Materials and Methods

Glass wares cleaning

All the glass wares were soaked in Potassium dichromate solution (100 g of Potassium dichromate in 1 liter of distilled water followed by slow addition of 500 mL concentrated Sulfuric acid) for about 12 hrs and rinsed in tap water. Finally, they were washed in distilled water, dried and used.

Sterilization

The glass wares were sterilized at 180ºC for one hour in hot air oven. All the media were autoclaved at 15 lbs pressure for 15 min.

Chemicals

All the chemicals and media used in this work were purchased from Hi-media, Ranbaxy and Sigma limited and distilled water was used throughout the study.

pH adjustment

The pH of the medium was adjusted with 0.1N NaOH or 0.1N HCl.

Chemicals used

1. Sodium Azide (Sigma) Chemical Mutagen.
2. Ethyl Methane Sulphonate (Sigma) Chemical Mutagen.
3. Ammonium Molybdate (Ranbaxy) for Chloromolybdic acid reagent preparation.
4. Stannous Chloride (Ranbaxy) for Chlorostannous acid reagent preparation.
5. Hydrochloric acid (Ranbaxy) Solution preparation and pH adjustment.
6. Phosphate Buffer (pH 7.0)
Dissolve 1.20g of sodium dihydrogen phosphate and 0.885g of disodium hydrogen phosphate in 1 liter volume distilled water.

7. Chloromolybdic acid reagent for Phosphate Estimation
Ammonium molybdate (15g) was dissolved in 400ml of warm distilled water. The solution was cooled and 300ml of 10.0N Hydrochloric acid was added slowly. This was diluted to 1000ml after cooling.

8. Chlorostannous acid reagent for Phosphate Estimation
Stannous chloride (25g) was dissolved in 50ml of concentrated Hydrochloric acid and was diluted to 500ml using recently boiled distilled water. This solution was diluted with 1.2N HCl to about one liter.

9. Sulphuric acid (Ranbaxy) (7N) for Phosphate estimation
196 ml concentrated sulphuric acid in 1000 ml distilled water.


11. Kovac reagent (Himedia) for Biochemical identification of bacteria.

12. Barrit’s reagent (Himedia) for Biochemical identification of bacteria.

13. Lacto Phenol Cotton Blue (Himedia) Spore staining of fungal cultures.


15. Paranitro phenyl phosphate (PNP-P) (Sigma) (1%) : Substrate for Acid Phosphatase assay.
Dissolve 1 g of paranitro phenyl phosphate in 100 ml of distilled water.

16. Para nitro phenol (PNP) (sigma): Preparation of standard graph for Acid Phosphatase assay
Dissolve 1.5 g of paranitro phenol in 100 ml of distilled water.

17. Magnesium chloride (Ranbaxy) (1mM) for Acid Phosphatase assay
Dissolve 20.331 mg of Magnesium Chloride in 100 ml of distilled water.
18. Sodium Hydroxide (Ranbaxy) (0.2M) for Acid Phosphatase assay
   Dissolve 8 g of pellets Sodium hydroxide in 1000 ml of distilled water.

19. Sodium Acetate buffer (Ranbaxy) (0.1 M, pH 5.8) for Acid Phosphatase assay
   a) Dissolve 0.6g of Sodium acetate in 100 ml of Distilled water.
   b) Dissolve 0.6 ml of acetic acid in 100 ml of distilled water.
   From ‘a’ take 90 ml and from ‘b’ take 10 ml make the final volume of 100 ml.

20. Gum Arabic (Ranbaxy) for Lipase Assay
   3g Gum Arabic in 100 ml distilled water.


22. O.1 M Phosphate buffer (pH 7.0) for Lipase Assay.

23. Alcoholic Phenolphthalein indicator (Himedia) for Lipase Assay.

24. Sodium Hydroxide (Ranbaxy) (0.02N)
   Diluted from 1.0N Sodium hydroxide stock solution. (20ml stock made up to 1000ml with distilled water.

25. Sodium Hydroxide (Ranbaxy) (1.0N)
   40 g Sodium Hydroxide pellets dissolved in distilled water and made up to 1000ml with distilled water.

