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Isolation and Characterization of Phosphate Solubilising Bacteria from a Discrete Ecological Niche in Navi-Mumbai, Maharashtra, India

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Abstract- Phosphorus usually occurs in insoluble form, but is an important element for plant growth. Some microbes can convert insoluble phosphate to soluble form making them available for plants. An effort on isolation and characterization of phosphate solubilizing bacteria from two different mangrove inhabitat sampling sites was attempted in this study. 30 phosphate solubilizing bacteria were isolated on Pikovaskaya's agar. Eight of these isolates showed sharp halos on Pikovaskaya's agar assay plate supplemented with 0.003% Rose Bengal. Maximum phosphate solubilisation in broth was shown by isolate S4RWB. Decline in pH value of media was observed from 7.2-2.5 within 48h for some of the isolate. The efficiency of Phosphate solubilisation of these isolates were also characterised in presence of various carbon and nitrogen sources and the effect of pH and temperature was also evaluated.

Keywords: Mangrove Microbiome, Pikovaskaya's medium; solubilisation index; phosphate solubilisation efficiency.

I. INTRODUCTION

Phosphorus is one of the essential mineral macronutrient for plant growth and development. It plays important role in many physiological activities of plant. Among the various ecosystems that plays pivotal role in nutrients cycling and regulating chemical environment, the mangrove ecosystems are unique. Diverse groups of microorganisms viz. free living bacteria (nitrogen fixers, phosphate solubilizers, cellulose decomposers, nitrifiers and denitrifiers, sulphur oxidizers, iron oxidizers and iron reducers), fungi and yeasts are present in micro niches which significantly help in the formation of detritus in the mangrove ecosystems (Thatoi et al 2012a). Mangrove inhabitat soil generally contains high proportion of organic phosphorus in comparison with the unvegetated saline deposit. While the insoluble form of phosphate in soil is not available for plants, the organic from in plant cell are limited to microbe utilization. Also the assimiable form of phosphorus is generally lost in the nature due to either its rapid incorporation in clay particles or its association with metal in both acidic and alkaline condition. This deficiency of available, assimiable Phosphorus is attributed due to formation of strong bonds between iron and aluminium in acidic while with calcium and magnesium in alkaline soil (Ranjan et al. 2013). Thus the mangrove soil functions as phosphorus sinks.

Nevertheless the deficiency of phosphorus in cultivated soil is overcome by the use chemical fertilizers, but phosphorus from chemical fertilizers is also lost as they form complex with elements (Aipova et al. 2010). Studies also shows that phosphorus uptake is tied up with that of iron and sulphur cycles. However unlike other soil, in mangrove ecosystem various mutualistic relationships between the microbiome and macrobiome govern the uptake of soluble phosphorus. Microbiota at the mangroves may consist of plant growth promoting rhizobacteria (PGPR) in rhizospheric soil, root surface and associated with roots. Some of these PGPRs can convert inorganic phosphate to organic form through release of phosphatase and organic acids. Such microorganisms are termed as phosphate solubilising microorganisms (PSM) (Rodriguez and Fraga 1999; Gulati et al. 2008). Utilization of PSB as biofertilizer has been studied for crop production and seed germination in many agricultural crops like chillies, chick pea, tomato, sugarcane, maize, wheat, lettuce (Kucey. 1987; Chabot *et al*. 1996; Sundara et al. 2002; Sharma et al**.** 2007; Khan et a*l*. 2007; Akhtar and Siddiqui 2009; Gupta et al. 2012; Suparat et al. 2013; Walpola and Yoon 2013).

In past, number of studies has also reported the presence of bacteria, fungi and actinomycetes from sediments, rhizospere and roots surface of various mangrove spps. (Thatoi 2012a, Ravi Kumar 2007). However, information about the diversity of phosphate solublisers and their efficiency in phosphate solubilization among Indian mangroves is scarce (Thatoi 2012a). Screening and isolation of PSB from soil involves detection of colonies with a halo around the colonies on media containing inorganic mineral phosphate as sole Phosphorus source (Rodriguez and Fraga 1999, Gupta et al. 1994).

