by Solute

Distance traveled by the Solvent

#### **Column chromatography**

The column was set with Silica gel and hexane. Then the dried concentrated sample with Silica gel was added. After settling of the column along with the sample various concentrations of solutions were prepared to elude the pure compound from the crude sample. The various solvents used were Hexane, Ethyl Acetate and Chloroform.

Various ratios between the solvents Hexane : Ethyl Acetate and Hexane : Chloroform was prepared such as 2:8, 4:6, 6:4, 8:2. Setting the column using Silica and hexane, then the different ratio mixtures were added to allow separation of compounds.

Best formation of bands was visible at 2:8 concentration of Hexane : Chloroform, using a TLC. This TLC was soaked in Salkowski reagent. The particular band indicating the presence of IAA changed into light pink color in the TLC.

**RESULTS AND DISCUSSION**

Halotolerant rhizobacteria is capable of promoting various effects on the inoculated plants such as Biological control, Disease resistance, Growth promotion and many others. This work on Halotolerant rhizobacteria has provided information on the enhancement of IAA production through alteration of various media parameter. It has been reported that IAA production by bacteria can vary among different species and strains, and it is also

influenced by culture condition, growth stage and substrate availability (Matsukawa *et al.*,2007).

Initially the best strain based on production of IAA was selected as AMET 7041(Graph 1). The production of this strain was then enhanced by using various media parameters such as Tryptone, Carbon Sources, Nitrogen Sources and Salinity. During the process of screening, Salkowski reagent was used for detecting the presence of IAA. This reagent is an important option for qualitative and semi-quantitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants based on the change of colour from yellow to pink, the presence of Indole Acetic Acid was confirmed. The amount of IAA produced by

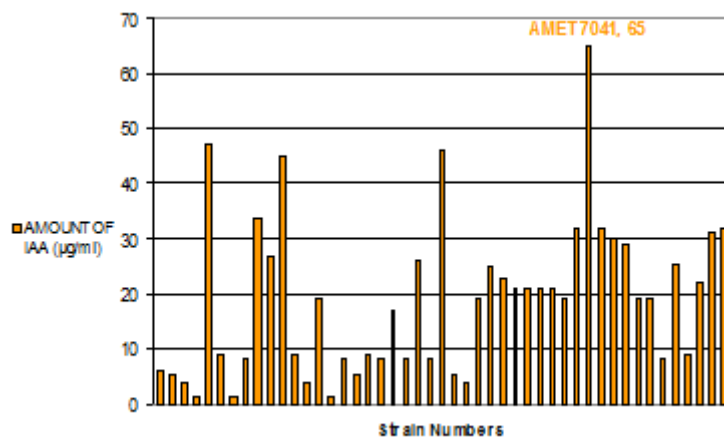


Fig. 1. Quantification of IAA production by HTRB strains

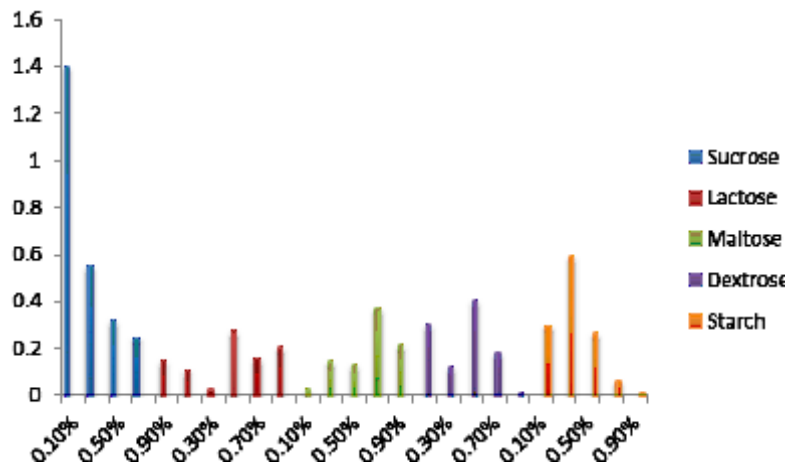


Fig. 2. Optimization of media with different carbon sources

the bacteria was within the detection limits of Salkowski reagent (Ehmann, 1977). The values of the various strains along with Salkowski reagent was checked using UV-VIS spectrophotometer and their values were noted down in terms of Optical density. From these OD values using a standard graph, the values were converted to  $\mu\text{g/ml}$ . The reagent gives reaction with IAA and does not interact with L-tryptophan and Na-acetyl-L-tryptophan and used by and large (Vaghasiat *et al.*, 2011). Among the isolates Strain No 41 was found to be the best producer of IAA with  $64\mu\text{g/ml}$ . Hence for further characterization of this isolates was done

