IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 4, Issue 6 (Nov. – Dec. 2018), PP 01-08
www.iosrjournals.org

Phosphate Solubilization Activity of Bacterial Strains Isolated from Gangetic Plains of North Bihar

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Abstract: *Phosphorous is considered as one of the vital macronutrients for the development and biological growth of plant. A survey report suggested that 98% of North Indian alluvial soil is deficient in phosphate because it not available in the form of minerals and even after secondary reaction it do not convert products into available form of soil nutrients unlike potassium (illite mineral). There are several phosphate solubilizing microorganisms (PSM) which have the capability to covert insoluble phosphate of soil into soluble forms by synthesizing organic acids. The present study was conducted to isolate bacteria from the alluvial soil of Gangetic plains of North Bihar and to evaluate their phosphate solubilization potential. A total of 18 microbial strains were isolated out of which 11 were bacteria and 7 were fungi. The two bacterial isolates S1(v)23 and S2(v)12 were selected for further study. The physiological, biochemical and molecular screening of purified isolates* ***S1(v)23*** *and* ***S2(V)12*** *identified as two distinct strains of Gram +ve Bacillus bacteria* ***Aneurinibacillus migulanus****. For phosphate solubilizing efficiency both the isolates and non-inoculated were cultured on PVK agar medium containing tri- calcium phosphate (TCP) for 15 days(360 hours).The maximum solubilization index was observed 4.19 in S1(v)23 and 3.94 in S2(v)12 PVK agar media. On the basis of halo zone formation, the qualitative and quantitative confirmatory analysis was done on Pikovskaya's-Bromo Phenol Blue agar (PVK-BPB) and PVK-BPB broth medium again for 15 days. The decrease in pH value ranged from 3.87 and 4.12 by S1(v)23 and by S2(v)12 from initial pH of 7.0 whereas control maintained its pH 7.0. The colour intensity (at O.D. $_{600}$) change of media taken as an indicator of phosphate solubilizing efficiency of bacteria. The O.D. of both the isolates S1(v)23 , S2(v)12 were 0.632 and 1.091 whereas the O.D. of control was 1.282.Phosphate solubilizing ability test of these two isolates also showed that they can solubilize TCP from 54.01 to 139.12 mg/l and 52.03 to 118.79 mg/l.*

Keywords: *North Bihar, Gangetic plains, phosphate solubilizing bacteria, Pikovskaya's Agar, Bromophenol Blue, TCP*

Date of Submission: 12-11-2018 Date of acceptance: 27-11-2018

I. Introduction

Long after the ball of fire started cooling down, our oldest ancestors- the microbes did a yeomen's job by playing the role of a catalyst and a facilitator in addressing the insalubrious conditions on planet earth which slowly but steadily became congenial for supporting life. While microbes like bacteria took upon the role of transforming the noxious gases in the earth's atmosphere into something more hospitable by accounting for almost half of the oxygen content besides, regulating the presence of nitrogen, methane and carbon dioxide, others of their ilk made the top of the terra-firma, soil their home and a center of ecological activities on the

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other. These activities have had a direct bearing on earth's food web, plant productivity and agriculture by promoting and unleashing a plethora of related biological activities. Because of their ubiquitous distribution microbial populations have a direct and major influence on agriculture. Important plant polymers as cellulose and lignin are degraded exclusively by soil microbes due to diverse enzymatic activities. A controlled balance of microbial activities is important for success in agriculture. Of late, the microbes, instrumental in the production of a seemingly diverse and growing variety of products - ranging from cheese, enzymes and biofertilizers, as a mark of their ever-growing footprints in the food and pharmaceutical industries- antibiotics, nanotechnology, vaccines, vitamins etc. along with agriculture sector, to name a few, have become indispensable to the development and sustenance of mankind on the globe **[Bashan 1998; Kloepper JW, 2004; Ryan RM et al. 2006; Rosenberg 2008; Tiwari M. et.al.2018]**.

With the human population rising exponentially in recent times, the global managers find themselves in an unenviable position to address the rising demand for food limited by an ever-decreasing acreage under agriculture around the globe. The domain experts have alluded to a paradigm shift from archaic to better land use practices, tillage, fertilizers, harvesting and storage facilities in making agriculture, an economically efficient venture. The use of microbial inoculants in the form of bio-fertilizers as against their traditional chemical but costly counterpart in various countries is suddenly in the limelight for the right reasons, both economic and environmental.