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Current study is an endeavour to attempt isolation and characterization of both rhizospheric and non-rhizospheric phosphate solubilising bacteria from two of the mangrove inhabited area in Belapur and Kamothe creek of Navi Mumbai, Maharashtra. The study also determines phosphate solubilising efficiency of selected isolates with respect to pH, temperature, carbon and nitrogen source so as to explore the potential of phosphate solubilising bacteria as bio-fertilizers for sustainability of mangroves in the satellite city.

II. MATERIALS AND METHODS

A. Isolation and characterization of phosphate solubilising microorganisms

The samples were collected from one sampling site at CBD Belapur creek- Site A (19° 1′ 25.33″ N, 73° 2′ 27.65″ E) Navi-Mumbai, India, and two different sampling sites at Kamothe creek- Site B and C; (19° 1′ 0.49″ N, 73° 5′ 47.26″ E), Navi-Mumbai, India. Soil at 0 and 20 cm depth, mangrove roots samples and water samples in triplicate from mangrove inhabited area at these three sites collected using metallic spoon and sterile sample collection bags. Sea water near roots of mangroves was collected in 500ml sterile polythene bottles. The samples were transported to laboratory in icebox within an hour. The adhering soil from the roots was removed carefully, followed by two consecutive wash of the roots with tap water. The roots were then macerated with 0.85% saline to isolate PSB from roots (Nautiyal C. 1999; Vazqeuz et al. 2000).

Serial dilutions were spread on Pikovoskaya's (PVK) agar plate and incubated at room temperature for 3-5 days respectively. Halo forming colonies due to solubilization of phosphate were picked from PVK agar and sub-cultured to obtain pure culture (Sharma et al. 2011; Reena et al.2013; Vazquez et al. 2000). PSB isolates were further characterized for their colonial, morphological and biochemical characteristic. Gram's nature and endospore staining was carried out according to Dubey and Maheshwari (2000). Biochemical characteristics like starch hydrolysis, gelatin hydrolysis, H_2S production, indole production, methyl red, Voges-Proskaeur, citrate, urea, catalase and oxidase were studied (Dubey and Maheshwari. 2000; Mahanttesh and Patil 2011; Reena et a*l* 2013).

B. Phosphate solubilisation efficiency

Solubilisation efficiency of all the selected isolates was carried out using PVK assay plate supplemented with 0.003% Rose Bengal. The diameter of halo and colony was measured on 3rd, 5th and 7th day of incubation (Nautiyal C. 1999). Phosphate solubilisation efficiency was assayed visually and the solubilisation index (SI) for each isolate was calculated as the ratio of total diameter of phosphate solubilization to colony diameter (Paul and Sinha 2013). Isolates exhibiting high solubilisation efficiency were selected for further studies.

C. Quantitative estimation of phosphate solubilisation using broth assay

PVK broth (10 ml) was inoculated with PSB isolates and incubated at room temperature on shaker at 180rpm for 5 days (Gulati et al.2008). Autoclaved un-inoculated media served as control (Islam et al. 2007). The cultures were harvested by centrifugation for 5 min at 5,000 rpm at 4°C. Available phosphorus in supernatant was evaluated with Vando-Molybdate colorimetric method at 420nm (Vazquez et al. 2000). The amount of phosphorus present was expressed as µg/ml (Gulati et al.2008).

D. Optimization of parameters effecting phosphate solubilisation efficiency

1) Cell density and pH of media: The cell density was estimated on PVK broth every 24 hrs at room temperature (Suparat et al. 2013). The change in the pH of the media was determined at 24 and 48 hr (Ramani and Patel. 2011).

2) Effect of carbon and nitrogen sources on phosphate solubilisation: The bacterial isolates were inoculated on PVK agar plate supplemented with 0.003% rose Bengal. Different carbon sources like lactose, sucrose and maltose were used instead of glucose. The plates were incubated for 7 days at room temperature. Solubilisation index was calculated as mentioned earlier.The effect of different nitrogen sources like $NaNO_3$, KNO_3 and urea on SI were also studied for selected PSB isolates.

3) Effect of pH and temperature on phosphate solubilisation: The isolates were also evaluated for phosphate solubilisation at varying pH (6, 7 ,8 and 9) and temperature (37°C, 45°C and 60°C) in media supplemented with 0.5% tri-calcium phosphate as sole phosphorus source. The efficiency of phosphate solubilisation was calculated on 3rd and 5th day by using Vando-Molybdate method.

