EFFECT OF DIFFERENT ABIOTIC FACTORS ON PHOSPHATE SOLUBILIZING ACTIVITY BY SPECIFIC ACTINOMYCETES STRAIN ISOLATED FROM GANGATIC PLAIN

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Abstract: *Actinomycetes with their sporulation, root colonization, bio-control, plant growth promotion and other activities could be potential Agricultural bio-fertilizers. Phosphorus is second most important macronutrients found in bound and immobilized form resulted less solubility and availability to the crops (Azziz et al., 2012, Sharma et. al., 2013). Hence, a phosphate solubilizing actinomycetes Sreptomyces cinnamoneus (AKR-18) was isolated from wheat, maize and paddy fields of Gangatic plain of Bhagalpur district, Bihar, India. Strain AKR-18 was capable to solubilize phosphate from P_2O_5, $AlPO_4$ and $FePO_4$ in NBRIP media. In the present study, effect of different abiotic factors such as pH, temperature, C-sources, N-sources and incubation period of this strain were standardized. Day wise kinetics of maximum P-solubilization against P_2O_5 by AKR-18 was 212.15 mg/L. During investigation it was found that lactose was the best C-source (315.20 mg/L) while $(NH_4)_2SO_4$ was best N-source (275.12 mg/L). The optimum pH and temperature were recorded 7 (286.12 mg/L) and 28˚C±2˚C (291.32 mg/L) respectively. The maximum activity of P-solubilization by AKR-18 was observed after 6 days of incubation (291.52 mg/L). Present study is helpful for future study on this strain (AKR-18). Hence if suitable conditions are available the application of P-solubilizing actinomycetes strain AKR-18 in agricultural field might be enhance the growth and yield of crop plants.*

Keywords: *Actinomycetes, Phosphorus, Gangetic plain, Abiotic factors, Sreptomyces cinnamoneus, P-solubilization.*

1. INTRODUCTION

Phosphorus is an essential macronutrient for plants growth but it is present in the soils as a highly insoluble forms hence it is unavailable to plants (Wakelin *et. al*., 2004). The insoluble phosphorus is accumulated in the soil as total phosphorus either in the inorganic or organic forms, whenever plants utilize only available phosphorus. Therefore, farmers use different types of chemical phosphatic fertilizers to take more yields while they are unaware regarding the accumulation of phosphatic fertilizers in soil. Phosphate makes a bond with different elements such as Al, Fe, Ca etc. and reproduces as complexes. The strategy is necessary to remove the low phosphate fertility production constraints, with corrective applications of phosphorus technically which cause high phosphate fixing capacity of the soil. Fungi, actinomycetes and bacteria have the capacity to solubilize and mineralize phosphorus from organic and inorganic pools of total soil phosphorus (Richardson, 20011; Wakelin *et. al*., 2004).
Hence the aim of present works were to isolates the phosphate solubilizing actinomycetes from different crop fields of Gangatic plain of North Bihar and evaluate the effect of different parameters like different media, different carbon, nitrogen sources, pH, temperature and incubation periods on particular phosphate solubilizing actinomycetes strain.

2. MATERIALS & METHODS

2.1 Sample collection and isolation of actinomycetes strains

The actinomycetes strain of *Streptomycetes cinnamoneus* i.e. identified after sequencing as **AKR-18** used in the present study was isolated from the three different crop fields like wheat, maize and paddy from Gangatic plain of Bhagalpur district, Bihar. Actinomycetes were enumerated from different crop fields by using serial dilution method. Dilution of 10^{-3}, 10^{-4} and 10^{-5} were used to isolate actinomycetes on Starch Casein Agar (SCA) plate. 100µl aliquot of diluted sample was poured on agar surface and is spread uniformly with a sterile glass spreader and incubated the plates at 37˚C±2˚C for 7-10 days. Isolates were identified by using 16S rDNA sequencing using 16S forward primer; 5'-AGHGTBTGHTCMTGNCTCAS-3' and 16S reverse primer; 5'-TRCGGYTMCCTTGTWHCGACTH-3'.

2.2 Screening for P-solubilization on PVK agar media:

Pikovaskaya's agar (PVK) agar medium was initially used to determine the P-solubilizing activity of selected strains on solid media.