Lee *et al* 2004 have reported that L tryptophan was more active for IAA production, though bacteria were able to produce IAA in absence of tryptophan. In presence of tryptophan, the microbes release greater quantities of IAA and related compounds. In present study tryptone being the basic substrate to find out the presence of IAA in a sample, was used in various concentrations. And the optimum condition was found to be at 1% giving  $12\mu\text{g/ml}$ . Though the bacteria were able to produce IAA in the absence of L tryptophan, they produced higher amount of IAA in culture media supplemented with tryptophan (Skerman *et al.*, 1959). Some

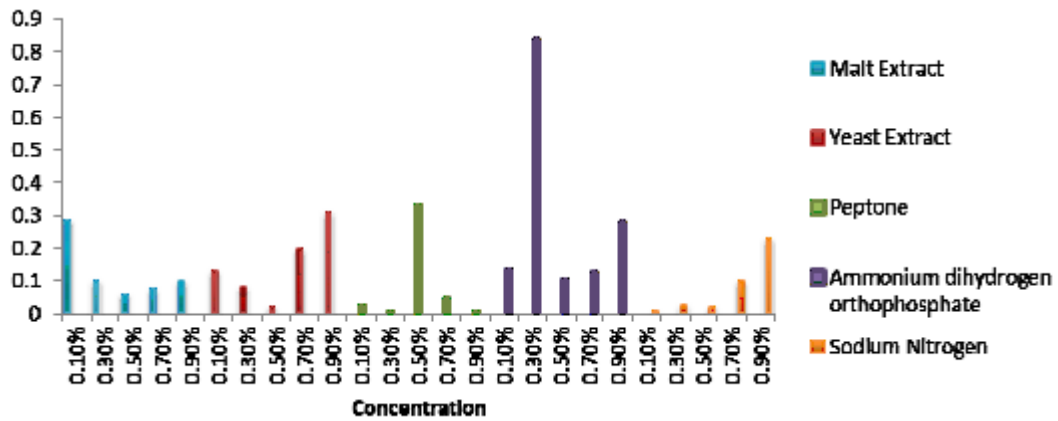


Fig. 3. Optimization of media with different nitrogen sources

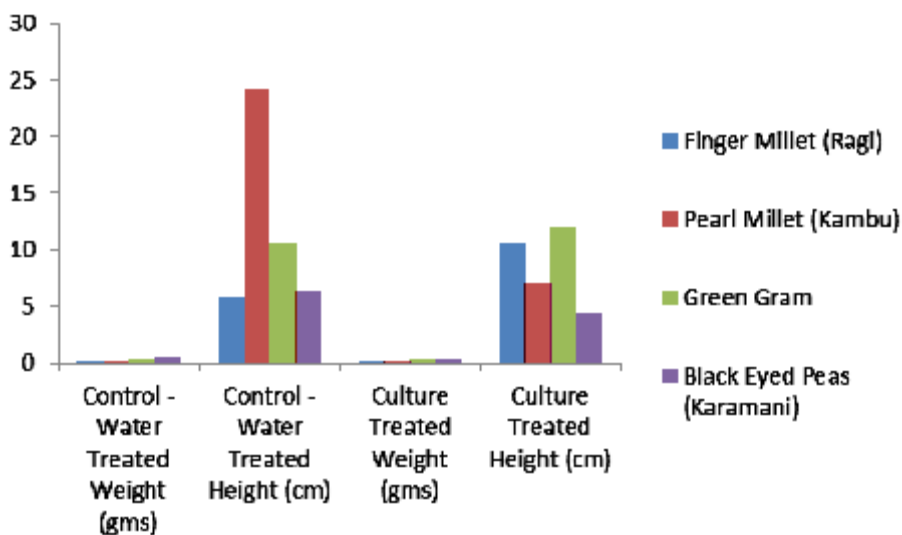


Fig. 4. Results obtained from plate and culture treatment

microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. The tryptophan increases the production of IAA in *Bacillus amyloliquefaciens* FZB42 (Tien *et al.* 1979).