III.RESULTS AND DISCUSSION

A. Isolation of PSB

A total of 64 isolates were screened for phosphate solubilisation of which 50 bacterial isolates and 8 fungal isolates were selected from site A and 14 isolates from site B and C for further studies. The isolates were named according to the sources from which they

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were isolated. Maximum number of microorganisms was observed in roots of mangroves. (Rodriguez and Fraga 1999). Although PVK agar with tri-calcium phosphate solubilisation causing halo formation (Sharma et al 2013) was used in this study for isolation of PSB, some reports suggest the inefficiency of agar method in isolation of PSB. The formation of halo around the PSB colony is attributed to decline in pH of the medium due to production of small molecular weight organic acids which are responsible for phosphate solubilisation (Ranjan et al. 2013). Nautiyal C. 1999, Chen et al. 2006 and Gulati et al. 2008 have standardised Vando-Molybdate, Fiske and Subbarow method and Mol-Blue method for screening phosphate solubilization as some isolates which do not produce halos may solubilise inorganic phosphate in liquid media (Rodriguez and Fraga. 1999; Nautiyal C. 1999).

B. Characterisation of PSB

All isolates were Gram negative in nature and non- endospore forming. Further characterization of the isolates showed all to be negative for urea, indole and catalase production. All isolates were negative for H_2S production except for S4RWB. Several strains of bacterial and fungal species have also been isolated by scientists (Thatoi et al 2012b). Dominant inorganic solubilizing species are *Pseudomonas* and *Bacillus* among bacteria and *Aspergillus* and *Penicillium* fungal species (Venketeshwaran and Natrajan. 1983; Sharma et al.2013). Based on biochemical results of the PSB isolated from this study we assume that they may belong to *Pseudomonas* and *Bacillus* species. Previous studies on isolation and screening of phosphate solubilizing bacteria from mangrove have also been described from Orissa coast (Gupta et al. 2007), Sundarban mangroves (Ramanathan et al. 2008) and Bhitarkanika (Thatoi et al 2012b)). Kothamasi et al. (2006) reported two strains of phosphate-solubilizing *Pseudomonas aeruginosa* (designated GM01 and GM02) in mangrove soils of Great Nicobar. Genera of phosphate solubilizing bacteria, like Pseudomonas, Bacillus, Corynebacterium, Vibrio, Micrococcus and Alcaligenes, were studied by Venkateswaran and Natarajan (1983) in mangrove biotopes in Porto Novo, Chennai water and sediment. Gayathri et al. (2010) have isolated PSB from leaf samples of mangrove plants of Pichavaram, Tamil Nadu. Adupidi et al (2012) has isolated two bacillus, fluorescent Pseudomonas and Azotobacter from Chollangi mangroves.

C. Phosphate Solubilisation efficiency

Out of the 64 bacterial isolates, 12 isolates showed development of sharp phosphate solubilisation zones (PSZ). Eight isolates (2 from Site A and 6 from site B & C) out of 12 showed PSZ within 24h of incubation, which were used for further studies. After 48h maximum PSZ was shown by isolates S1B0 followed by S1A06. Decrease in phosphate solubilisation efficiency was observed in all isolates except S1B0 and S1A06 on 5^{th} and 7^{th} day of incubation (Table 1). The decrease in phosphate solubilisation ability is due to increase in colony diameter which is not linearly proportional to increase in halo size due to phosphate solubilization.

Maximum phosphate solubilisation in broth assay was recorded in S4RWB followed by S1B0 on 5^{th} day of incubation. Only isolate WB showed same phosphate solubilisation from 3^{rd} to 5^{th} day of incubation (Fig 1). Increase in phosphate solubilisation was observed in all other isolates. While the non rhizopseric isolates showed maximum solubilisation efficiency in agar assay compared to broth assay, the rhizospheric samples showed maximum solubilisation efficiency in broth assay isolates. This may be attributed to plant metabolites regulating the different types of microbiota present in the two regions