2.3 Effect of different abiotic factors:

2.3.1 Estimation of P concentration in different media:

Three different broth media i.e. PVK, NBRYP (expansion) and NBRIP (expansion) medium were used to evaluate the best media for P-solubilization activity for AKR-18 strain. 5ml of AKR-18 culture were inoculated in 250 ml of broth medium and cultures were incubated at 28˚C±2˚C with 120 rpm rotation on orbital shaker. After 6 days of incubation available P concentration was measured by modified Olsen's method (Tandon, 2005).

2.3.2 Effect of different phosphate sources on P- solubilization:

The P-solubilizing capabilities of selected strain (AKR-18) towards P_2O_5, $AlPO_4$ and $FePO_4$ were studies after 6 days of incubation following the same method mentioned above. P source of NBRIP media i.e. $CaPO_4$ (5gm) was replaced by these sources.

2.3.3 Effect of different carbon sources on P-solubilization:

Four different carbon sources i.e. glucose, lactose, sucrose and galactose were used to determine their effect on P solubilization by AKR-18 after 6 days of incubation.

2.3.4 Effect of different Nitrogen sources on P-solubilization:

The effect of different N sources on P solubilization by AKR-18 were evaluated using $(NH_4)_2SO_4$, KNO_3 and urea in NBRIP broth media. P concentration was measured after 6 days.

2.3.5 Effect of different pH and temperature on P-solubilization:

pH was measured by using pH meter (*Hanna*). Three range of pH i.e. 4, 7 and 9 and temperature i.e. 20˚C, 28˚C and 37˚C were selected for evaluation of better P- solubilization in case of this particular strain.

2.3.6 Effect of incubation period on P-solubilization:

At interval of equal alternative days P- concentration was measured i.e. 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} day.

3. Statistical Analysis:

Every independent experiment was performed in duplicates and statistical calculations like analysis of variance (ANOVA) were calculated by using Graph pad prism ver-5.

4. RESULTS AND DISCUSSION

Actinomycetes are widely distributed in different habitats and involved in basic processes of recycling and rejuvenation of life. Numerous reports have been published on the phosphate solubilizing microorganisms (PSM) such as Bacteria i.e. *Bacillus subtilis*, *Pseudomonas sp* (Rodriguez & Fraga, 1999), Mesorhizobium sp (Peix *et. al*., 2001), many fungi like *Aspergillus*, *Penicillium sp.* etc (Singh, 2013) and arbuscular mycorrhiza (Sagar, 2004; Ouahmane *et. al*., 2007). Now a day focus has changed from discussing actinomycetes capabilities on biomolecules production rather antibiotic productions to utilization in other activities. Actinomycetes have the potential to solubilize organic phosphate (Ghorbani-Nasrabadi *et. al*., 2012) as well as inorganic phosphate (Hamdali, 2008). They have the characteristics to survive under extreme soil conditions such as low level of moisture or high salinity besides this they are also reported as plant growth promoter by solubilizing soil phosphate as well as rock phosphate (Hamdali *et al*., 2008; Jog *et al*., 2012).

4.1 Screening of AKR-18 for P-solubilization on PVK agar:

Unlike other phosphate solubilizing organisms actinomycetes also take part in solubilizing insoluble phosphorus present in soil (Salcedo *et al*., 2014; Farhat *et al*, 2015; Chacko *et al*., 2016 etc) and have the capability to convert them from insoluble phosphatic compounds to soluble forms in soil and make available to plants (Pradhan & Shukla, 2005; Nopparat *et al*., 2007; Khan *et al*., 2007; Deepa *et al*., 2010; Nenwani *et al*., 2010; Charana & Yoon, 2012) for their growth. During characterization it was observed that phosphate solubilizers usually forms clear zone around the colonies (Seshadri *et al*., 2002; Salcedo *et al*., 2014) but some time it was also observed that no any clear zone visible on the agar plates while solubilized insoluble inorganic phosphates in liquid medium (Nautiyal, 1999). The size of clear zone or halo zone varies on the basis of their potentiality.

Screening of AKR-18 showed 0.4cm/4mm diameter halo zone (**Fig.1**). The solubilizing index (1.4mm) showed the P-solubilizing potential of the strain. The strain AKR-18 was incubated at 28±2 °C and results were recorded at equal alternate day interval (i.e. 4, 6, 8, 10, & 12 day). It was observed that AKR-18 showed gradual increase in halo zone in respect to colony diameter. After few days it was also observed that inconspicuous decline in colonies as well as halo zone growth. That retardation in growth might be attributed to deficiency in nutrients in the medium.

