ISOLATION AND MOLECULAR CHARECTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM MARINE SAMPLE

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Abstract

Phosphorus is an essential nutrient for plant growth, yet its bioavailability in soil is often limited due to its fixation in insoluble forms. Phosphate-solubilizing bacteria (PSB) play a pivotal role in enhancing phosphorus availability by converting insoluble phosphates into bioavailable forms through the secretion of organic acids and phosphatases. The present study focuses on the isolation and characterization of PSB from marine soil samples collected from Kadalundi, Kerala, India was investigated. Experimental studies found that the strain B2 shows effective solubilization with solubilization index of 5 in Pikovskya's agar plate. Phosphate solubilizing bacterium was identified using physiological, morphological and biochemical characters as well as 16S rRNA gene sequencing. The phosphate solubilizing bacterium was identified as strain of *Staphylococcus warneri.* Acid phosphatase activity was determined by performing *para*- nitrophenyl 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; Pikovskya's agar

Introduction

Phosphorus is an essential nutrient that plays a pivotal role in plant growth and development, being a core component of nucleic acids, ATP, and phospholipids. However, its availability in soils is often limited due to its fixation in insoluble forms such as calcium, aluminum, or iron phosphates. Phosphate-solubilizing bacteria (PSB) have emerged as promising agents for biofertilizers, as they convert these insoluble forms into bioavailable phosphates, thereby enhancing phosphorus uptake by plants (Rodríguez & Fraga, 1999; Vassilev et al., 2006). Their ability to secrete organic acids and enzymes such as phosphatases plays a central role in this process.

Marine environments represent a vast reservoir of microbial diversity, including PSB with unique adaptations to extreme conditions such as high salinity, pressure, and limited nutrient availability. These characteristics make marine-derived PSB especially valuable for agricultural applications in saline and nutrient-poor soils, where traditional biofertilizers often fail (Zahran, 1999). Studies have shown that bacteria isolated from marine sediments, such as species of *Bacillus*, *Pseudomonas*, and *Vibrio*, exhibit efficient phosphate-solubilizing activity and plant growth-promoting traits (Chung et al., 2005; Hameeda et al., 2008).

The mechanisms of phosphate solubilization by PSB primarily involve the secretion of organic acids, such as gluconic and citric acids, which lower the pH of the surrounding medium and chelate cations that bind phosphates. Molecular studies have identified key genes, such as those encoding glucose dehydrogenase (gcd) and phosphatases, which play vital roles in these pathways (Kim et al., 2003). Furthermore, recent advancements in genomic and metagenomic tools have enabled the identification of novel PSB strains and their metabolic pathways, providing deeper insights into their ecological functions and potential applications (Mendes et al., 2013).

Despite significant progress in understanding terrestrial PSB, the exploration of marine-derived strains remains relatively limited. Marine PSB not only contribute to phosphorus cycling in aquatic ecosystems but also offer untapped potential for biotechnological applications. Studies in the Arabian Sea and Mediterranean regions have demonstrated the ability of marine PSB to improve phosphorus availability under saline conditions, highlighting their relevance for sustainable agriculture (Rusch et al., 2007; Gao et al., 2014).

The increasing salinization of arable land and the environmental impacts of chemical fertilizers underscore the need for eco-friendly alternatives. Marine PSB, with their dual capacity to tolerate salt stress and enhance phosphorus solubilization, present an ideal solution. The present study aims to isolate PSB from marine samples, identify them through molecular methods, and evaluate their potential in phosphate solubilization.

Materials And Methods

3.1 Sample Collection

Marine soil samples were collected from the coastal area of Kadalundi, Kozhikode, Kerala, India (11.13600°N, 75.82720°E). The samples were transferred to the laboratory in sterile polythene bags under aseptic conditions for further study.

