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In vitro Phosphate Solubilization by *Enterobacter* spp. Isolated from Wheat Rhizosphere

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Phosphorus is one of the very important minerals required for the proper plant growth. The availability of phosphorous to plants for uptake and utilization is impaired in alkaline and calcareous soil due to the formation of poorly soluble calcium phosphate minerals. Adding fertilizer phosphorous at normal rates and with conventional methods does not result in optimal yield and crop quality in these soils. The use of phosphate solubilizing bacteria can prove to be effective measure to provide phosphorous to the wheat plants to increase the productivity. In the present investigation, a total of 15 isolates were obtained from wheat rhizosphere soil samples. The isolates were subjected to primary and secondary screening and $IKas_4$ and $IH3_7$ which showed highest phosphate solubilization during secondary screening were selected for subsequent studies. The condition for *in vitro* phosphate solubilization by the selected isolates was optimized. The isolate $IH3_7$ and the isolate $IKas_4$ showed maximum phosphate solubilization of 0.070 μg and 0.99 μg P/ml respectively. The bacterial isolates were gram negative, non-spore forming rods. On the basis of the 16SrDNA sequencing isolates $IKas_4$ and $IH3_7$ were identified as *Enterobacter aerogenes* and *Enterobacter* sp. respectively.

Keywords: Enterobacter, Phosphate solubilizing bacteria, phosphate solubilization, wheat.

Phosphorus is one of 17 nutrients essential for plant growth. Phosphorus is classified as a major nutrient that is frequently required by crops in relatively large amounts. The total P concentration in agricultural crops generally varies from 0.1 to 0.5 percent[1]. In various plant energy reactions P plays a vital role in virtually every plant process that involves energy transfer[2]. P is a vital component of the substances that are building blocks of genes and chromosomes. Large quantities of P are found in seeds and fruit where it is believed essential for seed formation and development. P is also a component of phytin, a major storage form of P in seeds. About 50 percent of the total P in legume seeds and 60 to 70 percent in cereal grains is stored as phytin[3]. Movement of nutrients within the plant depends largely upon transport through cell membranes, which requires energy in the form of ATP and other P compounds to oppose the forces of osmosis[4]. P is taken up mostly as the primary orthophosphate ion ($H_2PO_4^-$), but some is also absorbed as secondary orthophosphate ($HPO_4^=$). P may be stored in the root or transported to the upper portions of the plant[5].

Plants acquire phosphorous from soil solution as phosphate anions which are extremely reactive and get immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of the soil. Several soil microorganisms known as phosphate solubilizing bacteria (PSB) have the ability to solubilizing insoluble mineral phosphate by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO_2 and H_2S.This results in acidification of the surrounding

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soil, releasing soluble orthophosphate ions ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) which can be readily taken up by plants[6].

Almost 75 to 90% of added P fertilizer in agricultural soils is precipitated by iron, aluminum and calcium complexes present in soils. Furthermore, phosphatic fertilizers are expensive, and excessive use of rock phosphate (RP) is potentially and environmentally undesirable[7].

Wheat takes up P throughout the growing season. Total P uptake by wheat is about 0.5 to 0.6 lb P_2O_5/bu (https://www.cropquest.com/2013/09/01/importance-phosphorus-fertilizer-wheat/). Deficiency of P leads to various side effects in the plant growth like reduction in leaf expansion and leaf surface area, as well as the number of leaves. It also leads to a decrease in the shoot root dry weight ratio. The processes of carbohydrate utilization become slow, while carbohydrate production through photosynthesis continues. This results in a buildup of carbohydrates and the development of a dark green leaf color. Since P is readily mobilized in the plant, when a deficiency occurs the P is translocated from older tissues to active meristematic tissues, resulting in foliar deficiency symptoms appearing on the older (lower) portion of the plant. Other effects of P deficiency on plant growth include delayed maturity, reduced quality of forage, fruit, vegetable, and grain crops, and decreased disease resistance[8].

