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Screening, Optimization and Assessment of PGPR Bacterial Species and Influence on *Jatropha* Cultivation in Wasteland

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***Abstract* –The aim of our study was investigation of PGP potential of halotolerant bacterial flora for *Jatropha* cultivation in wasteland. *Jatropha* cultivation in wasteland helps the soil to regain its nutrients and will be able to assist in carbon restoration and sequestration. Plant growth promoting rhizobacteria (PGPR) are competent to colonize all the ecological niches that originate on plant root and promotes plant growth. They have profound effects on productivity of plants. In this study total 3 isolates (A, B and C) were productively isolated from *Jatrophacurcas* rhizosphere of waste land soil collected from stretch of Bagodra to Bhavnagar highway with an objective to study PGPR activity. Various physicochemical parameters of this soil were determined such as pH, temperature, salinity and water holding capacity. PGP activities like Phosphate solubilization, Indole acetic acid production and Siderophore production was carried out using selected halotolerant isolates.**

***Keywords* –*Jatrophacurcas*, PGPR, Rhizosphere.**

I. INTRODUCTION

Close at hand is an eternally escalation, necessitating for bio-fuels owing to far above the ground outlay in oil prices. The inclusive biofuel assembly tripled in after everything else and it is vague that the stipulate for bio-ethanol and bio-diesel will supplementary twice over by the closing stages of this decade. In the midst of the assorted bio-energy crops, *Jatrophacurcas* L. is for the most part promising as it has been prevalently subjugated for the production of bio-diesel [1]. The reassessment discusses pervasiveness of imperative microbial groups implicated in nutrient cycling such as phosphate-solubilising bacteria, arbuscular mycorrhizal fungi, and plant growth promoting rhizo-bacteria in the rhizosphere. Auxiliary, have another look for identifying precincts, and addresses to delve in to research gap of relevance to typify rhizospheric micro-biota pertinent for sustainability of bioenergy crop *J. curcas* L. *Jatrophacurcas* is a versatile shrub with noteworthy fiscal magnitude and having the capabilities to recuperate the besmirched lands [2]. In view of the fact that its seed oil can be converted to biodiesel, it is emerging as a renewable energy source, and a substitute to petro-diesel. Several hearsay have demonstrated better concert of the *Jatropha* biodiesel compared with the conventional petro-diesel [3]. Globally *J. curcas* is promoted for large acreage cultivation in a big way for biodiesel production [4].

II. MATERIALS AND METHODOLOGY

A. Collection and Sample Preparation

For identification and characterization of PGP organisms for *Jatropha* cultivation in waste land, two types of soils, saline waste land soil forms Bagodra and rhizospheric *Jatropha* soil were collected. The samples were collected during summer so the soil texture was dry and its analysis was done according to Alef and Nannipieri [5]. *Jatrophacurcus* plants were also washed with care to fade out the excess microflora. The measurement of pH and salinity was done according to the method described in Alefand Nanniperi [6]. Salinity was measured with Eutech instrument - Oakton, Model No. 35425-05, from soil supernatant.

B. Phosphate Solubilisation Assay

Qualitative and Quantitative assays for Phosphate solubilization was performed to screening of bacteria. For qualitative assay, active culture of organisms were inoculated and tested by Pikovskaya's agar plate method [7]. For determination of phosphate solubilization under salt, pH and temperature stressed conditions, Pikovskaya's broth with $Ca_3(PO_4)_2$ was used. Pikovskaya's broth (100 ml) with different concentrations of NaCl (0.5%, 1.0%, 1.5%, 2% w/v) was prepared for assessment of phosphate solubilization in saline conditions. To investigate influence of pH, Pikovskaya's broth was adjusted to 6 different levels (pH 4, 5, 6, 7, 8, 9) by 1N HCl or 1M NaOH. Flasks with Pikovskaya's broth having various salt and pH level were inoculated with the three bacterial strains and incubated at 35ºC (35±2ºC) for six days. For determination of influence of temperature induced phosphate solubilization, Pikovskaya's broth (100ml) inoculated with three strains were incubated for 6 days at three different temperatures (30ºC, 35ºC and 40º C). In all cases, the quantity of solubilised phosphate was measured colorimetrically at 820nm as described by Ames method [8].

