ISOLATION AND MOLECULAR CHARECTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM MARINE SAMPLE

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Abstract

Phosphorus is a necessary component of all living things. Through a process of solubilization and mineralization, phosphate solubilizing bacteria can transform phosphate into a bioavailable form. Hence in the present study, a phosphate solubilizing bacteria isolated from Kadalundi, Kozhikode, Kerala, India was investigated. Experimental studies found that the strain TK033 was effective solubilization with solubilization index of 5 in Pikovskya's agar plate. Phosphate solubilizing bacterium was identified using physiological, morphological and biochemical charecters as well as 16S rRNA gene sequencing. The phosphate solubilizing bacterium was identified as a strain of *Staphylococcus warneri.* The maximum phosphate solubilizing activity of the strain was found to be high at pH 4 Acid phosphatase activity was determined by performing *para*- nitrophenyle phosphate assay (pNPP) of the bacterial culture. Optimum acid phosphatase activity was observed at 48 hour of incubation temperature of 37^0C and pH of 6.0. Bacteria play a significant role in the biogeochemical cycle of phosphorus and plant growth in coastal habitats, as evidenced by the existence of phosphate solubilizing microorganisms and their ability to solubilize phosphate.

Keywords: Phosphorus; solubilization; biogeochemical cycle; marine; halophilic

Introduction

Phosphorus is a proscribing nutrient, regardless of being a main factor of seawater. Major bureaucracy is both certain to or strongly adsorbed to silt and clay. Phosphorus commonly happens as phosphates in each inorganic and natural compounds [1]. Mainly phosphorus is solubilized enzymatically or with the aid of using acid hydrolysis [2]. It is a critical macronutrient for plant life, that is carried out to the soil withinside the shape of phosphatic manure. However, a big part of the carried-out phosphorus is swiftly immobilized, being unavailable to plant life [3]. The loose phosphatic ion in soil performs a vital function; the orthophosphate ion is the simplest ion which may be assimilated in a considerable quantity with the aid of using plant life [4]. Most of the phosphate and nitrate incorporated into the aquatic plant life, animals and sediments are because of the motion of the microbes. They also are accountable for decomposing lifeless natural remember and recycling of phosphorus [5]. However, a more a part of soil phosphorous, about 95-99% is gift withinside the shape of insoluble phosphates and for this reason cannot be used by the plant life [6]. Phosphorus is the second one maximum vital macro-nutrient required with the aid of using plant life, subsequent to nitrogen.Upon utility as inorganic phosphorus swiftly converted into much less to be had bureaucracy with the aid of using forming a complicated with Al or Fe in acid soils or with Ca in calcareous soils accordingly turns into unavailable to plant life [7]. In order to make amends for this herbal poverty in phosphate, highly-priced chemical phosphate fertilizers are utilized in agriculture to enhance crop yield. However, the chemical fertilizer enterprise is now taken into consideration as extraordinarily polluting [8].

The improvement of sustainable agriculture calls for a sturdy discount in agrochemical inputs and their substitute with the aid of using greater ecological, green and reasonably-priced herbal products [9]. The phosphorus being a structural factor of many coenzymes, phospho-proteins, phospholipids [10], additionally shape part of genetic memory “DNA” of all dwelling organisms. It is worried in switch and garage of strength that is used for the boom and reproduction [11]. This phosphorus is without difficulty translocated in the plant life, transferring from older to more youthful tissues because the plant life bureaucracy cells and develops roots, stems, and leaves [12]. Phosphorous is a one of the maximum ample metal factors observed withinside the earth’s crust [13]. Phosphorus in soils can exist in each natural (Po) and inorganic (Pi) bureaucracy; the inorganic types of phosphorus were calculated to account for 35 – 70% of general P in soil. While a few P minerals, like apatites and strengites, have very slow-launch prices, different P minerals, complexed with calcium, aluminium, or iron, have quicker dissolution prices which can be depending on the pH of the encircling soil and on the dimensions of the particles [14].

The phosphate solubilizing microorganism (PSB) are able to dissolving the insoluble inorganic phosphorus into soluble natural phosphorus. Microorganisms particularly microorganism play an

essential function withinside the biogeochemical biking of vitamins withinside the marine environment [13]. Compare to terrestrial microorganism Marine microorganism having one-of-a-kind biochemical, physiological properties. Hence on this have a look at we goal to isolate phosphate solubilizing marine microorganism and to have a look at the phosphate solubilizing activity. This study also aims to identify the phosphate solubilizing bacteria using 16SrRNA.

