2. MATERIALS AND METHODS

2.1 Sample

Sea water and sediment samples were used as source of samples for screening polyphosphate accumulating bacteria in the present study. Both the samples were collected from the coastal areas of southern part of Kerala State (Arabian Sea) and from coastal area of Rameswaram in Tamil Nadu (Bay of Bangal).

2.2 Collection and transport of Samples

Samples were collected (during January - April 2000), aseptically in sterile containers. Water samples were collected in sterile bottles. Sediment samples were collected using Peterson grab and the middle portion of the sediment collected in the grab was transferred aseptically in to sterile polyethylene bags. Samples were transported in icebox to the laboratory and subjected to microbiological analyses.

2.3 Medium

Zobell's Marine agar (HIMEDIA, India) medium was used for isolation of heterotrophic bacteria present in seawater as well as sediment. The medium was supplemented with 500 \text{mM} phosphate, using KH$_2$PO$_4$ (BDH) in addition to the phosphate already available in the readymade medium. Final concentration of phosphate in the medium, after addition of 500 \text{mM} of (KH$_2$PO$_4$) was 1800 \text{mM} (after autoclaving).

Zobell's Marine Broth (ZMB) and Zobell's Marine Agar (ZMA) (Hi-Media) were used throughout the course of the study unless otherwise specified. Cultures were stored in Zobell's Marine Agar slopes (with an additional 500 \text{mM} phosphate) at 4° C and sub cultured periodically. Zobell's Marine Broth without additional Pi was used for inoculum preparation, and the same was used with an additional 500 \text{mM} Pi for growth studies.

2.4 Isolation of Heterotrophic bacteria.

The water and sediment samples collected from different locations were plated on Zobell's Marine agar employing pour plate technique, after appropriate serial dilution.
The inoculated plates were incubated at 30°C for 3-5 days. Individual colonies developed on the agar medium were isolated, after recording their morphological characteristics, purified on ZMA plates, and stored at 4°C as slope cultures on Zobell's Marine Agar. Another set of cultures was used as working cultures further screening. All the isolates were subcultured periodically at regular interval of two weeks.

2.5 Screening of the cultures for phosphate uptake, and polyP Accumulation

2.5.1 First phase screening

All the isolates obtained were screened for their ability to uptake inorganic phosphate (Pi) from environment, and accumulate the phosphate as polyP in the cell. This screening was done in two phases.

First phase of screening included evaluation of all the isolates obtained for Pi uptake and polyP accumulation after 24 hours of growth.

2.5.1.1 Inoculum preparation:

A loopful of 24hrs. Agar slope culture was inoculated into 5ml of Zobell’s Marine Broth (HIMEDIA), without any additional phosphate in the medium and incubated at room temperature (28 ±2°C) and at 120 rpm for 18 hrs.

2.5.1.2 Inoculation and Incubation

After 18 hrs of incubation, 0.5ml of the culture broth was transferred into a 50ml of fresh Zobell’s Marine broth in a 250 ml conical flask (1% inoculum v/v) which was prepared with an additional 500μM phosphate (Pi) in the medium. After inoculation, the flasks were incubated at room temperature (28±2°C) at 120 rpm for 24 hrs. After growth the culture broth was used for all assays.

2.5.1.3 Assay

One ml of the culture broth was centrifuged at 10,000 rpm at 4°C, for 10 min. The supernatant was used for estimation of the residual phosphate in the medium and the cell pellet was used for polyphosphate and free inorganic phosphate in the cells.
2.5.1.3.1 Estimation of various Phosphates

The residual phosphate remained in the spent broth was estimated following the Ascorbic acid method described by Kato et al. (1993). The intracellular phosphates were estimated based on the method of Harold (1966). The cell pellet obtained was resuspended in one ml of 10% ice-cold TCA solution and allowed to stand for 30 min, after mixing well by vortexing the contents. Later, the contents were centrifuged at 15,000 rpm for 10 min. at 4°C. The supernatant was collected and used for estimation of free inorganic phosphate in the cell. The pellet was resuspended in 1 ml of IN HCl, vortexed, boiled for 7 min., cooled rapidly in cold water, centrifuged at 15000 rpm at 4°C for 10 min., and the Pi in the supernatant was estimated as polyP.

2.5.1.3.2 Total Cellular Protein

Biomass was estimated in terms of total cell protein. One ml of the sample was centrifuged at 10000 rpm for 10 min. (at 4°C) and the total protein of the sedimented cell pellet was estimated by the modified Lowry's method described by Herbert et al. (1971).

2.5.2 Second Phase Screening

Based on the results obtained during First phase screening, the potential strains were ranked according to their Pi uptake capacity. 26 isolates, representing different locations of sampling and both water and sediment, were selected and subjected to second phase screening towards selection of potential strains.