3.2 Screening

3.2.1 Isolation of phosphate solubilizing bacteria

The collected soil samples were serially diluted and plated onto Pikovskaya's agar medium for the isolation of phosphate-solubilizing bacteria. The plates were incubated at room temperature (28 ± 2 °C) for three days. Colonies forming clear zones indicative of phosphate solubilization were recorded as colony-forming units (CFUs). Morphologically distinct colonies with clear zone were subcultured onto fresh agar plates to obtain pure isolates.

3.2.2 Estimation of phosphate Solubilization Index

The ability of isolated bacteria to solubilize tricalcium phosphate (TCP) was assessed on Pikovskaya's agar. The selected isolates were spot inoculated on to pikovskaya's agar medium and incubated at room temperature. After incubation the diameter of the zone clearance was measured and the solubilization index (SI) was calculated using the formula:

$$\text{Phosphate solubilisation index (SI)} = \frac{\text{(Colony diameter + halo zone diameter)}}{\text{Colony diameter}}$$

All the observations were recorded in triplicate.

3.2.3 Conformation of extracellular nature of phosphatase enzyme

Agar well diffusion method was performed to confirm extracellular phosphatase activity, pure bacterial isolates were grown in Pikovskaya's broth at 28 °C with shaking (150 rpm) for 48–72 hours. Culture supernatant was prepared by centrifugation (10,000 rpm for 10 min) and filtration through a 0.2 µm membrane. Aliquots of the cell free supernatant was plated onto well of 6 mm diameter made on Pikovskaya's agar and incubated at 28 °C for 72 hours, with zone formation around the wells indicates the extra cellular nature of the enzyme.

3.2.4 Quantitative estimation

The isolate with the highest SI was further evaluated using *para*- nitrophenyle phosphate assay (pNPP). Cultures were grown in triplicate, with uninoculated broth as a control, incubated under shaking conditions (100 rpm) at 28 °C for 120 hours. pH changes were recorded at regular intervals. Phosphate

solubilization was assessed by pNPP assay method by reacting culture supernatants with para-nitrophenyl phosphate substrate. The universal buffer, borate buffer (Boric acid: 14.7g, NaOH: 2.4g, D.H_2O: 200ml) 4ml, 1ml cell free supernatant and 1ml 0.025 mM para-nitrophenyl phosphate mixed and incubated at 37^0C for 1hr. After 1hr incubation, the reaction was stopped by adding 4ml 0.5M NaOH and 1ml 0.5M $CaCl_2$. This mixture was again filtrated to using Whatman No.1 filter paper and OD was measured in colorimeter at 450nm.

3.3 Identification of Potent Strain

3.3.1 Morphological and Biochemical Identification of the selected strain

The selected strain was characterized based on colony morphology, motility, and biochemical tests including catalase, urease, indole production, H_2S production, methyl red, Voges-Proskauer, citrate utilization, and carbohydrate fermentation tests.

3.4 Molecular Identification of The Selected Strain

3.4.1 Genomic DNA Isolation

Genomic DNA was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's protocol. DNA quality and quantity were verified via agarose gel electrophoresis.

3.4.2 16S rRNA Gene Amplification and Sequencing

PCR amplification of the 16S rRNA gene was performed using specific primers. Amplified products were analysed on a 1.2% agarose gel, purified, and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit. The obtained sequences were processed using Geneious Pro software and analysed for homology using BLAST (NCBI). Phylogenetic relationships were inferred based on sequence alignments and the sequence was submitted to NCBI using BankIt submission portal.

3.5 Effect of pH and Temperature on activity of isolate

The growth of the selected strain with the highest SI was optimized by culturing in PKV broth across varying pH levels (2–9) and temperatures (4 °C, 28 °C, 37 °C, and 55 °C).