C. IAA Production Assay

Qualitative and quantitative assays for IAA production were performed to screen the bacterial culture. Qualitative estimation of IAA was performed for all three bacterial cultures by Luria-Bertani agar plate method, containing 5mM tryptophan [9]. Quantitative estimation of IAA production was done using selected bacterial cultures using LBT and Salkowski reagent method [9]. The amount of IAA produced was estimated from the standard curve of

IAA and expressed inμgl^{-1}. For determination of IAA production under salt, pH and temperature stressed conditions, Luria-Bertani broth (100 ml) with different concentrations of NaCl (0.5%, 1.0%, 1.5%, 2% w/v) was prepared for salt induced IAA production. To investigate pH influence of various pH conditions, pH of LB broth was adjusted to 6 different levels (pH 4, 5, 6, 7, 8, 9) by 1N HCl or 1M NaOH. Flasks with various salt and pH levels were inoculated with the three bacterial strains and incubated at 35ºC (35±2ºC) for six days. For determination of influence of temperature on IAA production, LB broth (100 ml) was inoculated with three strains and incubated for 6 days at three different temperatures (30ºC, 35ºC and 40ºC). In all cases, the quantity of IAA production was measured colorimetrically at 530 nm described by Gorton and Weber [9].

D. Siderophore Estimation

To check the presence of catechol type siderophores, Arnow assay was used. The cultures were grown in Fiss Glucose Minimal Media prepared in deionized distilled water. To 1 ml culture supernatant in an assay tube, 1 ml 0.5 N hydrochloric acid was added followed by addition of 1 ml nitrite-molybdate (production of a yellow colour at this point was checked for catechols). Thereafter, 1 ml of 1 M sodium hydroxide was added, the colour should change from yellow to red, if catechol type siderophores are present (OD at 521 nm). The amount of siderophore produced was estimated from this method and expressed inμgl^{-1}. For investigation of hydroxymate type siderophore production, organisms were grown in medium as described in early research [10]. The amount of siderophore produced was calculated using given formula. The amount of siderophore produced was expressed inμgl^{1} [10]. Siderophore concentration was calculated using equation (Eq.1)

$$\text{Siderophore concentration} = \frac{0.D400 \times 1500 \times 1000\ \text{mg}_{\overline{1}}}{16500}$$

Where,
1500 = Molecular weight of siderophore
16,500 = Extinction coefficient

E. Pot Study and Seed Sterilization

Seeds of *Jatrophacurcas* were washed warily on the trot of tap water for 10 minute followed by distilled water for 5 minutes. Seeds were surface sterilized for 1 minute by method described by [11]. Two kilogram soil was filled in each plastic pots in which 10 ml of the bacterial culture dilute to 50% water holding capacity was inoculated with various combinations of four organisms (A, B, C and D) having 108 colony forming units (CFU) per ml [11]. The combinations were A, B, C, D, A+B, A+C, A+D, B+C, B+D, C+D, Mixture of all four selected strains (consortium) and Control (uninoculated). All pots were labelled and kept in duplicates. Five sterilised seeds were sown directly in each pot at equal depth of 1cm. Soil in each treatment was moistened with an equal volume of autoclaved tap water for daily watering [12].

III. RESULTS

A. Physico-Chemical Characterization of Soil Samples and Microbial Diversity Analysis

Soil samples of wasteland and *J. curcas* rhizosphere is having neutral pH to slightly alkaline pH. The salinity of wasteland sample (between Bagodra and Bhavnagar) was 2.5%. The water holding capacity is 20%. According to microbial diversity study of Jatropha plant rhizosphere from Nirma University was performed out of fourteen functional groups, Total viable count of soil obtained was 105-106 log CFU g^{-1} soil. Azotobacter and Phosphate solubilizers Prototrophic bacteria and ferric reducers were found in range of 105-106 MPN g^{-1} soil. Which proves to help in better plant growth promotion. In microbial diversity study of waste land soil out of fourteen functional groups, Total viable count of soil obtained was 102-103 log CFU g^{-1} soil. *Azotobacter* and Yeast were 102-103 log CFU g^{-1} soil, while Phosphate solubilizers were also present in good amount 103 log CFU g^{-1}soil. Prototrophic bacteria and ferric reducers were found in range of 105-106 MPN g^{-1} soil. While Gram negative and *Actinomycetes* (mesophilic) were present in very less amount. Moreover there were no traces of *Rhizobium, Actinomycetes* (thermophilic), *Azospirillum*, fungi and Sulphate reducers. Hence the microbial population present in the waste land soil was not in good amount. On bases of above diversity test three superior pure bacterial strains namely A, B and C were isolated, collected, persevered and were found to be more dominant in CFU of total viable count. Different plant growth promoting activities such as phosphate solubilisation, IAA production, siderophore production and nitrogen fixation was carried out on selected bacterial strains.