All the selected isolates were evaluated for their efficiency for Pi uptake from medium, accumulation of cellular free Pi, polyP, Low Molecular Weight phosphates (ATP, ADP etc.), and the Nucleic acid phosphates in their cells during growth. Different phosphates were estimated at regular intervals up to 7 days. Biomass was also estimated in terms of total cell protein.

Inoculum for each selected culture was prepared as mentioned in the previous section. From the pre-culture prepared one ml was drawn and transferred into 100 ml of freshly prepared ZMB added with Pi (500ΜM) and incubated at room temperature (28±2°C), 120 rpm, for a total period of 7 days. Samples were drawn at regular intervals and the samples were analysed for (i) Total cell protein (ii) Residual phosphate in the medium for computing phosphate uptake, (iii) Cellular free inorganic Pi, (iv) Low Molecular weight phosphates (v) Polyphosphate and (vi) Nucleic acid phosphate (all estimated as inorganic phosphate Pi).
2.5.2.1 **Total cell protein:**

Total cell protein was estimated in 0.5ml of the broth. The sample was centrifuged at 10000 rpm at 4°C for 10 min. The pellet was re-suspended in 0.5 ml of 1N NaOH, boiled for 5 min., and the protein was estimated using the method explained by Herbert *et al* (1971).

2.5.2.2. **Estimation of the phosphates**

One ml of the culture was centrifuged at 10000 rpm, the supernatant was used for estimating the residual Pi using the Ascorbic acid method of Kato *et al* (1993) and the pellet was used to estimate the various intracellular phosphates based on the method of Harold *et al* (1966). All forms of phosphates were ultimately estimated as Pi using the Ascorbic acid method.

1. The pellet was re-suspended in 1ml 10% ice-cold TCA, vortexed, centrifuged at 15000 rpm. for 10 min.
2. 0.5 ml of the supernatant was used directly for Pi estimation as the cellular free Pi
3. The balance 0.5 ml of the supernatant was added with 1ml 5N sulfuric acid and a pinch of ammonium peroxodisulphate, autoclaved for 30min. and estimated Pi as the low MW phosphates.
4. The cell pellet was added with 1ml 1N HCl, boiled for 7min, centrifuged at 15000 rpm, for 10 min. and the supernatant was estimated for Pi as the polyphosphate.
5. The pellet was further added with 1 ml 5N sulfuric acid and a pinch of ammonium peroxodisulphate, autoclaved for 30min and estimated for Pi as the nucleic acid phosphate.

2.6 **Identification of Bacteria**

All the 26 isolates, selected based on their performance during the First phase screening and subjected to Second phase screening towards selection of potential strains for further study, were identified up to their generic level. Morphology of colony and cell, biochemical characteristics and physiological characteristics of the isolates were studied and based on the schemes suggested by the Bergy’s Manual of Determinative Bacteriology (1980) the cultures were assigned to various genera. No attempt was made to identify them up to their species level.

2.7 **Selection of Potential Strains for further studies**

Performance in terms of active phosphate uptake and release of Pi into the medium during their active growth, accumulation of inorganic phosphate as polyphosphate and various other forms
of phosphates during growth over 7 days of incubation, was evaluated for all the 26 cultures tested. Based on the results, two cultures were selected finally for further studies.

2.8 Impact of different conc. of phosphate in the medium on Pi uptake and polyphosphate accumulation by marine bacteria.

2.8.1. Medium

Zobell's Marine Broth (ZMB) (HI Media, India) was used throughout the study in order to have consistent and reproducible results besides providing otherwise optimal conditions for the growth of the selected marine bacteria. The medium was prepared by dissolving the dehydrated readymade medium in de-ionized water (DIW) and used after sterilization by autoclaving.

This ZMB was used as the basal medium and to this phosphate (KH₂PO₄) was added at different concentrations, in addition to the phosphate already present in the medium. Various levels of phosphate conc. in the medium tested included (1) 500 μM Pi (2) 3000 μM (3) 6000 μM (4) 9000 μM (5) 12,000 μM. ZMB without any additional Pi was considered as control, since the medium contain 1432 μM Pi (SD 12).

2.8.2 Inoculum preparation

A pre-culture of the selected strain was prepared first by inoculating 5 ml of Zobell's Marine Broth with a loopfull culture of 18 hrs. old ZM Agar slope culture and incubated for 18 hrs. in an orbitory shaker at 120 rpm, at room temperature (28 ±2°C). Later, using this pre culture, 50 ml of freshly prepared ZMB was inoculated at 1% (v/v) level and incubated at room temperature (28 ±2°C) on an orbitory shaker at 120 rpm for further period of 18 hrs. The culture broth obtained was centrifuged at 10,000 rpm, at 4°C for, 10 min. under sterile conditions, and the cells were harvested. Cell pellet was washed in physiological saline and suspended in the same. The prepared cell suspension (10 ml) was used as inoculum at 1% (v/v) level in all the subsequent experiments.