Alkaline soil is defined as soil with pH greater than neutral, typically 7.5 to 8.5 and calcareous soil is defined as having the presence of significant quantities of free excess lime (calcium or magnesium carbonate). Lime dissolves in neutral to acid pH soil, but does not readily dissolve in alkaline soil and, instead, serves as a sink for surface adsorbed calcium phosphate precipitation[9]. At low pH fixed forms of the P precipitate with calcium ions and at low pH they precipitate with iron and aluminum.

The phosphate solubilizing microbes can be a potential solution to the P deficiency problem. These microbes convert the insoluble and unavailable phosphates into soluble and available form by production of acids, exchange reactions, acidification, chelation etc.[10] A wide range of phosphate solubilizing bacteria (PSBs) have been isolated range of soil samples like from soil of mangroves [11], saline –alkaline soil[12], groundnut rhizosphere soil[13], black pepper rhizosphere soil [14], saltern[15], rhizospheres of vegetables like pea, spinach, etc. [16]

The effect of phosphate solubilizing bacteria on various plants has been assessed by various researchers. In most of the cases the growth has been enhanced by the PSBs Ramachandran *et al.* [14] found that the inoculation of PSB alone and PSB + rock phosphate has increased the shoot growth and dry matter production of the black pepper. The control plants on both the potting media have recorded the lowest shoot length. Similarly growth promotion has also been reported in case of pea plants inoculated with *Pseudomonas* isolate [17].

MATERIALS AND METHODS

Collection of soil samples

Soil samples from wheat rhizosphere were collected from the different areas of Bilaspur district of Himachal Pradesh (India) *viz*. Harnora, Kasol, Dhar, Bharathu and Kandrour.

Isolation of bacteria

Phosphate solubilizing bacterial (PSB) isolates were obtained by dilution plating the soil samples on Pikovskaya's [18] (PKV) agar medium plates (The composition is as follows (g/l): Glucose, 10 g; tricalcium phosphate (TCP), 5 g; ammonium sulphate, 0.5 g; sodium chloride, 0.2 g; potassium chloride, 0.2 g; magnesium sulphate, 0.1 g; yeast extract, 0.5 g; manganese sulphate, trace; ferrous sulphate, trace; agar, 15 g; the pH was adjusted to 7.0 ± 0.2). Pure culture of the isolates were made by repeated sub culturing on fresh PKV plates and maintained on PKV slants at refrigerated condition.

Screening of isolates for phosphate solubilization

Primary screening

All the suspected colonies were screened for phosphate solubilization on PKV medium. Isolates showing phosphate solubilizing ability were inoculated at the PKV plate and incubated at 37^0C. Diameter of clearance zone was measured. The phosphate solubilization efficiency (PSE) was calculated as PSE (%) = [Z–C/C] × 100; where, Z = Halo zone diameter, C = Colony diameter[6].

Secondary screening

25ml Pikovskaya's broth medium with Tricalcium phosphate was prepared and sterilized,

inoculated with the specific amount of the isolate. Then the inoculated sample was incubated for 48 hours on rotatory shaker at 37^{0}C, after incubation culture broth was centrifuged at 10,000 rpm for 5min and colour development done by using the John's method [19] and optical density calculated at 882nm and readings compared with the standard curve to calculate PSE for each isolate.

Characterization of bacterial isolates

Characterization of phosphate solubilizing bacterial strains was performed by morphogical characteristics and biochemical analysis. The microscopic identification was carried out by gram's staining and endospore staining using oil immersion microscope. Morphological and biochemical tests Bergey's Manual of Systemic Bacteriology[20]. For the bacterial isolates showing maximum phosphate solubilization activity, 16SrDNA sequencing was done by Samved Biotech, Ahmedabad (India).

RESULTS AND DISCUSSIONS

Isolation and screening of phosphate solubilizing bacteria

Wheat rhizosphere soil samples were collected from five different areas of Bilaspur district of Himachal Pradesh that are Dhar,Bharathu,Kandrour,Harnora and Kasol and a total of 15 morphologically distinct colonies were isolated (Table 1).