Nitrogen, phosphorous and potassium are the major plant nutrients obtained from soil are made biologically available to plants. Phosphorous is the second to nitrogen which is required by both plants and microorganisms **[Subba Rao, N.S, 1982].**Inorganically, it is present in the soil from phosphate rocks and the application of fertilizers whereas it is found organically in the form of residues of man, animals (bones of man and animals as calcium phosphate and as nucleic acid and nucleoproteins in the cytoplasm of cells) , plants after decomposing and in soluble form of water. After the cultivation, these minerals are present abundance in the soil -about 1100kg/ha but only 1% of total phosphorous is available to plants. Many soil bacteria (decomposers) play a potential role by breaking down phosphorous compounds and finally utilizes in cell synthesis. The mechanism involves the release of low molecular weight organic acids like citric, glutamic, succinic, lactic oxalic, maleic, fumaric, tartaric and α- ketoglutaric acids **[Goldstein, A.H;1995]** further through their hydroxyl and carboxyl group, chelates the cation bound to convert into soluble form of phosphate **[Kpomblekou, K. et.al;1994]**. The phosphate solubilization activity of bacteria and uptake of macronutrient by plants are generally observed, high in the oxygenated soil **[Meena R. K. et. al., 2015].**The role of phosphate solubilizing bacteria has been also well established in nutrient discrimination environments **[Goldstein, A.H;1995]**. Phosphate-solubilizing bacteria (PSB) mobilize insoluble inorganic phosphates from their surrounding soil mineral matrix to the bulk soil where they can be absorbed by plant roots for their growth and development **[Subba Rao, N.S, 1982]**. The phosphate solubilization bacteria are the key players in the rhizo-spheric zone and dominate the Plant Growth Promoting Rhizobacteria group. From last one-decade phosphate solubilization microorganisms have attracted the attention of agriculturalists of developing countries due to high price of synthetic fertilizer and environmental sustainability of soil, **[Glick B.R,1995]**. Now-a-days, phosphate solubilizing bacteria like Bacillus sp., Polymyxa and Azotobactor are getting mixed with organic compost to improve the quality of the manure and to achieve better yield and healthy growth of plants. Strains from these genera can be luxuriously cultured in different media, easily transportable from laboratory to field and can be utilized in phosphate deficient fields(as fertilizer) in more environment-friendly and sustainable manner.

The 255 million hectares fertile plain in the northern and eastern part of India known as the Indo-Gangetic plain, nestles Bihar, a geographical entity of 94.2 thousand square km in its bosom through which flows the eponymous river Ganges dividing the landmass into two separate geographical entities known as North Bihar and South Bihar with matching areas of 53.3 thousand km and 40.9 thousand km respectively. Similarly, three distinct Agro-climatic zones, namely Zone I (North West Alluvial Plain), Zone II(North East Alluvial Plain) and Zone III (South Bihar Alluvial Plain) each characterized by their own resources, potentials, challenges and prospects.

The biodiversity found in Indo-Gangetic heartland are very large. Overall Gangetic regions are highly productive. It is ironical that the Bihar located in this region has not been able to leverage its rich fertile soil and perennial rivers to become a major agricultural force in the country. Apart from socio-political reasons, there has been a major disconnect between the fields and the laboratory which is manifested by archaic tilling methods and indiscriminate use of chemical fertilizers. As a result, the productivity of the fields has begun to plateau, and the poisonous chemicals have found their way into the human diet, cattle feed and the environment damaging them irrevocably. With the burgeoning population becoming a nightmare and the natural resources dwindling at a faster pace as compared to the generation of new opportunities, sustainable development seems to be the answer to stem the rot and agriculture as a sector is no exception. Agriculture is the single largest private sector occupation in Bihar and can be considered the riskiest business.

With the winds of change blowing across the state and the Government of the day finally waking up to the ever increasing demand for food grains especially after its decision to implement the FSA from the current fiscal, the agricultural road map opens new vistas in the field of agriculture and it is believed that this sector shall witness a phenomenal growth with the use of improved seeds, new and eco-friendly technologies, system of organic farming, user friendly inputs, regular and timely Lab to Field transfers and development of requisite infrastructure.