In this study five different carbon sources were used for the process. It includes Sucrose, Maltose, Dextrose, Lactose and Starch respectively. The results were checked by using a UV-VIS Spectrophotometer and hence the optimum source and value was found to be Sucrose of 0.3% which

gave a value of 50µg/ml. Madhuri ,2011 have reported that *Rhizobium leguminosarum* and *R.loti* showed maximum IAA production in presence of lactose, mannitol in 48 hrs. Different sources of nitrogen were utilized for the process. It includes Malt Extract, Peptone, Ammonium dihydrogen orthophosphate, Sodium nitrate and Yeast Extract respectively. After checking the values by using Salkowski process, the best source and the best amount was found to be Ammonium dihydrogen Orthophosphate at 0.3% which gave

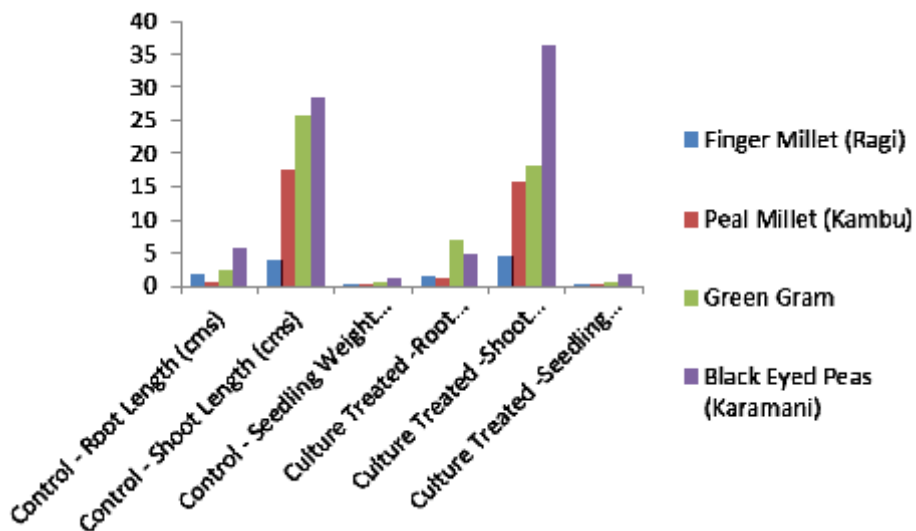


Fig. 5. Results obtained by cup and culture treatment

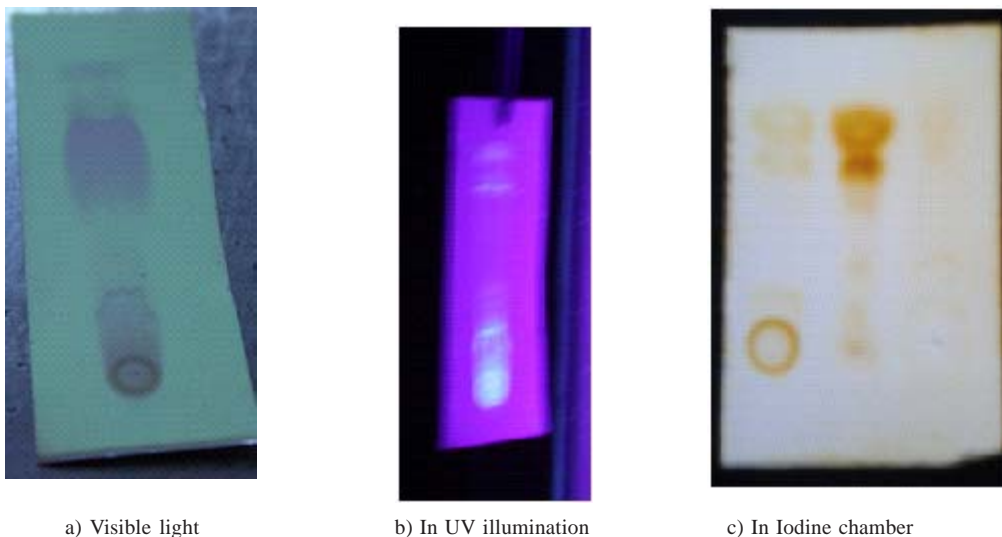


Fig. 6. Thin Layer Chromatography for tracking IAA separation at various solvent ratios in column chromatography

in 71µg/ml. Nita *et al.*, 2011 have reported that among the tested nitrogen sources, NH<sub>4</sub>Cl was found to be the most suitable nitrogen source for IAA production. 0.1% (w/v) NH<sub>4</sub>Cl was the optimum for highest IAA production. Similarly for salinity, various concentrations were considered, but of all the concentrations the best value was found out to be at 3% giving a value of 10µg/ml.