Till date many works have been done by various researchers, like Vaquez *et al*. [11] isolated PSBs from mangrove soil and reported that out of all isolates *V. proteolyticus* was found to be most active. Ramachandran *et al.*[14] isolated PSBs from rhizospheres of black pepper and *Pseudomonas* species showed maximum Phosphate solubilization efficiency. Also, Hameeda *et al.* [21] isolated bacterial strains from samples taken from compost and macrofauna.

Malboobi *et al*. [22] isolated *Pantoea agglomerans, Microbacterium laevaniformans* and *Pseudomonas putida* from potato rhizospheres. Similarly, Kannapiran *et al.* [23] isolated *Pseudomonas, Bacillus, Vibrio, Micrococcus, Flavobacterium, Corynebacterium, Alcaligenes* and *Enterobacter* from samples collected from different stations of the Thondi coast. Reena *et al.*[24] isolated PSBs from rhizospheres of banana plant and identified *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Micrococcus sp*. as major phosphate solubilisers. In the present investigation, the phosphate solubilizing efficiency (Qualitative) of isolates was determined by the plate assay where 15 isolates were spotted on Pikovskaya's agar plate as per the

Table 1. Colony characteristics of phosphate solubilizing bacterial isolates

Isolate	Sample (Distt Bilaspur, H.P.)	Shape	Edge	Opacity	Color	Elevation	Texture
$IH3_2$	Harnora	Irregular	Undulate	Opaque	White	Flat	Rough
$IKas_4$	Kasol	Irregular	Undulate	Opaque	White	Raised	Viscid.
$IH3_1$	Harnora	Irregular	Rhizoid	Opaque	White	Flat	Mucoid
$IH3_5$	Harnora	Irregular	Undulate	Opaque	White	Raised	Rough & Friable
$1H3_4$	Harnora	Irregular	Undulate	Opaque	White	Raised	Rough & Friable
$ID5_4$	Dhar	Irregular	Undulate	Opaque	White	Raised	Butyrous
$IH5_1$	Harnora	Round.	Entire	Translucent	Reddish	Raised	Smooth,Viscid
$IH3_7$	Harnora	Round	Entire	Transparent	White	Flat	Glistening and sticky
$IH3_6$	Harnora	Irregular	Lobate	Opaque	White	Flat,	Dry
$IKan4_4$	Kandrour	Irregular	Lobate	Transparnt	Yellow	Raised	Sticky
$IH4_8$	Harnora	Irregular	Undulate	Translucent	White	Flat	Butyrous
$IKan4_5$	Kandrour	Rhizoid	Filamentous	Translucent	White	Flat	Dry,Friable
$IKas6_2$	Kasol	Irregular	Lobate	Transparent	White	Flat	Sticky
$ID5_5$	Dhar	Irregular	Undulate	Opaque	White	Raised	Mucoid
$IH4_2$	Harnora	Round	Lobate	Opaque	White	Raised	Dry

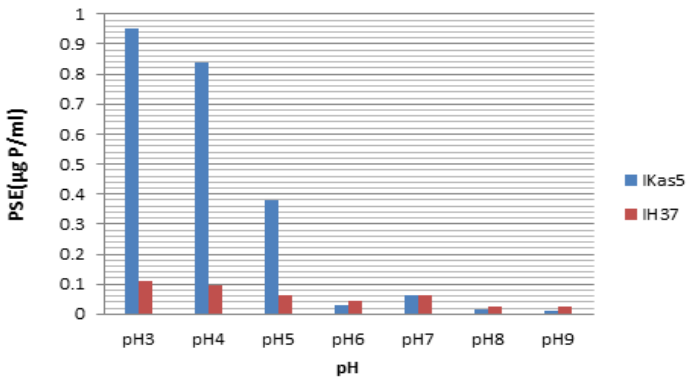

Fig. 1. Effect of pH on Phosphate Solubilization Efficiency (µg P/ml) of IKas5 and IH37