An appreciable work in soil microbiology and biodiversity has been done in Indo-Gangetic regions by researchers **[Kumar et al. 2002; Singh A. et al. 2015]**. A very little work has been done on potassium solubilizing microbial diversity and fertility of alluvial soil of Indo-Gangetic plains **[Maurya B.R et. al.; 2016]**.

II. Materials and Methods

2.1. Soil Sample Collection and Isolation of Phosphate Solubilizing Bacteria

The soil samples were collected from the five different zones of Gangetic Plains of North Bihar adhering to roots and allied areas mainly from banana*(Musa paradisiac)* field, dry crop land of mustard (*Brassica campestris*) and pea *(Pisum sativum*), wetland of rice *(Oryza sativa*) , and stagnant pond areas Latitude 25°37'55.29"N - 25°31'19.70"N, Longitude 85°13'7.67"E - 85°21'12.59"E (Near Gandhi Setu, Hazipur, Raghopur Diara and Vaishali Distt.). For the survival of microbes, proper protocol was followed while sampling the soil, transported to the research laboratory of the Environment and Water Management Department, A.N.College, Patna and stored at 4 °C ± 1 °C. Soils were analyzed for pH and organic carbon. A total 18 microbial strains were isolated from the soil sample out of which 11 were bacteria and 7 were fungi. The purified strains were kept in the sterilized environment of laboratory for further studies.

Fig: 1 Topographical Area of North BiharFig:2 GIS mapping of exploitation of PGPR from Indo- Gangetic plains of North Bihar

(Source: bih.org)

Identification of the Isolates

For the isolation of single bacterial colony, 100 mg. of each soil sample was dissolved in 10 ml of sterilized MiliQ saline water (0.85% NaCl w/v) and after several serial dilutions; sub- soil suspensions were prepared. The bacterial isolates were identified by certain physiological and biochemical screening method like colony morphology, shape, size, spore, motility, Gram's reaction ,growth at different pH and temperature following the standard methods described in Bergey's Manual of Determinative Bacteria and IMTECH catalogue**[Buchanan R.E. and Gibbons, N.E, 1974]** .The biochemical analysis was done on TM KB001 Enterobacteriaceae species Kit and TM KB 013 Hi Bacillus Identification Kit (both from Hi-media Laboratories Pvt. Ltd., Mumbai),following the manufacturer's instructions. The parameters investigated catalase production, urease production, Voges-Proskauer (V-P) reaction, Indole production, Nitrate reduction, citrate utilization, carbohydrate metabolism (acid-gas production), starch hydrolysis, Tributyrin (or vegetable oil) hydrolysis, Tween-80 hydrolysis, Cholesterol hydrolysis, gelatin hydrolysis, Casein hydrolysis. Based on prolific growth on Nutrient Agar isolates were selected and evaluated for their potential phosphate solubilizer on Pikovskaya's medium **[Pikovskaya R.E., 1948**].

Analysis of Phosphate Solubilizing Efficiency

Qualitative Estimation of Phosphate Solubilization-

For phosphate solubilization efficiency(TCP-tri-calcium phosphate)one loopful of pure culture of both the isolate suspensions S1(v)23 and S2 (v)12 were dispensed onto sterilized PVK agar medium petri plate separately , allowed to solidify and incubated at a temperature 28˚C±2 ˚C for a maximum period of 15 days. Based on the appearance of clear halo zone colonies, strains were visually assayed. The SI(solubilization index

)was determined by the calculation of halo (clear zone) diameter and the colony diameter**[Premono Edi M.et al.,1996]**.

The confirmatory analysis of phosphate solubilization efficiency was done by spreading bacterial strains on sterilized PVK-BPB(Pikovskaya's-Bromophenol Blue) agar medium with pH 7.0 and incubated again at 28˚C±2 ˚C for a period of 15days. The non inoculated PVK-BPB medium was taken as control.