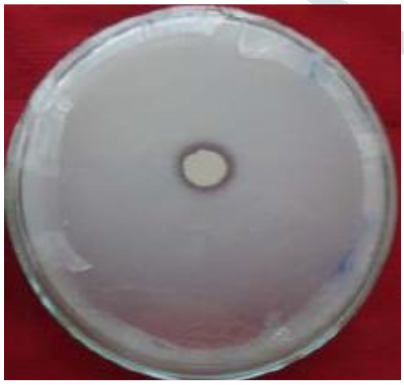


Fig.1 P-solubilizing activity on PVK agar by AKR-18

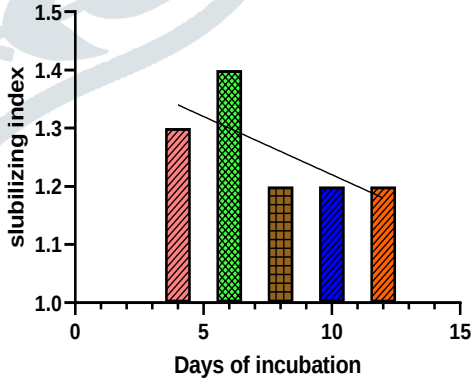
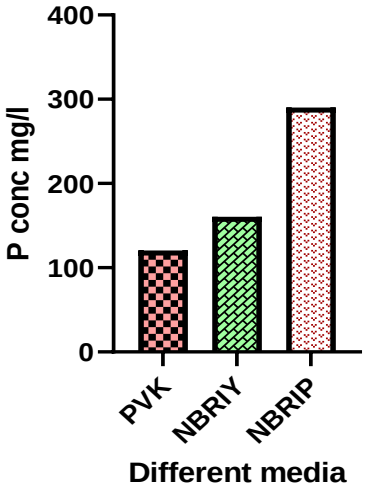


Fig.2 P-solubilizing Index by AKR-18

4.2 Effect of different media on P-solubilization potential of AKR-18;

To confirm the best broth media among PVK, NBRIY and NBRIP for selected strain (AKR-18) PVK was showed the concentration of P was 120.48 mg/L and pH drop was 4.9, in NBRIY media P was 160.35 mg/L and pH drop was 3.9 while in media NBRIP maximum P- solubilization was recorded i.e. 290 mg/L followed by minimum pH i.e. 3 and biomass of mycelium was 0.38g (Fig.3). Phosphate solubilization was accompanied by a decrease in pH of the medium. Out of all the three selected media i.e. PVK, NBRIY and NBRIP at constant pH 7, the drop in pH was correlated with rate of P-solubilization. Maximum P-solubilization was recorded at minimum pH. On the basis of P- solubilization it was concluded that chemical composition of media play a vital role because pH of all the three media was kept constant at neutral i.e. pH 7. The growth media may be graded as best fit to low grade from NBRIP> NBRIY > PVK for this particular strain (AKR-18). Therefore, for further studies NBRIP-medium was used.



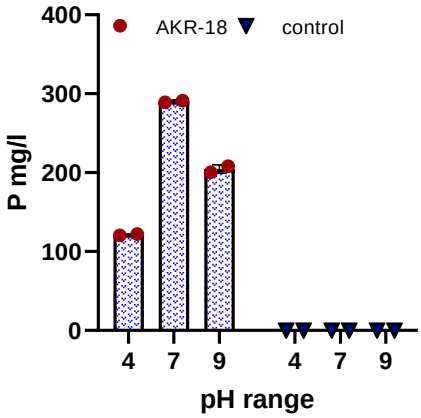


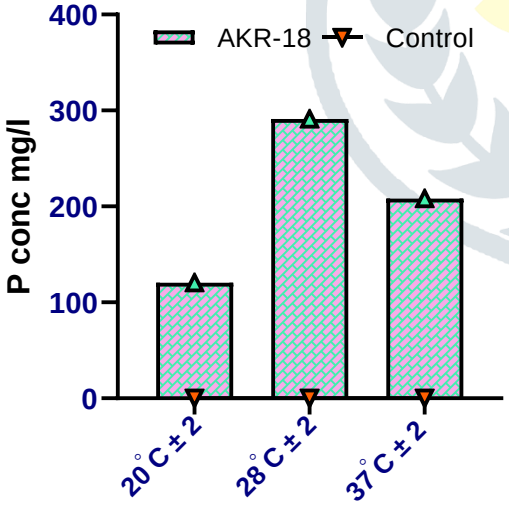
Fig.3 Effect of different growth media on P-solubilization **Fig.4 Effect of different pH on P-solubilization by AKR-18**