2.8.3 Inoculation and Incubation

Media prepared, in 100 ml aliquots, with different conc. of phosphates, were inoculated with the prepared inoculum at 1% (v/v) level, and incubated at room temperature (28 ±2°C) (unless otherwise mentioned), on an orbitory shaker at 120 rpm for a total period of 48 hrs. After incubation for the specified period, the culture broth was used for various assays.
2.8.4 Estimation of Biomass and Phosphates

Samples were drawn at 0, 1, 4, 8, 12, 20, 28, 36, and 48 hrs. of growth aseptically and subjected to analyses which was done in triplicate. The total cell protein, and Residual Pi and polyP were estimated as mentioned earlier, respectively, under sections 2.5.2.1. and 2.5.2.2.

2.9. Impact of pH on Pi uptake and polyP accumulation by marine bacteria

Impact of pH of the cultivation medium on the rate of inorganic phosphate (Pi) uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) adjusted to various levels of pH varying between 2 to 12, using 1 N NaOH and 1 N HCl. ZMB prepared in de-ionized water had a pH of 7.3 and this was considered as control for comparison purposes.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (section 2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.10 Impact of Incubation Temperature on Pi uptake and polyP accumulation by Marine bacteria

Impact of incubation temperature on the rate of inorganic phosphate (Pi) uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) at different incubation temperatures (RT (28 ± 2°C), 35, 40, 45, 50 and 55°C.).

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (section 2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.11 Impact of Additional Sodium chloride concentration in the medium on Pi Uptake and polyP accumulation by marine bacteria

Impact of NaCl in the cultivation medium on the rate of inorganic phosphate (Pi) uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of NaCl (0.5M,
0.7M, 0.9M, and 1.1M). The ZMB without additional NaCl was used as the control, since the medium already contained 0.33 M NaCl.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.12. Effect of other additional inorganic salts in the medium on Pi uptake and polyP accumulation by marine bacteria

2.12.1. KCl
Impact of KCl in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine *Vibrio* sp and *Achromobacter* sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of KCl (i) 0.014 M (ii) 0.021 M (iii) 0.028 M (iv) 0.035 M. The ZMB without additional KCl was used as the control, since the medium already contained 0.007 M KCl.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.12.2 CaCl₂
Impact of CaCl₂ in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine *Vibrio* sp and *Achromobacter* sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of KCl (0.03 M, 0.05 M, 0.07 M, 0.09 M). The ZMB without additional CaCl₂ was used as the control, since the medium already contained 0.016 M CaCl₂.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.
2.12.3 MgSO₄

Impact of MgSO₄ in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of MgSO₄ (0.1 M, 0.2 M, 0.3 M and 0.4 M). The ZMB without additional MgSO₄ was used as the control, since the medium already contained 0.073 M MgSO₄.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.12.4 Sodium Citrate

Impact of sodium citrate in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels using trisodium citrate (0.01 M, 0.02 M, 0.03 M, 0.04 M sodium citrate). The ZMB without additional trisodium citrate was used as the control, since the medium already contained 0.007 M Ferric citrate.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.12.5 Ammonium Nitrate

Impact of ammonium nitrate in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of ammonium nitrate (0.005 M, 0.01 M, 0.02 M and 0.03 M). The ZMB without additional ammonium nitrate was used as the control, since the medium already contained 0.002 M ammonium nitrate.
Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.13 Effect of other additional organic carbon sources in the medium on Pi uptake and polyP accumulation by marine bacteria

2.13.1. Peptone

Impact of peptone in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) adjusted to various levels of peptone (1%, 2%, 3% and 4% w/v). The ZMB without additional peptone was used as the control, since the medium already contained 0.5% peptone (w/v).

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.13.2 Yeast Extract

Impact of yeast extract in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of yeast extract (0.2%, 0.4%, 0.8%, 1.6% w/v). The ZMB without additional yeast extract was used as the control, since the medium already contained 0.1% yeast extract.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

2.13.3 Glucose

Impact of Glucose in the cultivation medium on the rate of inorganic phosphate uptake from the medium and polyphosphate accumulation by both marine Vibrio sp and Achromobacter sp was
studied in ZoBell’s Marine broth (ZMB) medium adjusted to various levels of Glucose (0.05 M, 0.10 M, 0.2 M) and added with 500μM Pi (KH₂PO₄) for each concentration of Glucose and for each culture, in order to understand the inhibitory effect of glucose on Pi uptake. The ZMB without additional glucose was used as the control.

Preparation of inoculum (section 2.8.2), inoculation and incubation (section 2.8.3), and estimation of biomass (2.5.2.1), residual Pi in the medium and polyphosphate in the cells (section 2.5.2.2) of the samples drawn at regular intervals (section 2.8.4) were all performed as mentioned earlier.

Residual Glucose in the medium was estimated by the Glucose Oxidase/Peroxidase (GOD/POD) Method (AUTOPAK, GLUC, Bayer Diagnostics India Ltd).

2.14 Statistical analyses

Standard deviation, and Mean were calculated using Microsoft Excel programme.