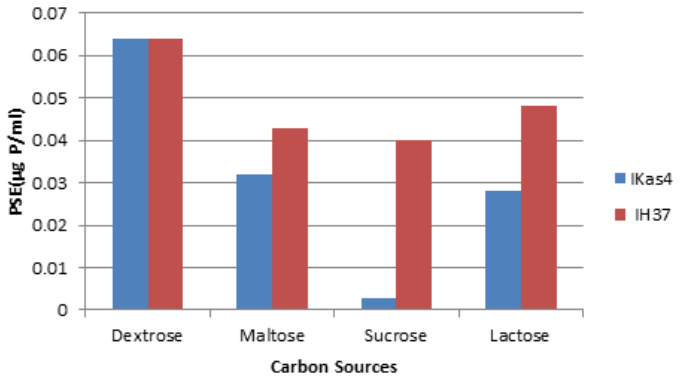

Fig. 2. Effect of Carbon sources on Phosphate Solubilization Efficiency (µg P/ml) of $IKas_4$ and $IH3_7$

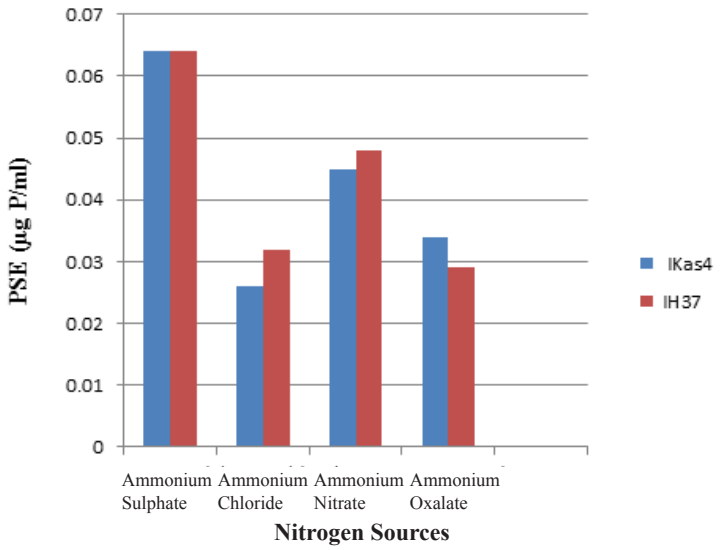

Fig. 3. Effect of Nitrogen source on Phosphate Solubilization Efficiency (µg P/ml) of $IKas_4$ and $IH3_7$

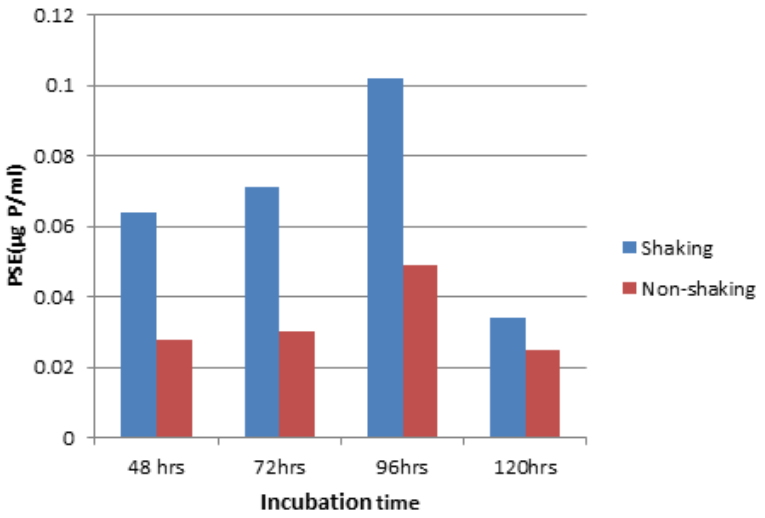

Fig. 4. Effect of incubation time and agitation on Phosphate Solubilization Efficiency (µg P/ml) of $IKas_4$