26. Silica gel G (Ranbaxy) for Thin Layer Chromatography.

27. Salkowski reagent for Indole Acetic Acid estimation
   2.03 g Ferric chloride dissolved in 500 ml water and 300 ml concentrated sulphuric acid.

28. Ethyl acetate, Chloroform and Formic acid (Ranbaxy) solvent system for Indole acetic Acid identification by Thinlayer Chromatography.

29. Glucose, Sucrose, Lactose and Mannitol (Ranboxy) act as carbon sources.

30. Ammonium sulphate, Potassium Nitrate, Sodium Nitrate and Urea (Ranboxy) act as nitrogen sources.
Media used

- Nutrient agar medium (Hi-media) for isolation and culture maintenance.
- Sabouraud agar medium (Hi-media) for isolation and culture maintenance.
- Starch agar medium (Hi-media) for biochemical identification of bacteria.
- Casein agar medium (Hi-media) for biochemical identification of bacteria.
- Certimide agar (Hi-media) for biochemical identification of bacteria.
- MR-VP broth (Hi-media) for biochemical identification of bacteria.
- Christensen citrate agar medium (Hi-media) for biochemical identification of bacteria.
- TSI agar medium (Hi-media) for biochemical identification of bacteria.
- SIM agar medium (Hi-media) for biochemical identification of bacteria.
- Urea agar base (Hi-media) for biochemical identification of bacteria.
- Gelatin agar (Hi-media) for biochemical identification of bacteria.
- Trypticase Nitrate broth (Hi-media) for biochemical identification of bacteria.
- Asparagine-Proline broth (Hi-media) for biochemical identification of bacteria.
- Pikovskaya medium (Hi-media) for isolation of Phosphate solubilizing bacteria.
- Pikovskaya broth (Hi-media) for Phosphate estimation and Acid Phosphatase assay.
- Czapek’s dox broth (Hi-media) Lipase and Indole Acetic Acid assay.
- Carbohydrate fermentation medium contains Glucose or Sucrose or Lactose or mannitol as a carbon source for biochemical identification of bacteria.
Soil sampling

Different types of rhizosphere soil were collected from the rice fields in and around Mannachanalur area of Trichy district, Tamilnadu for the isolation of bacterial and fungal cultures.

Isolation of phosphate solubilizing Microorganism

(Manjugupta and Bhriguvansji, 1997).

These soils were mixed and subjected to serial dilution technique using the stock soil suspension which contains 10 g of soil sample in 100 ml of sterile distilled water. From the serial diluted suspension, 1 mL of suspension from $10^{-3}$ to $10^{-6}$ dilution was taken for pour plate technique in Nutrient agar, Sabouraud agar and Pikovskaya agar medium. The Nutrient agar plates were observed after 2 days of incubation for enumeration of bacterial colonies, the Sabouraud agar plates were observed after 3 days of incubation for enumeration of fungal colonies. After 2-3 days of incubation, the plates were observed for phosphate solubilizing bacterial and fungal cultures from Pikovskaya agar medium. The bacterial cultures were then transferred to nutrient agar slants and fungal cultures to the Sabouraud agar slants for further studies.

Identification of Bacteria (Cowan, 1974) and Fungi (Josephgilman, 2001)

The bacterial culture was identified up to genus level by cultural, morphological and biochemical characteristics. From the 24 hrs broth culture of the isolated bacteria, 1ml of the culture was inoculated into Kings B agar, Cetrimide agar, Asparagine–Proline broth, MR-VP broth and carbohydrate fermentation tubes. One loopful of culture was inoculated into Christensan citrate agar, Urea agar, Gelatin agar, Starch agar, SIM medium (Stab) along with Oxidase and Catalase test.
After 24 – 48 hrs incubation, the results were observed in the Kings B agar and Cetrimide agar for colony morphology and pigmentation of the bacteria. Asparagine –Proline broth was observed for its fluorescents activity in UV transilluminator and other tests for the biochemical characterization purpose.

The bacterial culture was identified based on the observed results using bacteriological manual.

The fungal cultures were identified based on the colony morphology and spore structure. The spore structure was identified microscopically by Lacto phenol cotton blue method.