D. Effect of growth and nutrition parameters on phosphate solubilisation

Reduction in the pH of the culture medium was exhibited by all the eight isolates. Isolates S2RWB and WB showed maximum decline in pH to 2.5 (Table 2). Release of small molecular weight organic acids is one of the mechanisms for inorganic phosphate solubilisation (Goldstein et al. 1999). Results from the present study showed that the isolates which showed maximum drop in pH also showed maximum cell density. Chen et al (2006) reported positive correlation between the phosphorus solubilisation and decrease in media pH. Studies by Alam et a*l (*2002) showed a decline in pH value from 7 to 3.2 due to organic acids production leading to tricalcium phosphate solubilisation. Brempong and Aferi (2014) have also suggested that some of the organic acids released by the isolates might have chelating abilities and thus complex with metal ions thereby releasing phosphate.

Among the different carbon source, isolate (S1A08) gave maximum phosphate solubilisation on maltose (Fig 2A) However not all isolates were able to utilise maltose efficiently. All isolates were able to solubilize tricalcium phosphate in presence of lactose (Fig 5). The results obtained, contradict with the findings of Sagarvanshi et al (2012), where lactose showed minimum phosphate solubilisation compared with glucose, maltose and galactose. Carbon source plays an important role as production of acid is dependent of the carbon source available. Possibly the cultures that this study has isolated may be new isolates that needs further characterization and identification.

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Isolates when grown with $NaNO_3$ and KNO_3 as nitrogen source were able to solubilize phosphorus. S4RWB showed maximum phosphate solubilisation efficiency using $NaNO_3$. Only S2RWB and S1A06 were able to utilise Urea (Fig 2B). On 7th day of growth in presence of urea S2RWB showed maximum phosphate solubilisation. Researchers have reported that a number of fungi and bacteria are able to solubilise phosphate only in presence of ammonia as a nitrogen source (Illmer and Schinner 1992). However, observations from present study clearly indicate that the PSB isolated from the ecological niche at Navi Mumbai are able to utilize $NaNO_3$, KNO_3 and urea as efficient nitrogen source.

Maximum phosphate solubilisation was observed at pH 7 followed by pH 6 (Fig 3A). Isolates S4RWB and S1A06 showed maximum phosphate solubilisation at pH 6. Retardation in phosphate solubilisation was observed at pH 9. Rodriguez and Fraga (1999) documented the production of organic acids like gluconic, lactic, isovaleric, acetic, oxalic and citric acid by phosphate solubilising bacteria. The results obtained are similar with work performed by Sagarvanshi et al (2012). Nahas 1996 reported solubilisation of inorganic phosphorus depend on inorganic phosphate used, microorganisms and decrease in pH.

At optimum pH, maximum phosphate solubilisation was observed at 45°C followed by 37°C (Fig 3B). Sayer and Gadd (1998) reported most of bacteria solubilise phosphate from 25°C to 28°C. Nautiyal (1999) and Nahas (1996) reported phosphate solubilisation at 45°C in dessert soil. In the present study as the samples were collected from the mangrove ecological niche which experiences a lot of temperature, salinity and seasonal changes, it is possible that the isolates have acquainted to drastic change and thus were able to solubilize phosphorus at temperature range between 37-45°C. Only two of the isolates, S4RWB and S1A06 were able to show phosphate solubilisation at 60°C but with a 50 % reduced efficiency indicating them to be thermophilic in nature.

IV. CONCLUSION

From the present study it may be speculated that of the eight isolate characterised from the Navi Mumbai mangrove soil, the isolate S4RWB gave the maximum phosphate solubilising activity by the broth assay (40%). S1B0 and S1A06 isolate which gave higher phosphate solubilising efficiency are also important candidate for further studies. The Phosphate solubilising bacteria from both rhizospheric and non-rhizospheric mangrove soil needs to be further explored with respect to effect of other abiotic stress and usefulness as biofertilizer for restoration and growth promotion of mangroves in the satellite city.

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Table 1. Phosphate Solubilisation Efficiency at Different Days Of Incubation for Isolates (Agar Plate Assay).

Isolates	Day 3	Day5	Day 7
S1B0	5	15	**48**
S3B20	**20**	**39**	**47**
S2RWB	11	15	15
S4RWB	15	20	23
WB	13	14	20
S2B0	**20**	20	40
S1A06	10	**88**	**93**
S1A08	17	20	27

Table 2: Effect of Cell Density on the pH of the medium.