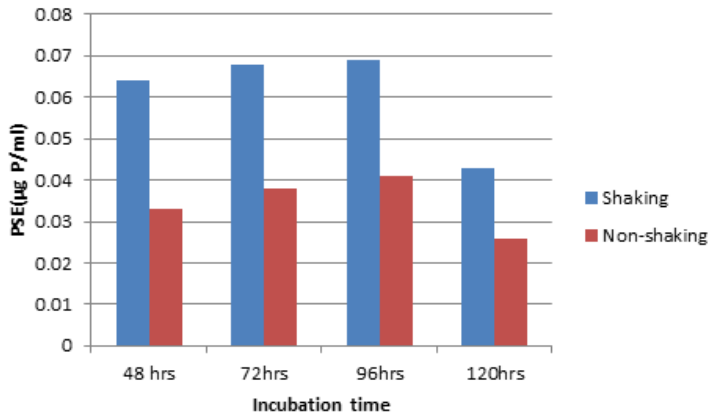

Fig. 5. Effect of incubation time and agitation on Phosphate Solubilization Efficiency (µg P/ml) of IH37

primary screening technique described by Kundu *et al.*[6] In the primary screening, only four isolates; $IKas_4$, $IH3_7$, $IKas6_2$ and $IH4_2$ showed halozones whereas all others did not show any zones. On the basis of the halozone and isolate diameter PSE % was calculated and $IKas_4$ showed maximum PSE% out of four isolates and minimum efficiency % was shown by $IKas6_2$.

Kumar *et al.* [25] also observed the halo zone of solubilization up to 20 mm on Pikovskaya agar plates. Kundu *et al.* [6] also described that 22 isolates exhibited the PSE less than 20% whereas 5 isolates showed PSE in between 50-100%. *Pseudomonas* sp. was most efficient phosphate solubilizers on Pikovskaya's agar plates with solubilization index 228±6.12 at 7th day incubation was shown by Kannapiran *et al.*[23]

Phosphate solubilization efficiency of various isolates in liquid culture was estimated on the basis of colorimetric determination of phosphorus in culture supernatant with ascorbic acid[19]. Phosphate solubilization efficiency was shown by all the 15 isolates during the secondary screening. Maximum efficiency was shown by $IKas_4$ and $IH3_7$ i.e. 0.064±0.0006 and 0.064±0.0005 whereas least PSE value shown by $IH3_1$. Kundu *et al.*[6] (2009) demonstrated the P-solubilization in PVK broth, maximum number of bacteria showed P-solubilization < 50 µg P/ml. The isolates were categorized in different classes on the basis of their

ability of the P- solubilization such as <50 µg P/ml, 100- 150 µg P/ml, 150-200 µg P/ml, etc.

As we compare the results of primary and secondary screening we find that all the 15 isolates showed PSE in secondary screening whereas only four formed zones of phosphate solubilization. $IKas_4$ and $IH3_7$ showed good PSE values both in primary and secondary screening.

In this research all the 15 isolates were subjected for the secondary screening even if they have not shown any halo zones in the primary screening. So, this shows that primary screening is not a very reliable technique for estimation of P solubilization activity. This is in agreement with observation made by several researchers [6, 26, 27]. Out of the 15 isolates highest phosphate solubilizing efficiency was shown by $IH3_7$ and $IKas_4$ and on its basis isolates showing maximum PSE were selected for subsequent studies.

Optimization

The conditions for maximum phosphate solubilization by the selected isolate were standardized by changing various parameters like pH, time of incubation, agitation, carbon source and nitrogen source.