4.3 Effect of pH:

The growth of microbes is affected by pH (Souchie *et al*., 2005), even minute variation is also affects the activity because it regulates the growth of microbes. To examine the role of pH, three range of pH was taken such as acidic (4.5), neutral (7) and alkaline (9) medium to observe the effect of P- solubilization at 28°±2°C in NBRIP medium (**Fig. 4**). During experiment it was observed that AKR-18 showed maximum P-solubilization at pH (7) 221.44 mg/L while changing in pH level from neutral to acidic reflects decrease (137.25 mg/L) in P-solubilization capacity. Contrary to that when it was tested at higher scale of pH at alkaline range the result was improved (200.37 mg/L) but not to the mark of neutral pH level. The rate of phosphate solubilization was most efficient at pH 7, moderate at pH (9) and poor at pH 4.5 as shown in Fig. – 4. This results shows consistency with work of Farhat *et. al*., 2015 and 2009. It was also observed that pH was decreased during incubation period as compared to initial pH.

4.4 Effect of temperature:

Three different range of temperature such as 20°±2°C, 28°±2°C and 37°±2°C was taken for examination. Aligning to the main objective of this study to examine the effect of temperature on P- solubilization by selected strain (AKR-18) and find out suitable temperature for maximum P-solubilization by AKR-18. The maximum P-solubilization was recorded at 28°±2°C (291.32 mg/L) followed by 37°±2° C (208.12 mg/L) and least at 20°±2°C (108.69 mg/L). Earlier reports also indicates that actinomycetes shows better growth & result at 28±2°C and 37±2°C (Sahu *et.al*., 2007; Hamdali *et al*., 2008; Mohan *et al*., 2013; Sreevidya *et al*., 2016). On the basis of above result, it was concluded that the AKR-18 showed maximum potential at 28°±2°C (**Fig.5**).



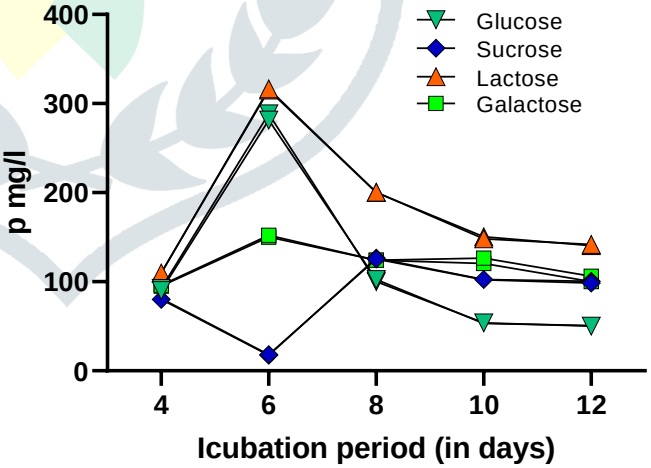


Fig.5 Effect of different temperature on P-solubilization **Fig.6 Effect of different C- sources on P-solubilization**

4.5 Effect of different carbon sources:

Phosphate solubilization mechanism is a complex process; this process is influenced by many factors like nutrients availability and growth status of the microorganisms. The amounts of glucose as a carbon source play a vital role in the P-solubilization. The rate of P-solubilization was increased with increasing concentration of glucose (Nautiyal, 1999). Phosphate solubilizing activity of selected actinomycetes was evaluated in the presence of four carbon sources by replacing glucose in NBRIP medium. For examination culture was grown in different carbon sources i.e. lactose, glucose, sucrose and galactose in NBRIP media by substituting glucose at an amount 10 g/L to evaluate the effect of different carbon sources on phosphate solubilization.

AKR-18 showed maximum solubilization (**Fig.6**) in lactose (315.20 mg/L) followed by glucose (288.40 mg/L), sucrose (170.41 mg/L) and galactose (150 mg/L). On the basis of above result it was concluded that lactose was the best carbon source for this strain to solubilize phosphorus. It might be due to the presence of lac operon system, because it is well known that actinomycetes have the characteristics of both bacteria as well as fungi.