Isolates	pH (Initial pH 7)		Cell density	
	24h	48h	24h	48h
S1B0	6.0	6.0	0.21	0.55
S3B20	6.8	5.5	0.19	0.51
S2RWB	5.5	**2.5**	0.48	**1.1**
S4RWB	6.5	6.0	0.22	0.58
WB	5.5	**2.5**	0.51	**0.99**
S2B0	5.5	3.0	0.20	0.40
S1A06	5.5	5.0	0.18	0.49
S1A08	6.0	3.0	0.21	0.89

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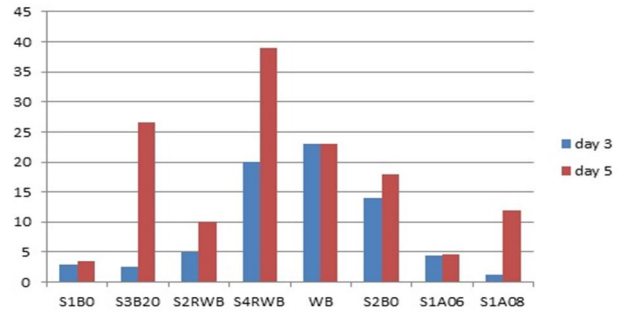
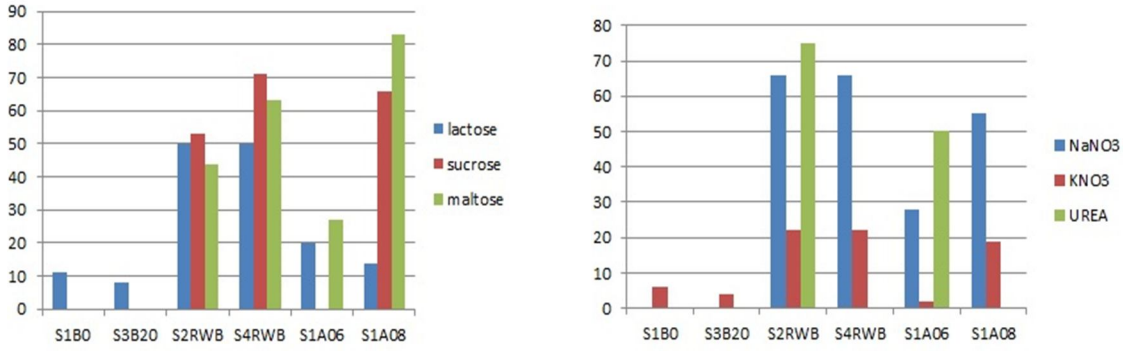
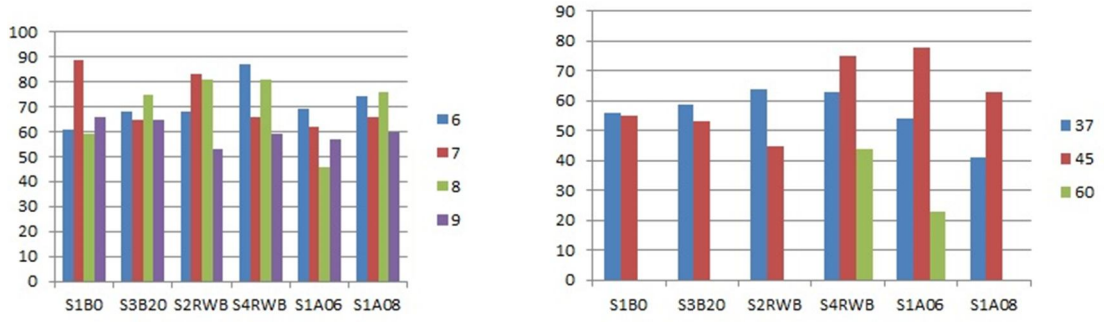


Figure 1: Phosphate Solubilisation (%) by the isolates in Broth assay evaluated on date 3 and day 5 of inoculation.