Effect of pH

Both the selected isolates were inoculated in PKV broth with different pH values; 3, 4, 5, 6, 7, 8 & 9 and incubated at 37^0C for 48 hrs. Maximum PSE was shown by isolate $IKas_4$ at 3 pH i.e. 0.949±0.003 µg P/ml and minimum at pH 8 i.e. 0.011±0.0006 µg P/ml. Similarly $IH3_7$ has shown maximum PSE value at pH 3.0 i.e. 0.107±0.007 µg P/ml and least value 0.023±0.003 µg P/ml at pH 8 (Fig. 1) According to the values it was observed that with the increase in the pH, the ability of phosphate solubilization by isolates was decreasing whereas the phosphate solubilization increased when pH was decreased. Afzal *et al.*[16] also observed that *Arthrobacter* sp.*, Bacillus* sp. *and Rhodococcus erythropolis* showed maximum phosphate solubilization at pH 3.0. However, Xiang *et al.* [28] also reported that the best Phosphate solubilization activity was found to be at pH 8. By Zhu *et al.* [15] it was found that phosphorus solubilization reached the maximum when the pH value of $Ca_3(PO_4)_2$ containing medium is 7.0.

Effect of Carbon source

Out of the four sets of the PKV broths with different carbon sources, isolates $IKas_4$ and $IH3_7$ inoculated in broth with the dextrose as the carbon source gave the maximum PSE value i.e. 0.064±0.0006 µg P/ml and 0.064±0.0005 µg P/ml respectively whereas minimum value was given by isolates when sucrose was used as carbon source i.e. 0.0027±0.002 µg P/ml and 0.040±0.002 µg P/ml (Fig. 2). Studies by Maheshwar and Sathiyavani[13] on different carbon sources like glucose, sucrose, lactose, mannitol and sodium acetate on phosphate solubilization revealed that incorporation of glucose followed by lactose increased solubilization of phosphate and enhanced acid production efficiently by phosphate solubilizing bacteria. Nautiyal[29] reported that the carbon source play an important role in the phosphate solubilization and rate of the phosphate solubilization was increased with increasing concentrations of glucose. Similarly, TCP solubilization activity by *Pseudomonas lurida* was also reported to be maximum with glucose by Kumari & Gupta[30].

Effect of Nitrogen source

Both the isolates were inoculated in the PKV broths having four different nitrogen sources; ammonium sulphate, ammonium chloride, ammonium nitrate and ammonium oxalate. For the isolate $IKas_4$ ammonium sulphate proved to be best nitrogen source as PSE value was maximum for it i.e. 0.034±0.0058 µg P/ml and minimum value of PSE was given with the ammonium chloride as the nitrogen source. For the isolate $IH3_7$ also ammonium sulphate was the best nitrogen source with PSE value 0.064±0.0005 µg P/ml whereas least PSE was shown when ammonium oxalate was used as the carbon source (Fig. 3). Ammonium sulphate has been found to be a suitable Nitrogen source for various phosphate solubilising bacteria by several workers. Sagervanshi *et al.* [31] reported that $(NH_4)_2SO_4$ with Pikovskaya medium showed maximum P solubilization followed by Casein whereas, Urea and Sodium Nitrate gave less activity. Thakker *et al*. [32] tested many nitrogen sources like ammonium sulphate, ammonium chloride, potassium nitrate, sodium nitrate, and urea for the solubilization of TCP and RP with *Enterobacter aerogenes* and again reported ammonium sulphate to be most suitable for the purpose. Similar observations were also made by Kumari & Gupta[30] in case of *Pseudomonas lurida.*