4.6 Effect of different nitrogen sources:

The effect of different nitrogen (N) sources i.e. $(NH_4)_2SO_4$, $NaNO_3$, KNO_3 and urea on P- solubilization was evaluated in NBRIP medium by replacing $(NH_4)_2SO_4$ in the culture media. But the amount of $(NH_4)_2SO_4$ was changed because it was reported that the amount of $(NH_4)_2SO_4$ affects the rate of phosphate solubilization (Nautiyal, 1999). In the present study, when NBRIP used as a growth medium (0.1 g) $(NH_4)_2SO_4$ used but when experiment was designed to evaluate the effect of N- sources on P-solubilization 0.5 g of $(NH_4)_2SO_4$ additionally mixed to the medium as nitrogen source. AKR-18 showed maximum activity in the presence of $(NH_4)_2SO_4$ (275.12 mg/L) followed by $NaNO_3$ (188.24 mg/L), KNO_3 (186.61 mg/L) and urea (56.10 mg/L). The selected strain showed minimum P- concentration in case of urea (**Fig.7**).

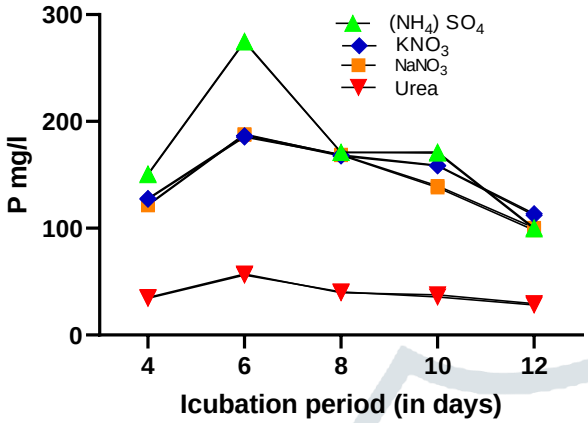


Fig.7 Effect of different N- sources on P-solubilization

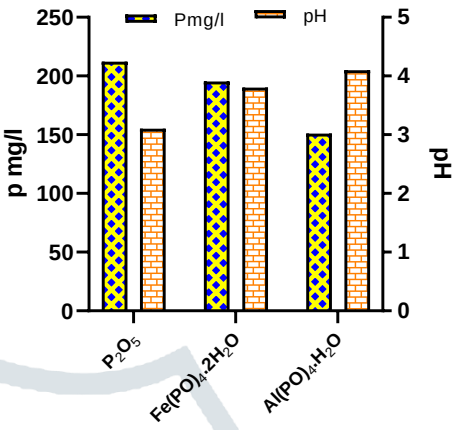


Fig.8 Effect of different P- sources on P-solubilization Potential of AKR-18

4.7 Effect of selected actinomycetes on P-solubilization when different phosphate source used:

Most of the literature reveals that in the natural soil comprise a significant amount of phosphatic compounds, ranging from 200 to 3000 mg/kg, averaged at 1200 mg/kg (Harrison, 1987). A wide variety of organic and inorganic phosphatic compounds are present in the soil (Sanyal *et al*., 1991). In spite of, only a small amount (i.e. < 1%) is immediately available to plants as free inorganic phosphate (Pi). The majority of inorganic compounds are commonly associated with calcium (Ca) in alkaline soils or with iron (Fe) and aluminum (Al) in acidic soil (Sanyal *et.al*., 1991).

Some workers explain the mechanism for Pi acquisition is the result of excretion of proton and organic acids either from plant roots or rhizospheric microorganisms which results in acidification of rhizosphere. The importance of this mechanism was not properly clear yet plasma membrane H^+ pumping ATPases were shown to be involved in plants adaptation to Pi starvation (Yet *et. al*., 2002). Acidification was also correlated with the up-regulation of novel membrane channels needed to transport anions such as citrate and malate from root cells into the rhizosphere (Diatoff *et al*., 2004). Excretion of organic acids results in the chelating of metal cations, that immobilizes Pi (e.g. Ca^{2+}, Al^{3+}, $Fe^{2/3+}$), thus, increasing free Pi concentrations in soil up to 1000-fold (Malboobi *et al*., 2012).