Quantitative Estimation of Phosphate Solubilization-

After qualitative observations, the purified isolates were estimated quantitatively **[Murphy J. et. al; 1962]**.Instead of PVK broth medium, the bacterial isolates S1(v)23 , S2(v)12were cultured on the combined PVK-BPB (Pikovskaya's-Bromophenol Blue) broth medium. The work followed the earlier defined procedure of **Nautiyal and Mehta; 2001**, in which combination of NBRIP-BPB broth was utilized for phosphate solubilizing efficiency by bacterial strains which were isolated from alkaline soil of Lucknow, India. The changes occurred during the process, decrease in pH of the medium and in the intensity of the colour of bromophenol blue, were taken as an indicator (the production of acid by bacterial activity in the medium) and was found more accurate correlation in the comparative study of phosphate solubilization instead of simple halo zone method. For the purpose, both the bacterial strains S1(v)23 and S2(v)12 were inoculated in Erlenmeyer flasks containing 100 ml of PVK-BPB broth medium and the non-inoculated medium was served as control. The flasks were incubated at 30^0C for 15 days at 100 rpm. To measure the bacterial growth, the pH of the medium as well as the estimation of released soluble phosphorus, aliquots of cultures were aseptically harvested, filtered through Whatman filter paper No. 2 centrifuged at 8000 rpm for 15 minutes at specific point (time interval of incubation).The assays were cultivated in the triplicates. The intensity of blue colour was measured on Thermo Scientific ™ GENESYS™10S UV-Vis Spectro-photometer. The final OD_{600} values of cultured isolates were compared with the non inoculated control value. The amount of Phosphate released by the both the isolates were compared with the standard curve of P (specific amount of Potassium di-hydrogen phosphate was used for the standardization).

Statistical Analysis

Statistical analysis was performed by JMP 14.0.1 Statistical Discovery from SAS (Microsoft Professional).

III. Result

In case of phosphate solubilization bacteria, most of the researchers have reported that the increase in the size of halo zone formed concurrently as with the formation of colony diameter .But in case of our study, the fluctuation in the size of halo zone were observed during the entire incubation period.

The solubilization of phosphate on PVK agar media by bacterial strains (cultured in triplicates) was accomplished in three-phases, in its initial phase of growth (till 48 hours) the bacterial strains gradually accumulated TCP whereas in the second phase (after 48 hours) the optimal assimilation of TCP was done by the isolates and about 65% of the halo zones were observed for next 12 days, finally a subsequent slow expansion in the halo zone were observed rest of the period.

Both the bacterial isolates were found capable of solubilization of TCP on solid PVK agar medium (Fig: 3). But the strain S1(v)23 with solubilization index of 4.12 (Table : 1) was found more efficient then the strain S2(v)12 with solubilization index of 3.82 (Table: 2) after 15 days of incubation. In the qualitative confirmatory analysis, the strains, S1(v)23 and S2(v)12also produces yellow halos by de-colorization of blue colour PVK-BPB agar media. Visually, the de-colorization of PVK-BPB media and formation of yellow halos by bacterial isolate S1(v)23 was larger than S2(v)12 It clearly defines the pH drop in the medium due to the release of organic acids by the activity of phosphate solubilizing bacteria. In quantitative estimation ,both the strains showed positive phosphate solubilizing efficiency in PVK-BPB broth media incubated for 15 days (Fig : 4 and Fig : 5). The decreased colour intensity of Bromophenol Blue (at O.D.=600 nm) medium was in the range of 0.632 to 1.091 by both the isolates S1(v)23 , S2(v)12 whereas the O.D. of Control was 1.282(the final OD was calculated =OD of control - OD of culture media). The soluble P concentration in the medium ranged from 54.01 mg/l to 128.10 mg/l by S1(v)23 as compare to 52.03mg/l to 110.32 mg/l by S2(v)12. After 15 days of inoculation the p solubilization by isolates were gradually slowed down. From the point of p assimilation by the isolates, the S2(v)12 signified a weak strain as compare to isolate S1(v)23.

It was observed that over a period of time the colour of the media changed from blue to purple and there after finally to yellow indicating that the media is becoming acidic from alkaline (Fig : 6 and Fig :7). Both the isolates instituted the drop in pH of PVK-BPB broth media ranged from pH 7.0 to 3.87 by S1(v)23 and 4.12 by S2(v)12 after 15 days of incubation(Fig: 8). The un-inoculated medium detected no soluble P and no drop in pH as well. The lowest pH was observed during maximum solubilization activity of Phosphate by both the strains.