Materials And Methods

3.1 Sample Collection

Marine soil samples were collected from coastal area of Kadalundi (11.13600N, 75.82720E), Kozhikode, Kerala, India. Collected samples were transferred to lab in a sterile polythene cover.

3.2 Primary Screening

3.2.1 Isolation of phosphate solubilizing bacteria

The serially diluted samples were plated on Pikovskaya's agar media to isolate the phosphate solubilizing bacteria. The plates were incubated in room temperature (28±2 ºC). After 3 days, the colony forming units (CFUs) were recorded. The cultures which showed clear zone formation around their colonies were considered to be the phosphate solubilizing bacteria and selected for further studies. The well-developed and morphologically different single colonies were picked out randomly and streaked on appropriate agar plates for obtaining pure culture.

3.2.2 Estimation of phosphate Solubilization Index

The quantitative estimation or abilities of the isolated phosphate solubilizing bacteria to solubilize tricalcium phosphate (TCP) on pikovskya's agar medium was determined in terms of solubilization index (SI). Phosphate solubilization index was calculated by measuring the colony diameter and the halo zone diameter and the colony diameter, using the formula SI = (Colony diameter + halo zone diameter) ×/ colony diameter

3.2.3 Conformation of extracellular nature of phosphatase enzyme

To check whether the bacteria can able to solubilize phosphate or not, pure colony of bacterial isolate was transferred into Erlenmeyer flask containing Pikovskya's broth and was incubated on rotary shaker (150 rpm) at 280C to produce secondary metabolites. After 48 and 72hrs, the broth culture was centrifuged at 10,000 rpm for 10 min. The supernatant was separated from the bacterial cells by 0.2µm Millipore membrane filter. This filtrate was spotted into the well on Pikovskaya's agar plate and incubate at 28oC for 72hr and observed the zone formation in every 24hrs.

3.3 Secondary Screening

3.3.1 Quantitative estimation of PSB by PKV liquid medium

The selected strain with highest solubilization index used for the quantitative estimation by PKV medium. Erlenmeyer flask containing 50ml PKV broth were inoculated with phosphate solubilizing bacteria in triplicate. Non-inoculated medium served as control. The flask were incubated in a shaker at 28oC for 120hr at a speed of 100rpm. The pH of the culture medium was measured at specific time interval. 1ml of bacterial culture samples was collected at every 24hr and centrifuged at 10,000rpm for 10min. The supernatant was separated from the bacterial cells by successive filtration through whatman No.1 filter paper followed by 0.2µm Millipore membrane. Here using para-nitrophenyl phosphate as a substrate for measuring phosphate solubilization. The universal buffer, borate buffer (Boric acid: 14.7g , NaOH: 2.4g, D.H2O: 200ml) take 4ml and add 1ml cell free supernatant then add 1ml 0.025mM para-nitrophenyl phosphate. It incubate at 370C for 1hr, one drop toluene was added to stop the microbial growth. After 1hr incubation, the reaction was stopped by adding 4ml 0.5M NaOH and 1ml 0.5M CaCl2. This mixture was again filtrated to using Whatman No.1 filter paper and its color intensity checked in colorimeter at 450nm.

3.4 Identification of Potent Strain

3.4.1 Morphological and Biochemical Identification of the selected strain

The cultural characteristics, such as colony appearance, spore formation and motility of selected strain determined by standard methods. Catalase, urease production, Indole production, H2S production, methyl red, Voges- Proskauer (V-P), citrate utilization and acid-gas production from sugar were also tested.

3.5 Molecular Identification of The Selected Strain

Molecular identification of selected bacteria was done by using 16S rRNA gene sequence. Genomic DNA isolation, PCR amplification and sequencing was done at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The final sequence was used for the analysis. The sequence similarity was search and phylogenetic analysis was done by BLAST of NCBI.

3.6 Optimization of pH and Temperature

To prepare PKV broth for optimizing the activity of selected strain have high solubilisation index with different pH (2, 4, 5, 6, 7, 8 and 9) and temperature (4oC, 28oC, 37oC, and 55oC).