Keeping these facts in mind, try to understand the effect of actinomycetes (AKR-18) on different P- sources i.e. P_2O_5, aluminum phosphate ($AlPO_4$) and iron phosphate ($Fe\ PO_4$) used as a sole P source in NBRIP medium replacing $CaPO_4$ (5g). The results clearly depicted in (**Fig.8**), indicates broth media supplemented with P_2O_5 showed minimum concentration of P (40.24 mg/L) in control condition (i.e. absence of AKR-18) whereas medium inoculated with actinomycetes showed release of considerable quantity of P in medium i.e. 212.15 mg/L while in $FePO_4.2H_2O$ containing media i.e. 195.31 mg/L and in case of Al $(PO)_4$. H_2O was 150.92 mg/L. On the basis of above results it was concluded that the amount of solubilized –P in $AlPO_4.2H_2O$ was less in comparison to the values obtained in media with P_2O_5 and $FePO_4.2H_2O$. The obtained graphs clearly indicate that P- release and drop in pH by actinomycetes follow the order of medium as P_2O_5> Fe-P>Al-P respectively. Ben Farhat *et al*., (2015) described the relation between concentration of released P and acidification of medium (i.e. drop in pH).

4.8 Effect of incubation period on P-solubilization in NBRIP medium:

To know the effect of incubation period **(Fig.9)**, concentration of soluble phosphate was measured at equal days of interval i.e. 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} day. The experiment was started from 4^{th} day because actinomycetes are slow growing organisms. In comparison to 4, 6, 8, 10 and 12 days, on the 6^{th} days of incubation showed drastic change in P-solubilization by selected strain (AKR-18) i.e. 291.52 mg/L while maximum pH drop was 3.0. The pH of the culture filtrate was compared with the P released by the isolates showed variations in concentration of soluble phosphate oscillating with pH (Illmer *et al.*, 1992). These changes in P concentration may be due to the organic metabolites (Henri *et al*., 2008).

Decreasing concentration of P in solution indicates that during incubation nutrition level also plays an important role. Thus it was concluded that, all the selected isolates showed their maximum P- solubilization on 6^{th} day of incubation but after that there was decline in rate of P- solubilization. It may be due to the utilization of P by isolates, resulting in the fluctuating level of phosphate release.

Although mechanism of P- solubilization is still not fully understood while solubilization kinetics of inorganic P-source is one of the important studies for P solubilization ability of an isolate. It is generally accepted that the microbes mediated acidification is one of the important mechanism of the overall P-solubilization process (Illmer & Schinner, 1995; Jog *et al*., 2014; Gaind, 2016).

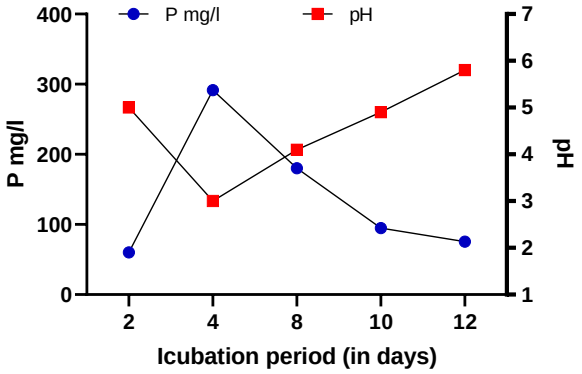


Fig.9 Effect of incubation period on P-solubilization in NBRIP medium

5. CONCLUSION

The present study revealed that the phosphate solubilising strain *Sreptomyces cinnamoneus* (AKR-18) isolated from the different crop fields such as wheat, maize and paddy is indeed able to solubilize phosphorus from insoluble inorganic phosphorus. The optimum conditions like pH, temperature, different sources of C, N, P and incubation period for maximum P- solubilization were standerized for particular strain i.e. AKR-18 and it was found that it is able to solubilize bound P- from P_2O_5, $Fe(PO_4).2H_2O$ and $Al(PO)_4.H_2O$. Hence, this study suggests that AKR-18 individually or in combination with any other fertilizers has great potential to lower the fixation of phosphorus and make it easily available to plants. After further study it will be confirmed that what is the proper mechanism and reason is behind the P-solubilization by this particular strain. The results of present investigation are expected to leads the development of novel multi-functional bio-phosphatic fertilizers.

6. ACKNOWLEDGEMENTS

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