Fig: 3 Bacterial Isolate Growth on PVK-Agar Medium

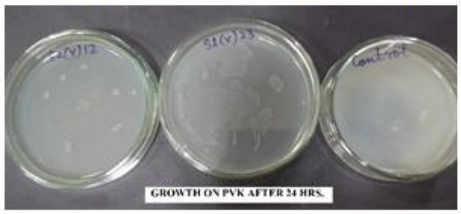


Fig : 4 Change in the Colour of PVK-BPB agar media culture after 48 hrs. of activity of Phosphate Solubilizing Bacteria

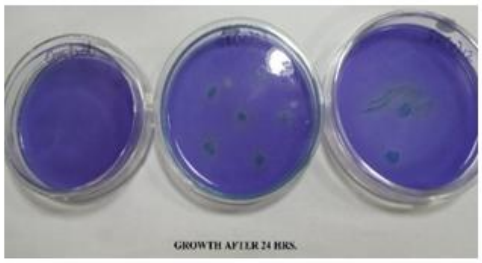


Fig: 5 Change in the Colour of PVK-BPB agar media culture after 216 hrs. of activity of Phosphate Solubilizing Bacteria

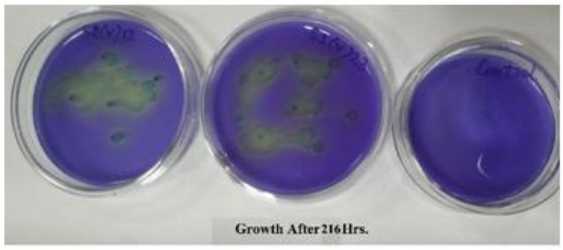


Fig : 6 Change in the Colour of PVK-BPB broth media culture after 48 hrs. of activity of Phosphate Solubilizing Bacteria

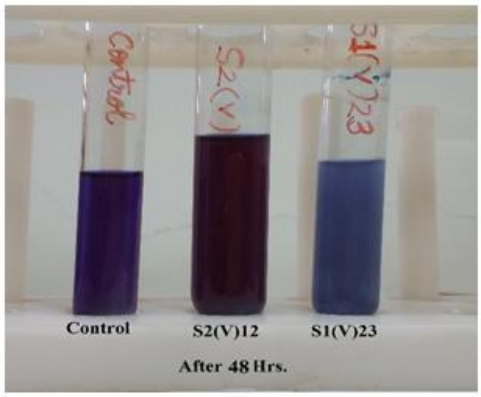
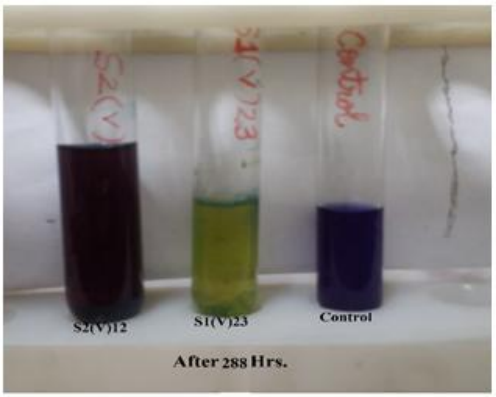


Fig: 7 Change in the Colour of PVK-BPB broth media culture after 288 hrs. of activity of Phosphate Solubilizing Bacteria



S1(v)23-

Incubation Period(Days)	Solubilization Index(SI)*	Soluble Phosphate(mg/l)	Reduction in pH
1	3.02	54.01 ± 0.27	7.00 ±.21
3	3.52	68.21 ± 1.24	5.75 ±.27
6	3.68	77.82 ± 2.59	5.12 ±.12
9	4.05	110.23 ± 2.80	4.01 ±.23
12	4.19	139.12 ± 1.33	3.95 ±.12
15	4.08	128.10 ± .96	3.87 ±.15

Table 1. Solubilization of Phosphate by S1(v)23 bacterial isolate and pH reduction

{ *SI (solubilizing suspended on Tricalcium Phosphate) =Colony diameter+ Halo zone diameter /Colony diameter}