The Culture filtrate of *AMET 7041* was then concentrated and the isolation of IAA separately from the crude sample was done. This IAA was then characterized. IAA that was isolated after concentration using a distillation unit was then studied using a Column chromatography to identify and locate the separate compound (Fig 2). This compound separation was found to be more accurate at a ratio of 2:8 using Hexane: Chloroform. The retention value was calculated further and was found to be 0.9675. **Sridevi *et al.*, 2008** reported TLC chromatogram of purified compound and standard IAA sprayed with salkowski reagent showed almost same Rf values.

#### REFERENCES

1. Abrol I. P., Yadav J.S. P., and Massoud F. I., Salt affected soils and their management, p39. Food and Agriculture Organisation (FAO), UN, Soils Bulletin Rome. (Glycine max (l.) Merrill) isolated from South Brazillian Fields, *Applied Soil Ecology* 1988; **38**: 261-269.
2. Bashan, Y., Moreno, M., & Troyo, E. Growth promotion of the seawater irrigated Oilseed halophyte *Salicornia bigelovii* inoculated with mangrove rhizosphere bacteria and halotolerant *Azospirillum* spp. *Biology and Fertility of Soils*. 2000; **32**: 265-272.
3. Belimov, A.A.; Safronova, V.I., Sergeyeva, T.A., Egonova, T.N., Matveyeva, V.A., Tryganov V.E., Characterization of plant growth promoting rhizobacteria isolated from polluted soils containing I-aminocyclopropane-I-carboxylase deaminase. *Journal of Microbiology*, 2001; **47**: 642-652.
4. Brick, J.M., Bostock, R.M., Silverstone, S.E., Rapid in-situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. *Applied Environmental Microbiology*. 1991; **57**:535-538.
5. Chen, C., Bauske, E.M., Musson, G., Rodriguez-Kabana, R., Kloepper, J.W., Biological control of *Fusarium* on cotton by use of endophytic bacteria. *Biological Control* 1994 **5**:83-91.
6. Das Sharma, S. and Arora, P., Halophiles: *Encyclopedia of life sciences* 2001; **1**.
7. Dave, S. R. and Desai, H. B., Microbial diversity at marine salterns near Bhavnagar, Gujarat, India. *Current Sciences* 2006; **60**(4): 497-500.
8. David, R. Arahal, M., Teresa, G., Carmen, V., David, C., Joaquin, J.N. and Antonio, V. *Chromohalobactersalexigens* sp. nov., a moderately halophilic species that includes *Halomonas elongate* DSM 3043 and ATCC 33174. *International Journal of Systematic and Evolutionary Microbiology* 2001; **51**: 1457-1462
9. Ehmann, The Van Urk-Salkowski reagent - a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *Journal of Chromatography* 1977. **132**, 267-276.
10. Frutos, C., Marhuenda, E., Sonsoles, P.V., Eduardo, Cadenas Enzymatic activity of an extremely halophilic phosphatase from the Archaea *Halobacterium salinarum* in reversed micelles. *Journal of Molecular Catalysis B: Enzymatic*. 2000; **10**(6): 555-563
11. Glick, B.R., The enhancement of Plant growth by free-living bacteria. *Journal of Microbiology* 1995; **41**:109-117.
12. Gothwal. R. K., V.K., Nigam, M. K, Mohan. D., Sasmal and Ghosh P., Screening of Nitrogen fixers from rhizospheric bacterial strains associated with important dessert plants. *Appl. Ecol. Environment Res* 2007; **6**: 101-109.
13. Gupta, A., Gopal, M., Tilak, K.V., Mechanism of plant growth promotion by rhizobacteria. *Indian Journal of Experimental Biology*, 2000; **38**: 856-862.
14. Hariprasad. P and S. R. Niranjana., Isolation and Characterization of Phosphate solubilizing rhizobacteriato improve planthealth of Tomato. *Plant Soil* 2009; **316**: 13-24.
15. Jaap H. Abbring, Gerard J. Van Den Berg., The Nonparametric Identification of Treatment Effects in Duration Models., *Econometrica.*, 2003; **71**(5): 1491-1517.
16. Lee ,S., Flores, E.M., contreras ,Z.M., Gracia ,F.L., Escamilla, J.E., and Kennedy ,C. IAA biosynthesis is deficient in *Gluconacetobacter diazotrophicus* strains with mutations in cytochrome c biogenesis gene. *Journal of Bacteriology*. 2004; **186**(16): 5384-5391.
17. Márcia do Vale Barreto Figueiredo, Lucy Seldin, Fabio Fernando de Araujo, and Rosa de Lima Ramos Mariano., Plant Growth Promoting Rhizobacteria: Fundamentals and Applications., *Plant Growth and Health Promoting Bacteria*, 2010.