Results and Discussion

4.1 Screening

4.1.1 Isolation phosphate solubilising bacteria

Phosphate solubilization by microorganisms plays a pivotal role in converting insoluble forms of phosphate into bioavailable forms, thereby enhancing soil fertility and influencing aquatic ecosystems

(Rodríguez and Fraga, 1999). In this study, nine phosphate-solubilizing bacterial isolates were obtained from marine water samples collected from Kadalundi, Kozhikode, Kerala, India. Among them, three isolates, identified as B2, B3, and A2, were selected based on their visible halo zones on Pikovskaya's agar plates, which is indicative of phosphate solubilization activity. Of the three strains, isolate B2 demonstrated the highest solubilization index, suggesting its superior ability to solubilize phosphate in comparison to the others (Figure 1). This finding is consistent with prior studies showing that halo zone formation is a reliable preliminary indicator of phosphate-solubilizing efficiency (Chen et al., 2006; Nautiyal et al., 2010).

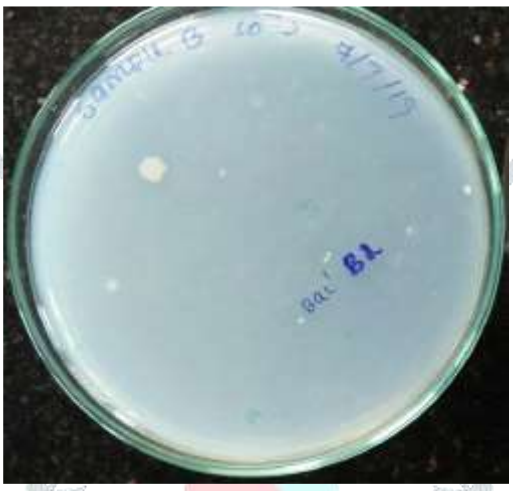

Figure 1 : B2 colony on PKV agar plate

4.1.2 Estimation of phosphate solubilisation index

The phosphate solubilization index (SI) for each isolate was calculated based on halo and colony diameters on agar plates. B2 exhibited the highest solubilization index of 5, while A2 and B3 had lower indices of 3.5 and 3.6, respectively (Table 1). The high SI of B2 reflects its enhanced ability to release phosphate, possibly through the production of organic acids, as reported by Bhattacharyya and Jha (2012). These organic acids are known to lower the pH in the surrounding environment, thereby facilitating the dissolution of phosphate (Kim et al., 2010).

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

PSB isolate	Colony diameter(mm)	Halo zone diameter (mm)	Solubilisation Index (SI)
A2	2	5	3.5
B3	1.5	4	3.6
B2	1	4	5

4.1.3 Confirmation of extracellular nature of selected strain

Extracellular nature of the phosphatase enzyme was confirmed by agar well diffusion method. The selected strain shows a wide zone around the colony (Figure 2).

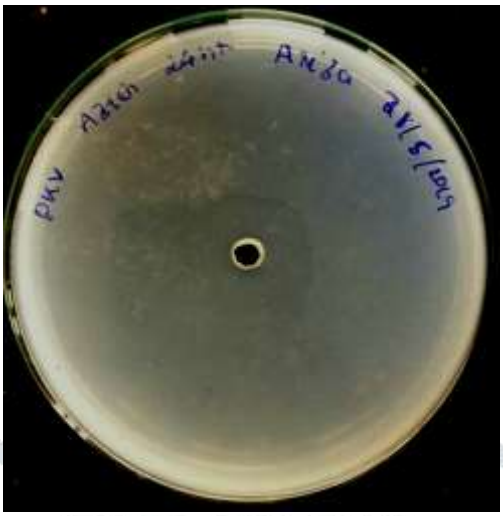

Figure 2 Extracellular nature of selected strain

4.1.4 Quantitative estimation of selected strain by liquid medium

Evaluation of phosphate solubilising activity of the strain B2 was carried out in the PKV broth medium at every 24 hours, for a period of 120-hour. The quantitative phosphate solubilization by isolate B2 was assessed colorimetrically at 450 nm by *para*- nitrophenyle phosphate assay (pNPP) method. B2 demonstrated an increase in solubilized phosphate concentration from 0.05 mg/ml to 0.10 mg/ml, correlating with a decrease in pH from 7.0 to 4.0. Notably, the highest solubilization level (0.10 mg/ml) was observed at 48 hours with a pH of 4. This decline in pH aligns with other studies that found a relationship between organic acid production and enhanced phosphate solubilization in acidic conditions (Pandey et al., 2006; Kumar et al., 2013). The observed pH reduction could indicate that B2 produces organic acids, which, as reported by Rashid et al. (2004), play a key role in phosphate solubilization by lowering the pH of the medium. This trend supports earlier findings that organic acid production by phosphate-solubilizing bacteria (PSB) lowers the pH, enhancing phosphate solubilization (Nautiyal, 1999). The subsequent increase in pH after 48 hours may indicate the consumption or neutralization of organic acids by metabolic processes (Table 2).