B. Phosphate Solubilization

For quantitative estimation free phosphate released after incubation were determined. The amounts of phosphate solubilized by bacteria ranged from 2 to 401.8μg ml^{-1}. The maximum phosphate solubilization was shown by **A** and was 401.8μg ml^{-1} at 144 h of incubation. While phosphate solubilisation by **B** and **C** gave 171 and 155.8μg ml^{-1} at 144 h of incubation. Hence **A** was found to be relatively better phosphate solublizers than **B** and **C**.

(a) Effect of Phosphate Solubilization at Different pH

Maximum soluble phosphate obtained at pH 7 was 79.3μgml^{-1} by **A** after 6 days of incubation (Graph 3.1).According to Graph 3.2.solubilization of phosphate at pH 7 and 8 was 32.2μg ml^{-1} and 33.1μg/ml respectively, after 6 days of incubation while at pH 9 no soluble phosphate was achieved. Maximum soluble phosphate at pH 6 i.e. 63μgml^{-1}, after 6 days of incubation. Below pH 6, phosphate solubilization was decreased as shown in Graph 3.7.From results it can be concluded that as compared to both culture i.e. **B** and **C**, **A** was found to solubilize more phosphate at neutral to slightly alkaline pH.

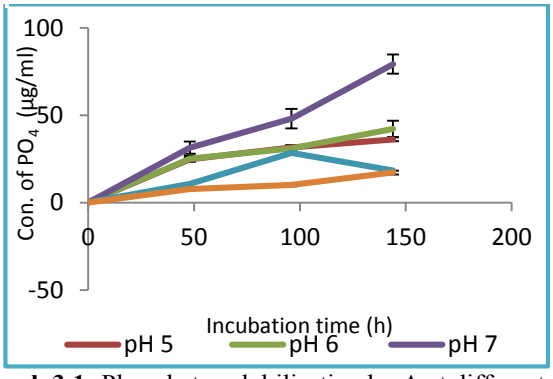
(b) Effect of Phosphate Solubilization at Different Temperature

At 24 h soluble phosphate at 35˚C was 7.8μgml^{-1} which increased toμgml^{-1}, after 6 days of incubation as depicted

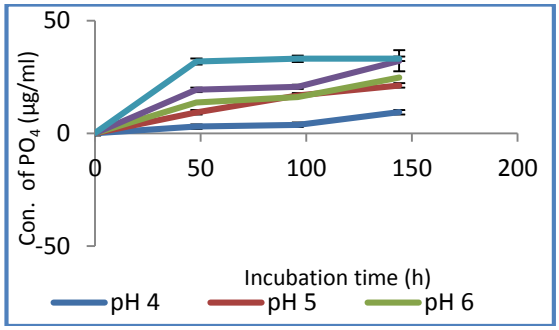
in Graph 3.4. Maximum soluble phosphate was obtained at 35˚ C i.e. 41.7µgml^{-1} after 6 days of incubation. While at 30°C phosphate solubilization was initially less which increased after 96 hrs of incubation were at 40°C soluble phosphate was initially more which decreased after 96 hrs of incubation as depicted in Graph 3.5. While by **C** maximum soluble phosphate was obtained at 35°C and 40° C i.e. 62.07µgml^{-1} and 62.7µgml^{-1} respectively, after 6 days of incubation, as compare to 30° C as shown in Graph 3.6.Hence from these studies, it is concluded that **A** and **B** can solubilize phosphate at moderate temperature and **C** can solubilize phosphate at higher temperature. Amongst all three strains showed highest phosphate solubilization.

