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Marine Yeast *Candida tropicalis* as Alternative Source of Biofertilizer

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ABSTRACT: Isolate and identify the phosphate solubilization ability of marine yeast *Candida tropicalis* and its alternative approach for bio fertilizer. The marine yeast was isolated and subjected to observe phosphate solubilization of yeast by plating in Pikovskaya medium and the characterization of yeast was done by the microbial and biochemical methods. The phosphate solubilization was measured by phosphate molybdenum method. The growth of *Sorghum bicolor* crop and seed was measured by using phosphate solubilized yeast in the field.

Keywords: *Candida tropicalis,* phosphate solubilization, biofertilizer, Pikovskaya medium.

INTRODUCTION

Soil generally contains adequate amount of organic and inorganic Phosphorus. But most of these remain unavailable to plants. Phosphorus is one of the most important nutrients limiting plant production. Despite Phosphorus being distributed widely and abundantly in soil in both its inorganic and organic forms, many soils throughout the world are deficient in Phosphorus. The efficiency of water-soluble Phosphorus is usually low, its recovery does not exceed 20%. But by the help of phosphate solubilizers, phosphate availability in soil and the utilization of phosphate by plants are increased. Microbial solubilization of insoluble phosphate especially low grade and its use in agriculture is receiving greater attention. This process not only compensates for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to the soil. Therefore, large amounts of costly phosphate fertilizer are applied to soils to satisfy the demands of plant growth. Occurrence and isolation of PSM Solubilization of insoluble Phosphorus by yeast was reported by Pikovskaya (1948). During the last two decades knowledge on phosphate solubilizing yeast increased. Several strains of bacterial and fungal species have been described and investigated in detail for their phosphate-solubilizing capabilities (Sharma *et al.*, 2013). Typically, such phosphate- solubilizing yeast has been isolated using cultural procedures with species of *Saccharomyces cerevisiae, Cryptococcus albidus* and *Candida tropicalis* (Gizaw *et al.*, 2017). These organisms are ubiquitous but vary in density and mineral phosphate solubilizing (mps) ability from soil to soil or from one production system to another. In soil Phosphorous solubilizing bacteria constitute 1-50% and fungi 0.1-0.5% of the total respective population. They are generally isolated from marine and non-marine soils, rhizoplane, hyllosphere, and marine Phosphorous deposit area soil and even from stressed soils using serial plate dilution method or by enrichment culture technique (Sharma *et al.*, 2013). The concentration of iron ore, temperature, and C and N sources greatly influence the P solubilizing potentials of these microbes. Among the various nutrients used by these P solubilizing microorganisms, ammonium salts have been found to be the best N source followed by asparagine, sodium nitrate, potassium nitrate, urea and calcium nitrate (Alori *et al.*, 2017). Since 1948, when Pikovskaya suggested that microbes could dissolve non-readily available forms of soil Phosphorus and play an important role in providing Phosphorous to plants. Numerous methods and media, such as Pikovskaya, bromophenol blue dye method and National Botanical Research Institute Phosphorus (NBRIP) medium (Joe *et al.*, 2018). The source of insoluble phosphate in the culture media to isolate PSY is a major issue of controversy regarding the isolation of Phosphate Solubilizing Yeast in true sense. Commonly used selection factor for this trait, tricalcium phosphate (TCP), is relatively weak and unreliable as a universal selection factor for isolating and testing phosphate solubilizing yeast (PSY) for enhancing plant growth. The use of TCP usually yields many (up to several thousand per study) isolates of "supposed" Phosphate Solubilizing Yeast When these isolates are further tested for direct contribution of Phosphorus to the plants, only

a very few are true Phosphate Solubilizing Yeast (Wan *et al.,* 2020).

MATERIALS AND METHOD

Marine yeast isolation and identification Marine yeast isolation from soil: The research area is in Parangipettai, on the southeast coast of India (Lat. 110.29'N; Long. 790.46'E), in a mangrove vegetation that has been raised artificially along the banks of the Vellar estuary. The sea soil was collected here. By applying the procedure with minor improvements, serial dilution and spread plate was used to isolate marine yeast (Zaky *et al.,* 2016). After being autoclaved to prevent growth of the bacteria, the yeast isolate was maintained on modified YEPD media (Yeast extract 0.3%, Peptone 0.5%, $(NH_4)3SO_4$ 0.1%, KH_2PO_4 0.025%, Dextrose 3%, supplemented with chloramphenicol (200 mg/l), and incubated at 30°C for 2 days.

Yeast morphological identification: Simple staining, colony color, cell shape, and size, as well as aerobic carbon and nitrogen utilization tests, and carbon fermentation assays are used to identify a single strain of yeast under the microscope.