Time interval (Hrs)	pH	Soluble P concentration (mg/ml)
24	4	0.05
48	4	0.10
72	5	0.09
120	5	0.08

Table.2 Phosphate solubilising activity of the selected isolate B2 at different interval

4.2 Identification of Potent Strain

4.2.1 Morphological and Biochemical Characterization

Morphological analysis revealed that isolate B2 formed small, dull white colonies with smooth surfaces and circular peripheries. Microscopic observations identified B2 as Gram-positive cocci arranged in chains or pairs. Biochemical tests indicated positive results for methyl red, Voges-Proskauer, and urease tests, while catalase and oxidase tests were negative. The strain fermented different carbohydrates, including glucose, lactose, maltose, sucrose, and mannose, producing acid without gas (Table 3).

S.no.	Characteristics	Isolate B2
1	Gram reaction	G +ve
2	Colony morphology	Cocci, Circular, entire, flat, punctiform, smooth, dull, white, opaque
3	Pigmentation	-
4	Indole	-
5	MR	+
6	VP	+
7	Citrate	-
8	Urease	+
9	Oxidase	-
10	Catalase	-
11	H_2S	_
12	Lactose fermentation	⊥
13	Sucrose	⊥
14	Mannose	⊥

15	Glucose	⊥
16	Maltose	⊥

Table 3: Morphological and Biochemical Characteristics of selected isolate

4.2.2 Molecular Identification and Phylogenetic Analysis

After various morphological and biochemical studies, the selected isolate was identified as Staphylococcus genus and Molecular characterisation results reveals that the selected isolate confirmed as *Staphylococcus warneri*. Thus, the isolate was further named as *Staphylococcus warneri* EMEA1and nucleotide sequence was deposited in GENBank with nucleotide sequence accession number PQ636904

4.3 Effect of pH and Temperature on activity of isolate

4.3.1 Effect of pH

The growth of isolate B2 was evaluated at different pH levels, with maximum activity observed at pH 6.0, (Table 4). The optimal pH range suggests that B2 has adapted to slightly acidic conditions, which may enhance its phosphate-solubilizing abilities. Similar findings were reported by Kumar et al. (2013), who noted that phosphate solubilization generally increases under acidic conditions, supporting the role of organic acid production in solubilization.

pH	Soluble P concentration (mg/ml)			
	24 hours	48 hours	72 hours	120 hours
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

Table 4: Optimisation of pH on Phosphate solubilisation

4.3.2 Temperature Optimization

B2 showed maximum growth at 37 °C (Table 5), which indicates its capacity to thrive in moderate temperatures. This optimum temperature aligns with prior research that found many marine phosphate-solubilizing bacteria exhibit peak activity at temperatures ranging from 25 to 40 °C (Pandey et al., 2006). The temperature-dependent solubilization pattern of B2 further supports its potential application in tropical and subtropical marine environments.

Temperature	Soluble P concentration (mg/ml)			
	24 hours	48 hours	72 hours	120 hours
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

Table 5: Optimisation of temperature on Phosphate solubilisation

Conclusion

In this study, the isolated phosphate-solubilizing bacteria from the marine environment of Kadalundi, Kerala, India, identified as *Staphylococcus warneri*, demonstrated a high phosphate-solubilization index and extracellular enzyme production capabilities. Optimal pH and temperature studies further confirmed that B2 is well-suited for nutrient cycling applications in marine ecosystems. The 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.

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