(C) Effect of Phosphate Solubilization at Different Salt Concentrations

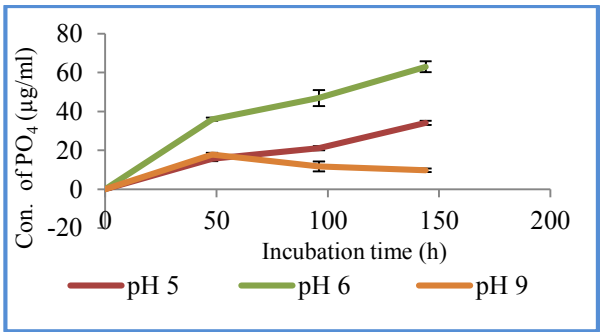
The selected isolate **A** gave 94.5µgml^{-1}of soluble phosphate at 1.5% salt concentration and 42.7µgml^{-1} of soluble phosphate at highest salt concentration of 4% after six days of incubation as shown in Graph 3.7. Both isolate **B** and **C** gave highest soluble phosphate at 2.5% salt concentration 37.8 and 19.7µgml^{-1}respectively, after 6 days of incubation. Phosphate solubilization capacity was found to decrease as salt concentration increased. But not able to solubilise higher amount of phosphate as salt concentration was increased more than 3.5% as depicted in Graph 3.7 and 3.8. In our case all three isolates gave high phosphate solubilization only at 2.5% salinity. As concentration of NaCl was increased, above solubilization by bacteria decreased as shown in Graph 3.7, 3.8 and 3.9. Maximum phosphate solubilisation of 94.5µgml^{-1}was given by **A.**



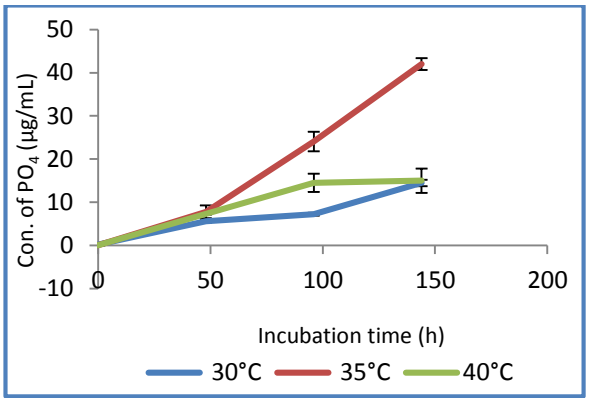
Graph 3.1: Phosphate solubilisation by **A** at different pH level



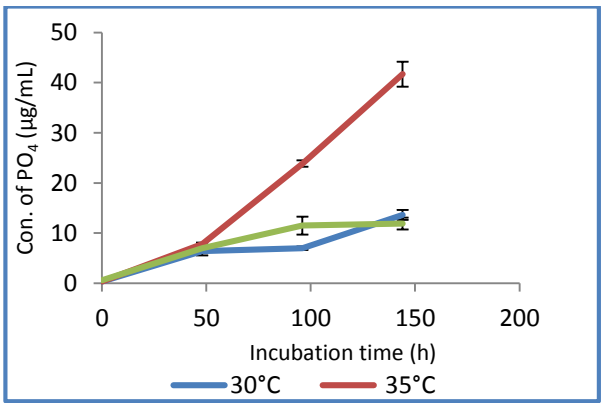
Graph 3.2: Phosphate solubilisation by **B** at different pH level



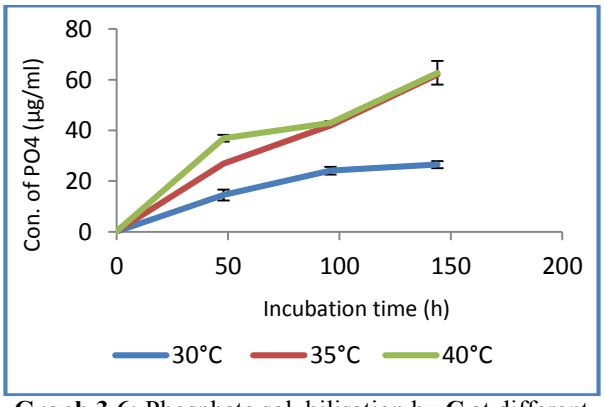
Graph 3.3: Phosphate solubilisation by**C** at different pH level