The above isolated bacterial and fungal cultures were tested for its Phosphate solubilization property and Phosphatase, Lipase and Indole Acetic Acid producton cabablity in liquid broth.

**Phosphate solubilization in liquid broth**

**Inoculation** (Sudhansupal, 1999; Varshanarishan et al., 1995)

The pure bacterial culture from nutrient agar slant was inoculated into nutrient broth. After 18 hrs of incubation, 0.5 mL of nutrient broth culture (1 O.D) was inoculated into 100 mL Pikovskaya broth containing 1g of Tricalcium Phosphate. The flask was incubated at 37 °C. The Pure fungal cultures from Sabouraud agar slants were transferred to Sabouraud agar plates. After four days of incubation at 27 °C, two 8 mm disc were cut from the plates by sterile borer from all three fungal plates and were inoculated into 100 ml Pikovskaya broth separately. The flasks were incubated at 27 °C. Uninoculated Pikovskaya broth served as control in each case. Each experiment was done in triplicate set.
**Phosphate estimation** (Artidave and Patel, 1999; Seshadri, 1995)

Growth medium was withdrawn aseptically at three days interval from each flask and centrifuged at 10,000 rpm for 20 min. The supernatant was analyzed for phosphate content by Chlorostannous Reduced Molybdophosphoric Blue Color Method. The clear 10 mL of supernatant was collected in a 50 mL volumetric flask and 10 mL of chloromolybdic acid reagent was added along the sides to the flask. The content was diluted to 40 mL and 5 drops of chlorostannous acid reagent was added. After thorough mixing the volume was made up to 50 mL quickly. The optical density was measured at 600 nm and the value was obtained with the help of the standard curve. Pigments produced by some of the cultures interfere with color development which was overcome by the addition of 1g of activated charcoal to each flask. The solution was filtered and the filtrate was taken for further analysis.

**Preparation of standard graph**

Potassium di-hydrogen orthophosphate (0.2195g) was dissolved in 400 mL distilled water and 25 mL of 7N H$_2$SO$_4$ was added. This solution was made up to 1000 mL with distilled water, which served as stock phosphate solution. From that 20 mL solution was taken and diluted to 500 mL, which was secondary phosphate solution. From the secondary solution 0.5 mL, 1 mL, 2.5 ml, 5 mL, 7.5ml, 10.0ml, 12.5ml, 15.0ml, 20.0ml and 25ml solutions was taken and Phosphate estimation was carried out and Optical Density (O.D) value was obtained as described above. The O.D values (X-axis) and phosphate in ppm (Y-axis) were plotted on a graph.

**Random UV mutational studies for Bacteria** (Kumar and Dhruv, 1990)

The 24 hrs old parent culture was used for the preparation of bacterial suspension, which was diluted with phosphate buffer (pH 7.0) contains $10^8$
cells/ml. The above culture was inoculated over the surface of nutrient agar plates. Then the plates were exposed to UV light (2600 Å) at a distance of 15cm in different time intervals and one plate was kept as growth control without exposing to UV light for control. After the above process the plates were covered with black paper to avoid light induced repair mechanism and were incubated at 37°C for 2 days.

Then the cultures were tested for phosphate solubilization as described above.

**Random UV mutational studies for Fungi** (Ellaiah et al., 2002)

The growth of 72 hrs old fungal cultures were scrapped off in 5ml sterile distilled water and diluted with 45 ml of sterile distilled water containing Tween 80 (1:4000). Sterile glass beads were added and were shaked on a rotary shaker for 30 min to break the hyphal mycelium. The suspension was filtered by using sterile cotton cloth to remove the mycelium. The spore suspension was prepared in phosphate buffer (pH 7.0) containing 10⁶ spores per ml. Five ml quantities of the spore suspension was pipette aseptically into sterile petri dishes of 90 mm diameter having a flat bottom. Then the plates were exposed to UV light (2600 Å) at a distance of 15cm from the center of the Germicidal lamp (UV light source) in different time intervals.