Based on PCR identification: Using the primers ITS4 (5'-TCCTCCGCTTATTGAT ATG-3') and ITS5 (5'GGAAGTAAAAGTCGTAA CAAGG-3') as described by Qvirist *et al.* (2016), selected marine isolates were identified using their internal transcribed spacer (ITS) region sequences (Zaky *et al.,* 2016). Each reaction's constituents for the PCR master mix for amplification includes 8 l of 10 PCR buffer, 1 unit of Taq DNA polymerase, 25 pmol of each forward and reverse primer, 100 M of each deoxy nucleoside triphosphate, and enough distilled water to make a 50-l total reaction volume. Using a micropipette tip, a tiny amount of yeast growth from a culture (24–48 h) was taken, suspended in 50 l of deionized water, and then incubated for 10 min at 95 °C. The warmed yeast suspension was then diluted by 4 l and added to the PCR tubes as a DNA template. The tubes were then put into a thermocycler with the following settings: initial denaturation at 94 °C for 30 s, then 35 cycles at 94 °C for 15 s, 52 °C for 45 s, and 72 °C for 30 s, with a final extension step at 72 °C for 10 min. Electrophoresis separation using 1% agarose gel with 4 l of ethidium bromide in TBE buffer was used to identify the PCR products (0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA, pH 8.3). Prior to sequencing, the MinElute Reaction Cleanup Kit (QIAGEN) was utilized for purification. For comparison with already accessible sequences, the acquired sequences were aligned using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

All PCR clones' core sequences were used for the ITS phylogenetic comparisons, and any lengthy sequences were shortened to ensure that all bases were included in the multiple sequence alignments. Too-short sequences were excluded. Megalign, DNA Star, and ClustalW embedded in Megalign were used to create the alignments and create the cladograms of the phylogenetic relationships. One thousand generated trees were used in the bootstrap analysis.

In vitro screening of yeast phosphate solubilizing activity: The selected yeast strain was subjected to plating in Pikovkaya's medium (yeast extract 0.5%, Dextrose 10%, Calcium phosphate 5%, Ammonium sulphate 0.5%, Potassium chloride 0.2%, Magnesium sulphate 0.1%, Manganese sulphate 0.0001%, Ferrous sulphate 0.0001%, Agar 15% /liter) Measured and mix thoroughly heat the agar medium and boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes. Then portion equally into petri plates, incubate into 30°C for 24-48 hours.

Phosphate Solubilizing by Phosphate Molybdenum blue assay: The phosphate solubilization assay, Pikovskaya broth is prepared and sterilized by autoclaving at 15 Lbs pressure (121°C for) 15 minutes. Then the media was transfer in conical flask. After the cooled in medium inoculate the yeast and incubate in orbital shaker at 30°C for 10 days. After agitation the phosphate solubilization measured by Phosphate molybdenum blue assay.

Reagents

Sulfuric Acid (2.5 M), Ammonium molybdate (20g into 500ml water), Potassium antimonyl tartrate (0.28g into 100ml water), Ascorbic acid (1.76g into 100ml water) Mixed reagent 1(MR1) for neutralized eluents. 10ml A + 3ml B + 1ml C + 6ml D.

Add 1ml the mixed reagent 1(MR1) into a 5 ml sample of eluent (diluted if necessary), wait for 15-20 minutes for color to develop and then measured in colorimeter at 570nm. The concentration in the eluent or its derived diluted solution can be measured by preparing a calibration curve using standard phosphate solution. Ensure that the standard solution contains the same concentration of salt as in the eluent, resulting from the neutralization. The same procedure as above is followed using 5 ml of standard solution.

Effect of Phosphate solubilization yeast on sorghum Plant and sorghum seed: To study the effect of Phosphates solubilizing yeast on the growth of sorghum plant crop and sorghum seed, an experiment was conducted with the seeds of sorghum which were purchased from Rasipuram, Namakkal district.

***Sorghum* Plant Crop:** The sorghum plant crop and soil were taken from the forming land. Then the soil was transfer into the soil these are respectively control, sample 1, sample 2, sample 3. Initial crop size was measured.

***Sorghum* seed:** The soil sample was taken from the forming land. Then the soil was transfer into pots. The pots are filled with equal kg of soil (750 grams). The sorghum seed was inoculated into the soil these are respectively control, sample 1, sample 2.

RESULTS AND DISCUSSION

Isolation of marine yeast: The marine yeast strains were kept alive on modified YEPD medium and were isolated from sediments of the Vellar estuary, Parangipettai, and the southeast coast of India (Fig. 1). According to a review of the literature, there have been very few studies on marine yeasts, and knowledge of marine mycota is still limited. The distribution of the species, their numbers, and their metabolic traits were discovered (Kutty *et al.,* 2008). Marine yeasts have a special potential and can survive in harsh environments.