Graph 3.4: Phosphate solubilisation by **A** at different temperatures



Graph 3.5: Phosphate solubilisation by **B** at different temperatures



Graph 3.6: Phosphate solubilisation by **C** at different temperatures

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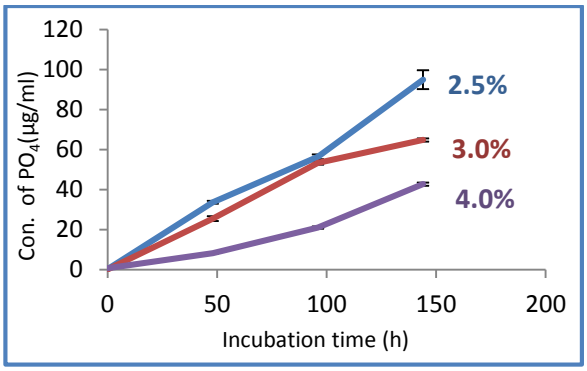
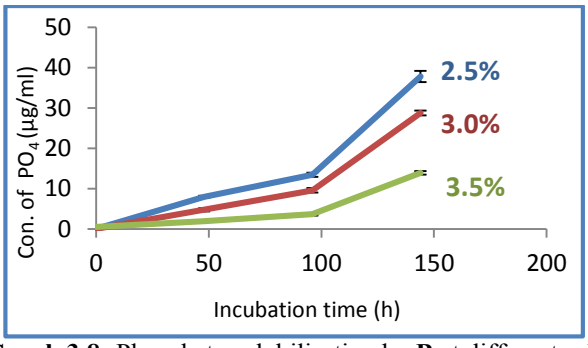
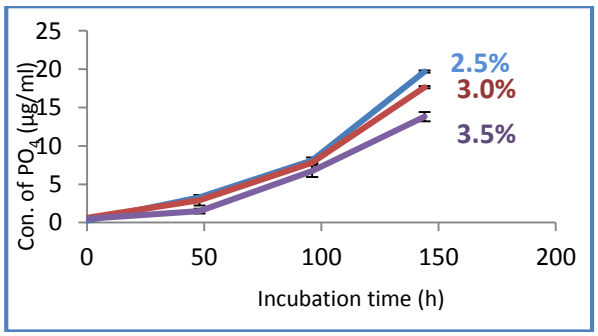


Graph 3.7: Phosphate solubilisation by **A** at different salt concentrations



Graph 3.8: Phosphate solubilisation by **B** at different salt concentrations



Graph 3.9: Phosphate solubilisation by**C** at different salt concentrations

C. *Indole Acetic Acid Production*

In quantitative estimation IAA production was determined after incubation. Highest IAA was produced by **A** and that was 89.25µg ml^{-1} followed by 66.6µg ml^{-1} by **B** after 144h of incubation in presence of 5mM L-trypyophan. **C** did not produce IAA.

(a) Effect of pH on IAA Production

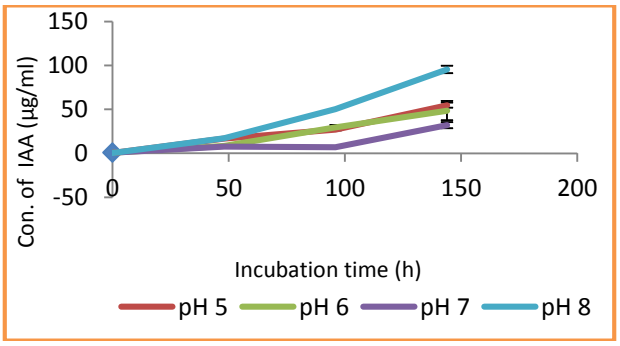
Highest IAA production at pH 8 and 5 i.e. 95.4µgml^{-1} and 54.8µgml^{-1} respectively, after 6 days of incubation. While at pH 4 and 9 no IAA production was obtained (Graph: 3.10). Highest IAA production was obtained at pH 6 i.e. 13.6µgml^{-1} and no IAA production was achieved at pH 8 and 9 after six days of incubation as indicated by Graph 3.11. Hence it was concluded that after 6 days of incubation period **B** showed better IAA production at slightly acidic pH and no IAA production at alkaline pH. While **A** showed better IAA production at both alkaline and acidic pH as compared to that of **B**.

(b) Effect of Different Temperatures on IAA Production

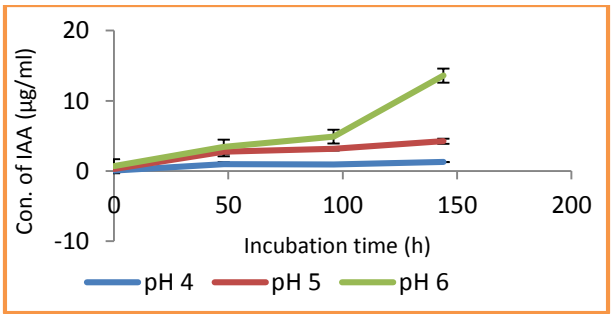
Highest IAA production was obtained at both the temperatures of 30˚C and 35˚C i.e. 17.6µg$ml^{-1}$and 17.2µg ml^{-1} respectively, but at 40˚C IAA production was found to be relatively less as shown in Graph 3.13.Isolate **B** could produce maximum IAA at 40˚C i.e. 17.2µgml^{-1} as compared to 30˚C and 35˚C as shown in Graph 3.12. Hence from both Graph 3.12 and 3.13, it can be concluded that at low and moderate temperatures **A** shows more IAA production and at high temperature **B** produces more IAA.