Results and Discussion

4.1 Primary Screening

4.1.1 Isolation phosphate solubilising bacteria

Total of 9 phosphate solubilizing bacteria isolated from marine water sample of Kadalundi, Kozhikode, Kerala, India. Out of those three colonies B2, B3and A2were selected based on their halo zone formation around the colony on PKV agar plate and evaluated for their solubilisation index. Among the three bacterial strains, only the B2show maximum solubilisation index. The A2 and B3 showed solubilisation index is lower than that of B2. The B2 colonies were selected for further studies (Figure1).

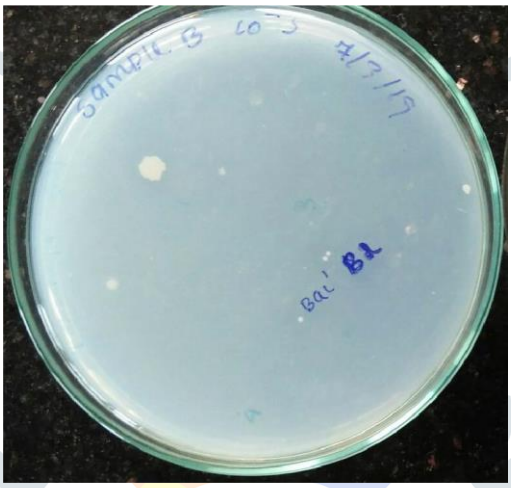

Figure 1 Spread plate of B2 colony on PKV agar plate

4.1.2 Estimation of phosphate solubilisation index

Solubilisation index is based on their halo zone formation around the colony and colony diameter. Among those three organisms, B_2 show maximum solubilisation that is 5 (Table 1).

Table 1 Qualitative estimation of phosphate solubilisation efficiency of selected strain.

PSB isolate No	Colony diameter(mm)	Halo zone diameter (mm)	Solubilisation Index (SI)
A_2	0.2	0.5	3.5
B_3	0.15	0.4	3.6
B_2	0.1	0.4	5

4.1.3 Conformation of extracellular nature of selected strain

Phosphate solubilising organism have an ability to produce secondary metabolites like enzymes. These enzymes play an important role in the degradation of phosphate and produce halo zone around the colony. This activity of those organism defined as extracellular nature. So, the selected strain shows a wide zone around the colony (Figure 2).

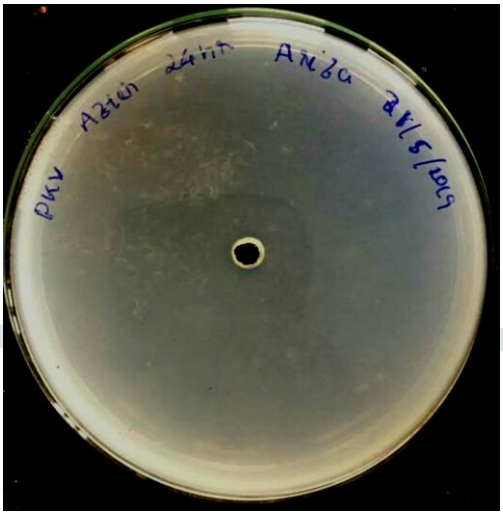

Figure 2 Extracellular nature of selected strain

4.2 Secondary Screening

4.2.1 Quantitative estimation of selected strain by liquid medium

Evaluation of phosphate solubilising activity of the strain B_2 was carried out in the PKV broth medium at every 24 hour, for a period of 120 hour. The phosphate solubilising ability was increased from 0.05µg/ml to 0.10µg/ml as the pH decreased from 7.0 to 4.0. The pH of the medium decreased steadily after 24h of incubation, but started to increase again after 48h of incubation. Maximum phosphate solubilisation was observed at 48h (0.10µg/ml) of incubation with a maximum drop in pH 4 of the medium.

4.3 Identification of Potent Strain

4.3.1 Morphological Characterization

Morphological characteristics of the isolate (Table-3) gave small colonies with dull white colour, smooth surface and a regular circular circumference.

Table 3: Observation of morphological characteristics of isolated strains

Isolates	Colony Morphology
B_2	Circular, entire, flat, punctiform, smooth, dull, white, opaque
B_3	Circular, entire, flat, small, smooth, dull, white, opaque
A_2	Irregular, undulate, raised, small, smooth, moist, shiny, cream, Opaque

4.3.2 Microscopic Identification

The microscopic examination revealed that the selected isolate (B_2) was Gram positive, with a cellular rods form associated in pairs or in chain (Figure 3).