18. M. Jayaprakashvel, P. Thiruchitrabalam, R. Muthazhilan, C. Kaarthikeyan, K. Karthik, and A. Jaffar Hussain. Plant Growth Promotion Potential of Halotolerant Bacteria Isolated from Kelambakkam Salterns, Tamil Nadu. In: National conference on Marine Explorations of the Natural Bioactive Compounds from the Marine Resources” during Feb-10th and 11th 2011 organized by Department of Marine Science, Bharathidasan University. (Awarded best presentation). 2011; 46-47.
19. Matsukawa, E., Nakagawa, Y., Iimura, Y., Hayakawa, M. Stimulatory effect of indole-3-acetic acid on aerial mycelium formation and antibiotic production in *Streptomyces* spp. *Actinomycetologica*. 2007; **21**: 32-39.
20. Munns. R Comparative physiology of salt and water stress. *Plant, Cell and Environment*. 2002; **25**: 239-250.
21. Nita B P., Milind G, Sangita S A., Aparna B G., Balasaheb ,P. Optimization of IAA production by *Acetobacter diazotrophicus* isolated from sugarcane. *International journal of environmental sciences*. 2011; **2**: 223-228.
22. Pfiser, R.W. Limits to growth of *Salicornia bigelovii* Torr. at suboptimal salinity. [PhD]. Tucson: University of Arizona. 1999
23. Rodriguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*. 1999, **17**: 319-339.
24. Shazia Afrasayab; Muhammad Faisal; Shahida Hasnain., Comparative study of wild and transformed salt tolerant bacterial strains on *Triticum aestivum* growth under salt stress., *Braz. J. Microbiol.* vol.41 no.4 São Paulo Oct./Dec. 2010.
25. Shen, W., Gomez-Cadenas, A., Routly, E. L., Ho, T-H. D., Simonds, J. A. and Gulick, P. J., The salt stress inducible protein kinase gene, Esi47, from the salt-tolerant wheatgrass *Lophopyrum elongatum* is involved in plant hormone signalling. *Plant Physiology* 2001; **78**: 1429-1441.
26. Siddikee, M. A.1, P. S. Chauhan1, R. Anandham2, Gwang-Hyun Han1, and Tongmin Sa1 Isolation, Characterization, and Use for Plant Growth Promotion Under Salt Stress, of ACC Deaminase-Producing Halotolerant Bacteria Derived from Coastal Soil . *Journal of Microbiology and Biotechnology* 2010; **20**: 1577-1584.
27. Sophie Mantelin and Bruno Touraine., Plant growth promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. *Experimental Botany* 2004; **55** : 27-34.
28. S. Prashanth and N. Mathivanan., Growth promotion of groundnut by IAA producing rhizobacteria *Bacillus licheniformis* MML2501. *Archives of Phytopathology and Plant Protection* 2010; **43** : 191-208
29. Sridevi ,M ., Yadav NCS. And Mallaiah KV., Production of IAA by rhizobium isolates from *Crotalaria* sp. *Research journal of microbiology* 2008; **3** : 276-281
30. Skerman VBD. A Guide to the identification of the genera of bacteria with the methods and digests of generic characteristics. 1st ed. Williams & Wilkins Co., Baltimore: Maryland, USA; 1959.
31. Tien, T.M., Gaskins, M.H., Hubbell, N.D.D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Applied Environmental Microbiology* **37**: 1016-1024.
32. Vaghasiat, H.L., Patel, G.M., Chudasama, R.S., Bhatt, K.R. Screening of IAA from rhizosphere microflora of field crops. *Bioscience Discovery*. 2011; **02**: 94-100.
33. Zhu, G. Y., Dobberlaere. S & Vanderleyden. J Use of green fluorescent protein to visualize rice root colonization by *Azospirillum irakense* and *A. brasilense*. *Funct Plant Biology* 2002; **29**: 1279-1285.