(c) Effect Salinity on IAA Production

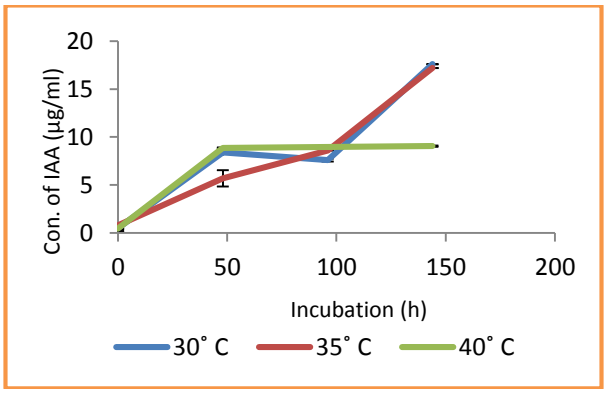
More IAA was obtained at 2.5% i.e. 42.5µgml^{-1} after six days of incubation as shown in Graph 3.14. More IAA was obtained only at 2.5% i.e. 15.7µgml^{-1} after six days of incubation as shown in Graph 3.15. At different salt concentration (NaCl), IAA production was obtained. **A** and **B** gave high IAA production at low salinity. As concentration of NaCl was increased, IAA production by bacteria was decreased.



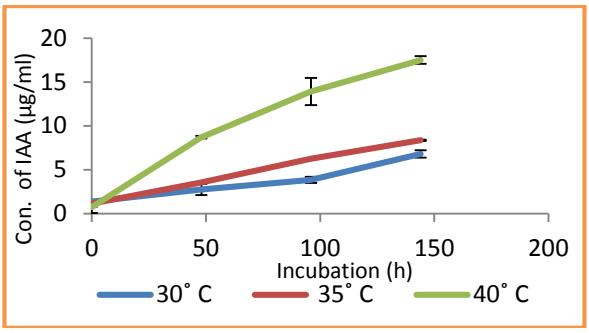
Graph 3.10: IAA production by **A** at different pH level



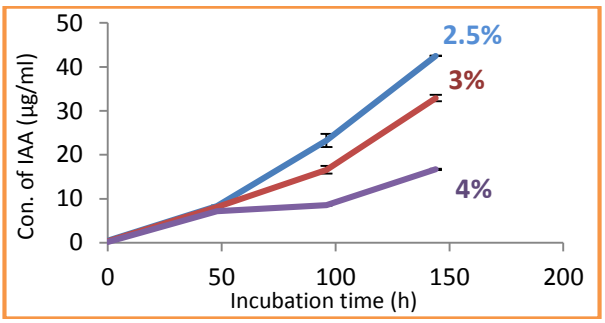
Graph 3.11: IAA production by ***B*** at different pH level



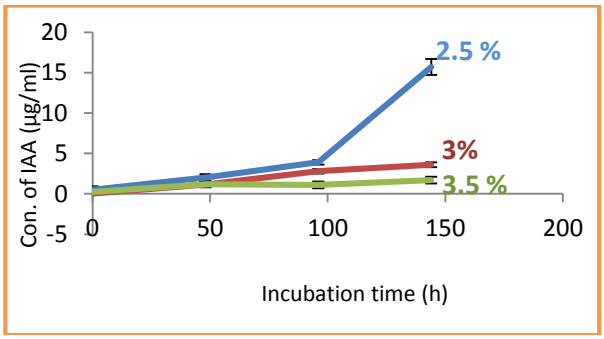
Graph 3.12: IAA production by *A* at different temperature



Graph 3.13: IAA production by **B** at different temperatures



Graph 3.14: IAA production by **A** at different salt concentrations



Graph 3.15: IAA production by **B** at different salt concentration

D. Siderophore Production from Isolates

Hydroxymate type of siderophore was produced by **C** and **A** which is 2.45 and 3.18µgml^{-1} respectively, calculated using formula given by Meyer & Abdallah [13]. In catechol type of siderophore colour changes from yellow to red after addition of NaOH but it was not obtained in our case, hence it was concluded that none of our bacteria produced catechol type siderophore. It is also reported in literature that "Majority of the Organisms produced hydroxymate type of siderophores" [13].