The exposure times were 10, 20, 30, 40, 50, 60, 70 and 80 min. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in phosphate buffer and plated on Sabouraud agar medium. The plates were incubated for 5 days at 27°C and the number of colonies in each plate were counted. Each colony was assumed to be formed from a single spore.
Then the cultures were tested for phosphate solubilization as described earlier.

The unexposed fungal suspension was inoculated into the Sabouraud agar medium that served as a growth (positive) control.

**Random Chemical mutational study** (Bapiraju *et al.*, 2004)

Sodium Azide and Ethyl Methane Sulphonate (EMS) were the chemical mutagens used in the present study for the strain improvement of phosphate solubilizers. The spore suspension of fungal cultures was prepared by using phosphate buffer pH 7.0 as described earlier. To 9 ml of spore suspension, 1 ml of sterile solution of Sodium Azide (250 μg ml⁻¹ in phosphate buffer) and Ethyl Methane Sulphonate (EMS) (150 μg ml⁻¹ in phosphate buffer) was added. The 24 hrs old bacterial culture was used for the preparation of bacterial suspension, which was diluted with phosphate buffer (pH 7.0) contains 10⁸ cells/ml. To a 9 ml of bacterial suspension, 1 ml of sterile solution of Sodium Azide (250 μg ml⁻¹ in phosphate buffer) and Ethyl Methane Sulphonate (EMS) (150 μg ml⁻¹ in phosphate buffer) was added. The above culture was inoculated and Samples were withdrawn from the reaction mixture at an interval of 30, 60, 90, 120 and 150 min. and centrifuged for 10 min. at 5000 rpm. The cells were washed three times with sterile distilled water and again re-suspended in 10 ml sterile buffer. The samples were serially diluted in the same buffer and plated over Nutrient agar and Sabouraud agar medium for bacteria and fungi respectively.

Then the cultures were tested for phosphate solubilization as described earlier.
The unexposed fungal suspension was inoculated into the Sabouraud agar medium that served as a growth (positive) control.

**Effect of different carbon sources**

To study the effect of different carbon sources on phosphate solubilization, the carbon source of Pikovskaya broth i.e. Glucose was replaced by Lactose, Sucrose and Mannitol. The mutated cultures which were superior in Phosphate solubilization were inoculated, incubated and Phosphate solubilization assay was carried out as described earlier.

**Effect of Different Nitrogen Sources**

To study the effect of different nitrogen sources on phosphate solubilization, the nitrogen source of Pikovskaya broth i.e. Ammonium Sulphate was replaced by Potassium Nitrate, Sodium Nitrate and Urea. The mutated cultures which were superior in Phosphate solubilization were inoculated, incubated and Phosphate solubilization assay was carried out as described earlier.

**Acid phosphatase Production**

**Inoculation** (Sudhansupal, 1999; Varshanarishan *et al.*, 1995)

The pure bacterial culture from nutrient agar slant was inoculated on nutrient broth. After 18 hours incubation, 0.5 ml of nutrient broth culture (1 O.D) was inoculated in 100 ml Pikovskaya broth containing 1g of Tricalcium phosphate. The flask was incubated at 37°C. The pure fungal cultures from Sabouraud agar slants were transferred to Sabouraud agar plates. After four days incubation at 27°C, two 8 mm disc were cut from the plates of all three fungal plates and were inoculated into 100 ml Pikovskaya broth. The flasks
were incubated at 27°C. Uninoculated Pikovskaya broth served as control in each case. Each experiment was done in triplicate set.

**Acid phosphatase assay** (Eileen ingham *et al.*, 1979)

The enzyme Acid phosphatase was assayed using para- nitrophenyl phosphate (PNP-P) as substrate. The reaction mixture contained 2.5 ml (0.1M) sodium acetate buffer (pH 5.8), 1 ml (1mM) Magnesium chloride, 0.5 ml 1% PNP-P and 0.5ml of a suitable dilution of enzyme preparation. One ml of the reaction mixture was transferred to 2ml of 0.2M Sodium Hydroxide before and after 15 min incubation at 37°C to stop the reaction. The Sodium Hydroxide solution added before incubation act as a control sample for each analysis. The amount of Para- nitro phenol (PNP) liberated was measured by recording the absorbance at 420 nm using an appropriate calibration curve. Activity is expressed as μmol PNP liberated min⁻¹. The blank was run in a similar manner using distilled water.