S2(v)12-

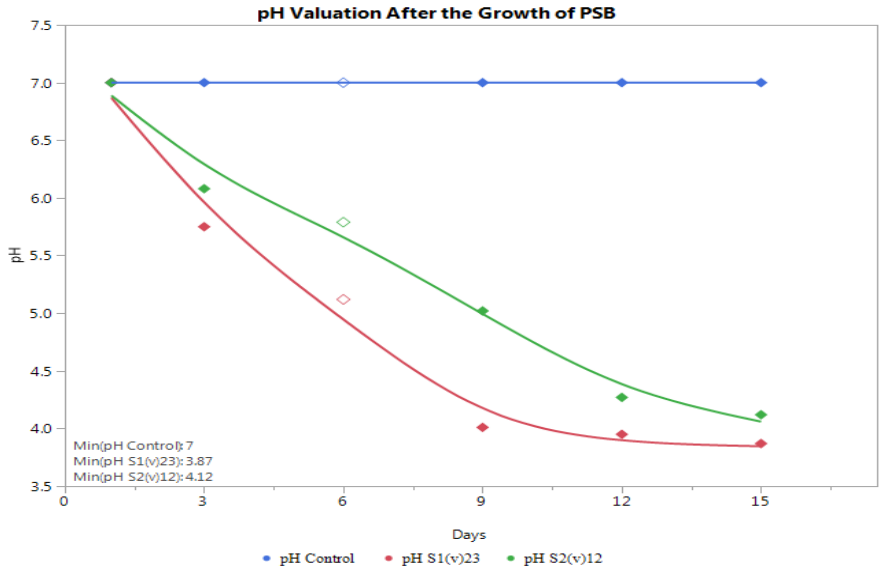
Incubation Period(Days)	Solubilization Index(SI)*	Soluble Phosphate(mg/l)	Reduction in pH
1	2.70	52.03 ± 0.18	7.00 ± .18
3	3.14	64.74 ± 1.83	6.08 ±.21
6	3.43	71.46 ± 2.01	5.79 ±.20
9	3.61	97.84 ± 2.37	5.02 ±.18
12	3.94	118.79 ± 1.42	4.27 ±.15

15	3.53	110.32 ± 1.35	4.12 ±.15

Table 2. Solubilization of Phosphate by S2(v)12 bacterial isolate and pH reduction

{ *SI (solubilizing suspended on Tricalcium Phosphate) =Colony diameter+ Halo zone diameter /Colony diameter}

Fig: 8 Valuation of pH Level after phosphate insolubilizing activity of isolates S1(v)23 and S2(v)12 on PVK-BPB Nutrient Broth Media



IV. Discussion

Recently, there had been a surge in the use of improved HYVs (high yielding varieties) along with high dosage of chemical fertilizers in agricultural production which spiked the quantity of insoluble phosphates in soil because of chemical fixation. It was only after some sustained and relentless research effort(s) of **Gerrestson (1948)** that microorganisms capable of converting insoluble phosphate in the soil into their soluble counterparts were identified which led to a paradigm shift to sustainable agriculture.

A subsequent spell of ongoing research helped isolate microbes known for their prowess as efficient biofertilizers which opened new vistas for attractive and environmental friendly alternatives in agriculture. Several bacterial species, like Pseudomonas, Azospirillum, Azotobacter, Enterobacter, Bacillus, Rhizobium, proficient in solubilizing phosphate in soil have been identified henceforth by **Kumar et al, 2012; Kumar A. et al, 2016.**

From our study, out of a total of 11 isolates from the soils of the Gangetic plains, two were selected for studies related to ability to grow and mineralize insoluble Calcium tri-phosphate on Pikovaskaya's Agar medium after IAA test and HCN production. The role of PSBs in solubilization of phosphates and ensuring its availability for plants is well established **[Vikram et al.,2007]** and is subject to production of organic acids and presence of phosphate enzymes in microbes **[Park et al, 2010]**. There are several bacterial isolates which did not produce any visible halo zone on agar plate though they were found more capable of solubilizing insoluble inorganic phosphate in liquid medium **[Louw, H.A. et. al; 1959]**.