Effect of incubation time and agitation

The selected isolates were inoculated

in Pikovskaya broth and incubated for different time periods such as 48, 72, 96 and 120 hours to check the optimum incubation period for highest P solubilization both at shaking and non-shaking conditions. It was observed that the selected isolates $IKas_4$ and $IH3_7$ were showing highest P solubilization; 0.102±0.005 µg P/ml and 0.069±0.0006 µg P/ml respectively after 96 hrs of incubation in shaking conditions. Whereas less PSE was observed for both isolates $IKas_4$ and $IH3_7$ at specific time intervals in non-shaking conditions as compared to shaking conditions the maximum values at 96 hrs of incubation i.e. 0.49±0.0005 µg P/ml and 0.041±0.002 µg P/ml respectively. For the isolate $IH3_7$ P solubilization was almost similar at both 72 and 96 hrs. It was reported that PSE values kept increasing as the incubation time increased up to 120 hrs for both shaking and non-shaking conditions but after that when PSE observed at 96 hrs its value decreased in both the conditions for both isolates However the values were found to decrease with increase in incubation time (Fig 4 and 5). Higher Phosphate solubilizing activity under shaking conditions could be attributed to proper aeration and good mixing of nutrients and tri calcium phosphate. Nath *et al.* [33] also reported that with increase in the incubation time *Penicillium sp.* showed the increase in the phosphate solubilization efficiency from second to the eighth day but after that phosphate solubilization by the *Penicillium sp.* started decreasing. Kannapiran *et al.* [23] (2011) showed that the *Pseudomonas sp.* solubilized phosphates from the medium containing tricalcium phosphate and it solubilized a maximum of 1670 mg ml^{-1} by 10th day of incubation and beyond which no further solubilization was seen.

Effect of all optimized conditions

The isolate $IKas_4$ was cultured under the optimized condition (pH- 3, C source – dextrose, ammonium sulphate as N source, shaking conditions, and incubation period- 96 hrs) and the Phosphorus solubilization activity was assessed. It was observed that the optimized conditions resulted in increase of phosphorus solubilization efficiency of $IKas_4$ and the final efficiency was recorded to be 0.99±0.0005 µg P/ml while initially it was showing phosphate solubilization activity of 0.064±0.00058. Whereas isolate $IH3_7$ was when cultured under optimized conditions pH- 3, C-source-dextrose, ammonium sulphate as N-source, shaking conditions, and incubation period- 96 hrs gave a slight enhancement in the PSE 0.070±0.0051 µg P/ml from the initial value of 0.064±0.00051.

Characterization of selected isolates

Selected isolates were characterized on the basis of various biochemical and morphological tests. They were also identified on the basis of 16s rDNA sequences.

Isolate $IKas_4$ colonies were opaque and white in color. According to the gram reaction results $IKas_4$ was gram negative and rod shaped. Isolate gave negative results for indole and methyl red test whereas positive results were shown for vogues proskauer, citrate utilization and Catalase test. On the other hand, the isolate $IH3_7$ colonies were white in color and translucent. According to the gram reaction it was rod shaped gram negative bacteria. It also showed negative results for indole, methyl red, citrate utilization and catalase test, whereas positive result was shown for vogues proskauer test.

On the basis of the 16srDNA sequence, the isolates were identified as *Enterobacter aerogenes* IKaS4 (Genebank Accession Number : KJ921704) and *Enterobacter* sp. IH37 (Genebank Accession Number : KJ921705).

CONCLUSION

In the present study isolation of Phosphate solubilizing bacteria was carried out from wheat rhizospheres. A total of 15 isolates were obtained from five rhizosphere soil samples. The isolates were subjected to primary and secondary screening and IKas4 and $IH3_7$ showing highest Phosphate solubilization during secondary screening (0.064±0.00058 µg P/ml and 0.064±0.00051µg P/ml) were selected for subsequent studies. The conditions for *in vitro* Phosphate Solubilization by the isolates were optimized. The isolates showed maximum Phosphate solubilization under optimized condition with dextrose, ammonium sulphate, pH- 3, incubation period 96 hrs with agitation. The bacterial isolates were Gram negative rod, catalase positive, Indole negative, Methyl Red negative, VP positive, and non-spore former. $IKas_4$ was identified as *Enterobacter aerogenes* and $IH3_7$ was identified as *Enterobacter* sp. by 16s rDNA sequencing.

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