**Preparation of Standard curve**

Dissolve 1.0 g of Para- nitro phenol in water, and dilute the solution to 1000 ml. This is standard Para nitro phenol solution. Store the solution in a refrigerator. To prepare the standard graph, dilute 1 ml of the standard Para-nitro phenol solution to 100 ml in a volumetric flask and mix the solution thoroughly. Then pipette 0, 1, 2, 3, 4 and 5-ml aliquots (equaling to 0, 10, 20, 30, 40 and 50 μg of Para- nitro phenol) of this diluted standard solution into 5 ml volumetric flask and made up to 5 ml by addition of water, and proceed as described above. The O.D value (Y axis) and corresponding Para nitro phenol concentration (X axis) were plotted in a graph.
**Random mutational studies**

Physical and chemical mutations were carried out as described earlier. The bacterial and fungal cultures, obtained after Physical and chemical mutation were tested for Acid Phosphatase as described earlier.

**Effect of Different Carbon Sources**

To study the effect of different carbon sources on Phosphate solubilization, the carbon source of Pikovskaya broth i.e. Glucose was replaced by Latose, Sucrose and Mannitol. Then the mutated cultures which were superior in Acid Phosphatase were inoculated, incubated and Acid Phosphatase assay was carried out as described earlier.

**Effect of Different Nitrogen Sources**

To study the effect of different nitrogen sources on Phosphate solubilization, the nitrogen source of Pikovskaya broth i.e. Ammonium Sulphate was replaced by Potassium Nitrate, Sodium Nitrate and Urea. The mutated cultures which were superior in Acid Phosphatase activity in Pikovskaya broth were inoculated, incubated and Acid Phosphatase assay was carried out as described earlier.

**Lipase Production**

**Inoculation** (Sudhansupal, 1999; Varshanarishan *et al.*, 1995)

The pure bacterial culture from nutrient agar slant was inoculated on nutrient broth. After 18 hours incubation, 0.5 ml of nutrient broth culture (1 O.D) was inoculated in 100 ml Czapek’s dox broth containing 1% olive oil as inductor and also 10% olive oil in 100ml Czapek’s dox broth instead of Sucrose. The pure fungal culture from Sabouraud agar slant were transferred to Sabouraud agar plates. After four days incubation at 27°C, two 8mm disc were cut from all three fungal plates and inoculated into 100ml Czapek’s dox broth
containing 1% olive oil as inductor and also 10% olive oil in 100ml Czapek’s dox broth instead of Sucrose. The flasks were incubated at 27° C. Uninoculated Czapek’s dox broth served as a control in each case. Each experiment was done in triplicate set.

**Lipase assay** (Ray *et al.*, 1999)

Growth medium was withdrawn aseptically at three days interval from each flask and centrifuged at 3000rpm for 15 minutes and the supernatant was collected and used for lipase activity.

The enzyme solution prepared by mixing the supernatant solution with 1ml of olive oil and 3% gum Arabic in 250ml conical flask and the contents were emulsified using magnetic stirrer for 5 minutes at top speed.

After emulsification, 10ml of enzyme solution was taken in a flask followed by 20ml of double distilled water and 5ml of 0.1M phosphate buffer (pH 7). This mixture was kept on rotary shaker for 30 minutes at 120rpm. Simultaneously the same protocol was followed except for the enzyme solution and this was treated as a control. After 30 minutes a drop of 1% alcoholic phenolphthalein solution was added and titrated against 0.02 N Sodium Hydroxide (NaOH) till the appearance of pale pink colour.

**Lipase activity**

One unit of lipase activity was defined as the amount of enzyme required to release one µ mol of free fatty acid in one min under standard assay conditions.

\[
\text{Lipase activity} = \frac{\text{Volume of NaOH consumed} \times \text{Normality of NaOH}}{\text{Time of incubation} \times \text{Volume of enzyme solution expressed as Unit/ Substrate}} \times 1000
\]
Random mutational studies

Physical and chemical mutation was carried out as described earlier. The bacterial and fungal cultures, obtained after Physical and chemical mutation were tested for Lipase as described above.