The variation in the TCP accumulation by both the strains can be defined as kinetic changes in the growth of bacterial strains during the formation of halo zone **[Gyaneshwar P. 1999]**. The variation in diffusion rate of organic acids and cellular respiration at different stages of growth by isolates may be the outcome of specific enzyme or coenzyme accountable for triggering the catabolism of chemicals of PVK-TCP agar media. The specific coenzyme ATPase - adenosine tri-phosphatase is primarily responsible for catalyzing ATP in ADP and free phosphate ion after dephosphorylation. The broken-down minerals are thus utilized in transportation and consumption of chemical energy in the cell. Similar results were reported by **Trivedi P.et.al.; 2008** in Pseudomonas corrugate during phosphate solubilizing activity whereas **Rodriguez et. al., 2000** have testified that the production of soluble phosphate witnesses a peak during incubation only to be offset by its consumption by the cascading bacterial population and the plants could absorb tri-calcium phosphate (Ca3PO4) of media in the ion ($Pi,HPO4^{2-}$, H^2PO^{4-}) form.

Though both the strains were from same sp. Bacillus sp. assimilation of P was less by S2(v)12 as compare to S1(v)23.The result can be supported by the findings of many researchers previously worked on the assimilation of p soluble bacillus sp. like **Park J. H. et.al 2011**; who have analyzed P solubilization 217–479 mg/L by Bacillus sp. isolated from lead amended soil. Two strains of Bacillus sp. isolated from Trans

Himalayan region of Himachal Pradesh, the rate of maximum phosphate solubilization may be different and less active in TCP **[Chatli A. S. et. al.; 2008].**

The decreasing pH was primarily due to the production of acids (organic acid) and it has also caused the solubilization of Phosphate by both the strains from the PVK-BPB broth media **[Gerke J. et. al. 1992** and **Hoberg, E. et. al. 2005]**. Our findings on Phosphate solubilization reaffirmed that bacterial isolates are involved with the production of organic acids either it is gram positive or gram negative bacteria **[Goldstein A.H. in 1995]**. By observing the inverse relationship between the pH Value and soluble-Phosphate media it can be inferred that the production of organic acid using such bacterial strains is an important aspect in the acidification of the medium causing the solubilization of Phosphate. There had been other cases as reported by Illmer, P. in 1995, where it was established that there is a direct inverse relationship between pH and soluble phosphate. The findings on the drop of pH level from initial value of 7.0 to 3 to 4 times down may be due to the acidification of supernatant's culture **[Perez E. et al. 2007]** and the synchronization of phosphate solubilization with the acidification of broth can be recognized **[Collavino M. M. et al , 2010].**

A spectro-photometry measurement (quantitative) study of the ability of each PSB to dissolve phosphates for a fortnight reveals that the ability of the isolates to produce organic acids and the presence of an effective phosphate enzyme is a function of the pH of the liquid media. After 48 to 72 hours high acidic pH, inhibition of the bacterial growth which was detrimental to the solubilization efficiency of the isolates was observed **[Perez et al., 2007 and Abd-Alla M.H., 1994]**. The performance of the bacterial strain, S1(v)23 stood out for its ability to solubilize the phosphates as compared to S2(v)12.

V. Conclusion

The socio-environmental challenges has fuelled the search for PSBs, albeit, with potential to be exploited, for crop production besides, shedding light on the properties of soil and opening new avenues for diversifed group of microbes.The new Agricultural road-map of Government of Bihar is expected to leverage an initiative from such studies which reveal that Phosphate Solubilizing Bacteria(s) (PSBs) from the Gangetic soil of North Bihar can be isolated and exploited as Biofertilizers for the improvement of crop production. The research work could also be helpful to taxonomists, agriculturalists and even industries for the production of biofertilzer, in the state without adversely affecting the fertility of soil. Further research work is required to explore the performance of these competent PSB isolates either alone or in combination with other biofertilizers such as potassium-solubilizing and nitrogen-fixing bacteria to increase the yield and growth of crops under different field condition.

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IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) is UGC approved Journal with Sl. No. 4033, Journal no. 44202.

Mini Tiwari. " Phosphate Solubilization Activity of Bacterial Strains Isolated from Gangetic Plains of North Bihar." IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) 4.6 (2018): 01-08.