2A 2B

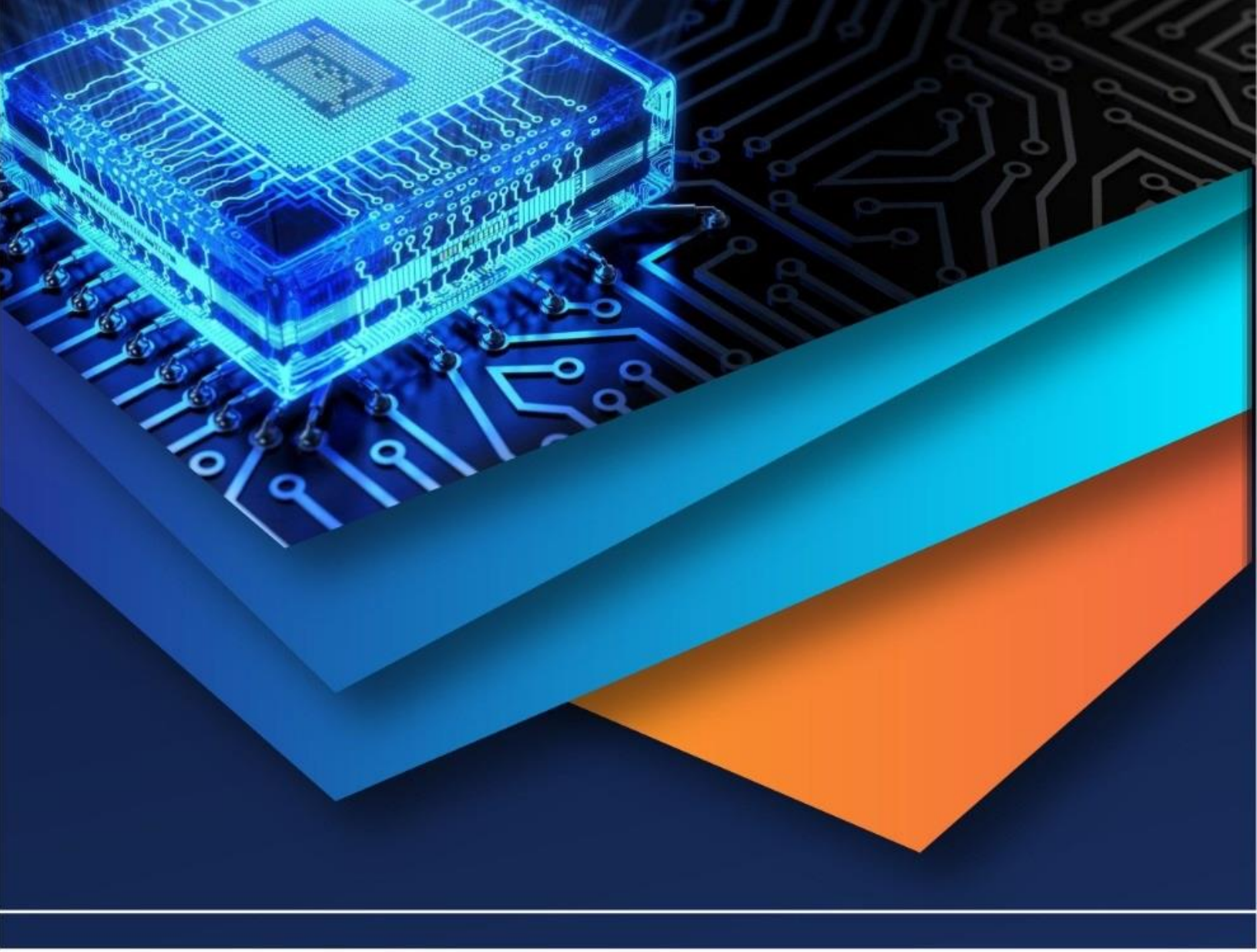
Figure 2: Effect of various carbon and nitrogen sources on phosphate solubilisation. 2A shows the effect of carbon sources viz Lactose, Sucrose and Maltose, while 2B indicates the effect on $NaNO_3$, KNO_3 and urea on solubilisation of phosphate.



3A 3B

Figure 3: Effect of pH and temperature on phosphate solubilisation efficiency of the eight isolates.3A shows the effect of Phosphate solubilization at pH values 6,7,8 and 9, while 3B indicates the effect of temperature of incubation at 37°C, 45°C and 60°C on solubilization effeiciency.

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