Effect of different Carbon Sources

To study the effect of different carbon sources on lipase production, the carbon source of Czapek‘s dox broth i.e. Sucrose was replaced by Lactose, Glucose and Mannitol. The mutated cultures which were superior in Lipase activity were inoculated, incubated and Lipase assay was carried out as described earlier. When Sucrose, Lactose, Glucose and Mannitol as a carbon source in Czapek’s dox broth, 1% olive oil was used as a inducer.

Effect of different Nitrogen Sources

To study the effect of different nitrogen sources on lipase production, the Nitrogen source of Czapek’s dox broth i.e. Sodium Nitrate was replaced by Potassium Nitrate, Ammonium Sulphate and Urea. The mutated cultures which were superior in Lipase activity were inoculated, incubated and Lipase assay was carried out as described earlier. When Sodium Nitrate Potassium Nitrate, Ammonium Sulphate and Urea as a nitrogen source in Czapek’s dox broth, 1% olive oil was used as a inducer.

Indole Acetic Acid (IAA)

Indole Acetic Acid Production (Maria Guineth et al., 2000)

The isolated bacterial and fungal cultures were subjected to Indole Acetic Acid production by inoculating the cultures in Czapek’s dox broth.
The pure bacterial culture from nutrient agar slants was inoculated on nutrient broth. After 18 hrs incubation, 0.5 mL of nutrient broth culture (1 O.D) was inoculated in 100 mL Czapek’s dox broth and incubated at 28°C for 3 days.

The pure fungal cultures from sabouraud agar slants were transferred to Sabouraud agar plates. After 5 days incubation at 27°C two 8mm discs were cut from the fungal plates & were inoculated separately into 100ml Czapek’s dox broth. The flask were incubated at 30°C for 4 days. (Mittal et al., 2008)

**Screening of Indole Acetic Acid producer** (Maria Guineth et al., 2000)

Culture broth was withdrawn aseptically after three days incubation for bacteria and five days incubation for fungi from each flask and centrifuged at 3000 rpm for 15 minutes and the supernatant was collected and used for IAA assay by Thin layer chromatography. The centrifuged sample of each broth were brought to pH 3.0 and extracted three times with ethyl acetate. The organic phase was concentrated to dryness and the diluted with 0.5 ml methanol. Application of this solution on silica gel G plate (20cm X 5cm) and chromatogram was developed with Chloroform –ethyl acetate-formic acid (5:3:2) solvent system and then developed with Salkowiski reagent.

**Quantitative estimation of Indole Acetic Acid** (Tsavkelova et al., 2007)

The quantitative estimation of Indole Acetic Acid in broth cultures were determined by a colorimetric assay technique using Salkowski reagent. The two part of the supernatant, one part of the Salkowski’s reagent was added. The O.D was determined at 530nm in a spectrophotometer. A standard curve was prepared from serial dilutions of IAA stock solution.
Preparation of standard graph for Indole Acetic Acid

1. 100mg of Indole Acetic Acid was weighed and dissolved in 100ml of distilled water by slight heating. From this stock solution, Indole Acetic Acid standard solutions were as prepared in different concentrations.

2. 8ml of the Indole Acetic Acid standard solution was taken with 2ml Salkowski reagent. It was incubated at room temperature in dark for 30 minutes. Blank was prepared by taking 8ml of distilled water with 2ml of Salkowski reagent.

3. Optical density was read at 530nm and plotted a graph for the concentration of Indole Acetic Acid against optical density.

Estimation of Indole Acetic Acid

1. 8ml of culture filtrate was taken and 2ml of Salkowski reagent was added to it. It was incubated at room temperature in dark for 30 minutes for the development of pink colour.

2. The optical density was read at 530nm.

3. The results were read from standard graph and expressed µg/L

Random mutational studies

Physical and chemical mutation was carried out as described earlier. The bacterial and fungal cultures, obtained after Physical and chemical mutation were tested for IAA qualitatively and quantitatively as described above.