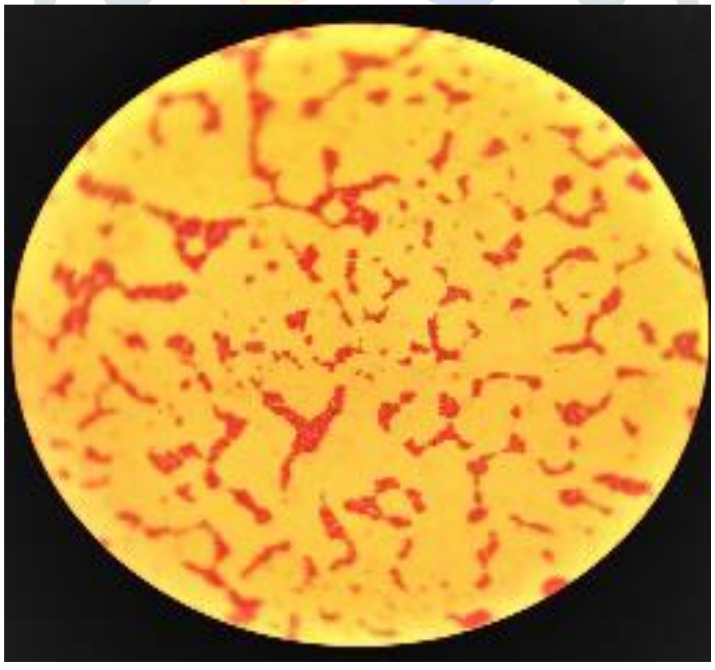

Figure 3 Microscopic examination of the selected strain

4.3.3 Biochemical test

The selected colony was identified based on macroscopic, microscopic as well as biochemical tests. Table:4 give the results of intracellular enzymatic activities of the organism.

Table 4 Intracellular enzymatic activity of the selected strain

Test	Result
Indole production	Negative
Methyl red	Positive
Voges-proskaure	Positive
Urease test	Positive

4.3.4 Carbohydrate fermentation

The ability of organism to degrade and ferment carbohydrates with the production of acid and gas were determined. Table 5 shows the carbohydrate fermentation result of the organism.

Table-5 Carbohydrate fermentation of the selected strain4.4

Added sugar	Result
Glucose	Acid, no gas
Lactose	Acid, no gas
Maltose	Acid, no gas
Sucrose	Acid, no gas
Mannose	Acid, no gas

4.4 Molecular Identification

Molecular identification using 16S rRNA gene clearly revealed that, the isolated bacterium B2 has 100% similarity to Staphylococcus warneri.

4.5 Optimization of pH and Temperature

4.5.1 Estimation of optimum pH

The growth rate of the organism at different pH were measured based on optical density at 645nm. The organism showed maximum activity at pH 6.0 (Table 6).

Table 6: Growth of isolated organism on different pH

pH	Optical density at 645nm			
	24 hour	48 hour	72 hour	120 hour
2	0.01	0.03	0.03	0.03
4	0.05	0.05	0.04	0.05
5	0.04	0.05	0.04	0.04
6	0.07	0.08	0.08	0.05
7	0.05	0.06	0.06	0.06
8	0.04	0.05	0.04	0.05
9	0.05	0.05	0.04	0.05

4.5.2 Estimation of Optimum temperature

The growth rate of the organism at different temperature were measured based on optical density at 645nm. The organism showed maximum activity at temperature 37$_0$C (Table-7).

Table 7 Growth of isolated organism on different pH

Temperature	Optical density at 645nm			
	24 hour	48 hour	72 hour	120 hour
4^0C	0.05	0.05	0.04	0.04
28^0C	0.05	0.06	0.05	0.04
37^0C	0.06	0.08	0.07	0.07
55^0C	0.03	0.05	0.03	0.03

Conclusion

In this project work, concluded that isolation and characterization of phosphate solubilizing bacteria from marine sample of Kadalundi beach, Kozhikode, Kerala, India. The isolated strain TK033 show maximum halo zone around the colony and it indicate maximum phosphate solubilisation. In order to other identification steps evidently proved the organism's morphology and other characteristics. As well as the 16S rRNA gene sequencing strongly proved the organism is *Staphylococcus warneri.* And its maximum growth observed at optimum pH and temperature. Hence the present phosphate solubilisation and acid phosphatase activity may have greater probable use for industrial, agricultural and biotechnological application.

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