E. Nitrogen Fixation

An efficient strain of nitrogen fixing organism is important for plant growth promotion by fixing atmospheric nitrogen. Nitrogen fixation capabilities of organisms were detected by their growth on nitrogen free medium. All selected organisms were able to grow on Ashby's nitrogen free medium, hence they all were found to be nitrogen fixers.

F. Pot Experiment

After autoclaving soil, sterilized seed were sown with combinations of selected strain; results concluded that germination was observed even after 27 days of sowing as shown in Figure 1. Hence it is clearly seen that practically also isolate A shows maximum plant growth promoting activities and is more superior to B and C. Also compared to control pot (without inoculums) all pot shows growth of plant. Moreover in pot containing mixture of all inoculums best growth of *Jatropha* plant was observed as compared to other pots. So it can be concluded that all the three isolated selected for study were best Plant growth promoters and were also able to withstand the high salinity of waste land soil. *Jatropha* also proved to be the best plant that can grown in such saline area and could grown more better if inoculated with such good plant growth enhancing bacteria's.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figures 6

Figure 7

Figure 8

Figure 1 to 8: Germination of Jatropha seeds after inoculating with selected isolates in combination

IV. Discussion

The PGPR are known to partake in many imperative bionetwork processes, such as the biological control of plant pathogens, nutrient cycling and / or seedling growth. PGPR can impinge on plant growth either directly or indirectly. Direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. Indirectly promote plant growth by preventing the deleterious effects of phytopathogenic bacteria, fungi, nematodes and viruses [14]. Motile rhizobacteria may colonize the rhizosphere more profusely than the non-motile organisms resulting in enhanced rhizosphere activity and nutrient makeover. Phosphate in the soil predominantly exists in insoluble form and the concentration of soluble phosphate in soil is very low where plants are able to absorb only the soluble forms as mono and dibasic phosphates. Microorganisms enhance the phosphorus (P) availability to plants by mineralizing organic P in soil and by solubilising precipitated phosphates. A number of rhizobacteria were able to produce volatile compounds such as ammonia and hydrogen cycnide, which are reported to play an essential role in biocontrol. HCN produced in the rhizosphere seedlings by the selected rhizobacteria is a potential and environmentally compatible mechanism for biological control of weeds and minimizing harmful effects on the growth of desired plants [15]. Accumulation of ammonia in soil may increase the pH and thus by creating an alkaline condition of soil at 9-9.5 that suppresses the germination and growth of fungi as well as nitrobacteria due to its potent inhibiting effect. EPS production is an important trait of bacteria because it protects the cells against phagocytosis, phage attack and helps in nitrogen fixation by preventing high oxygen tensions [16]. IAA producing PGPR strains can able to enhance the growth and development of plants by interfering in the concentration of known phytohormones [17]. The salt concentration, temperature, acidity and alkalinity activities of rhizobia in the rhizosphere are likely to be influenced by the plant genotypes and their symbiosis status. The present study was designed to identify and develop a suitable strain of PGPR which can be used in wastelands for the establishment of seedling of *Jatrophacurcas* facilitating high yield.

V. Conclusion

The present study reflects that isolate A was proved to be the best PGPR strain. It showed highest phosphate solubilisation at neutral to slightly alkaline pH, 35˚C and 1.5% salinity i.e. 79.3µgml^{-1}, 42µgml^{-1}and 94.5µgml^{-1}respectively. In case of salinity all selected isolates gave high phosphate solubilisation at 1.5% salinity. As concentration of NaCl was increased, solubilisation by bacteria decreased. IAA when estimated quantitatively isolate A showed highest IAA production i.e. 89.25µgml^{-1} at 144 hrs of incubation. It produced more IAA at pH 8, 30˚C and 1.5% salinity i.e. 95.4µgml^{-1}, 17.6 µgml^{-1} and 42.5µg/ml respectively. For siderophore production bothisolates produced hydroxymate type of siderophore 2.45 and 3.18µgml^{-1} respectively, none of the isolates produced catechol type siderophore.

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