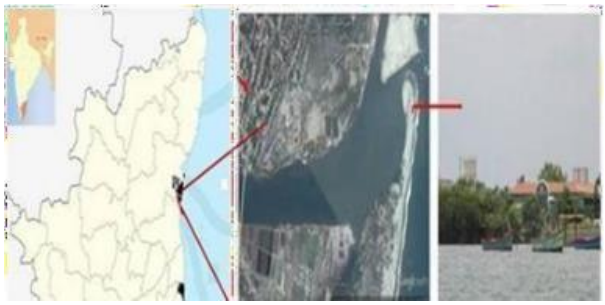

Fig. 1. Map of study area: Vellar estuary, Parangipettai, Southeast coast of India.

Morphological identification of *MYCt*: Isolated yeast was morphologically identified by Trinocular microscope followed by simple staining (Fig. 2). Initially, yeast isolate *MYCt* was inoculated into modified YEPD media (Murthy *et al*., 1975).

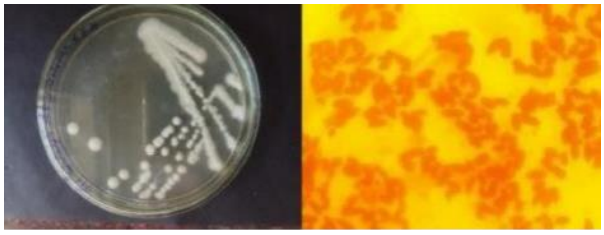

Fig. 2. (i) Morphology of yeast (*MYCt*) on plate (ii) Microscopic view of yeast.

Phylogenetic analysis of the isolate: The yeast isolate MYCt was shown by phylogenetic analysis of 18S rRNA to belong to the genus Candida tropicalis (Fig. 3). Candida tropicalis (MYCt) isolate MCAS01, together with the nucleotide sequence of the marine yeast isolate MYCt, has been added to the NCBI database with accession number MZ723324.1.

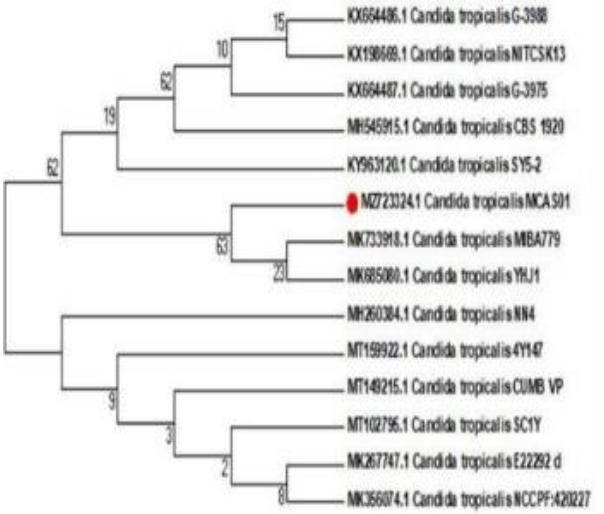

Fig. 3. The Phylogenetic position of Yeast isolate *MYCt* obtained with neighbor-joining method. The optimal tree with the sum of branch length = 0.37221527 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The tree was constructed by a neighbor-joining method using the Mega 6 tools and the sequences were aligned by clustal W and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 6. Accession number MZ723324.1 and the name of *Candida tropicalis* isolate (*MYCt*)MCAS01.

Isolation and Identification of phosphate solubilizing yeast: The isolate showing clear halo-zone were observed on Pikovskaya's agar and were single streaked on Pikovskaya agar Fig. 4. Use of phosphate solubilizing yeast has been reported promising in reducing phosphate fixation and increasing the phosphorous availability from soluble and insoluble phosphatic fertilizers. Beneficial effect of inoculation of phosphate solubilizers on the uptake of nutrients and on the yield of crops has been reported by many workers (Amri *et al.*, 2022).

Fig. 4. Clear halo-zone were observed on Pikovskaya's medium.

Phosphate Solubilizing by Phosphate Molybdenum blue method: The role of yeasts in agriculture has been a matter of interest due to their abundant population and potential as plant growth promoters. In the present investigation results of solubilization by these yeast isolates in Pikovskaya liquid medium. The isolate could effectively solubilize phosphate in the broth, and the percentage of soluble phosphate released by the isolate increased significantly during after 3 days of P-solubilizing experiments, while there was no significant change in the percentage of soluble phosphate released under control conditions. The results indicate that these yeast isolates have a great potential for use as potential P solubilizers for use in natural soils. Isolates varied with respect to levels of P solubilization achieved. *Candida tropicalis* was the most efficient strain for P solubilization and released the largest percentage of soluble phosphate after 3 days. Phosphate solubilization in Pikovskaya's broth medium for both isolates were analyzed quantitatively. Use of phosphate solubilizing yeast has been reported promising in reducing phosphate fixation and increasing the Phosphorus availability from soluble and insoluble phosphatic fertilizers. Beneficial effect of inoculation of phosphate solubilizers on the uptake of nutrients and on the yield of crops has been reported by many workers. There is increasing evidence that Phosphate yeast improve plant growth due to biosynthesis of plant growth substances rather than their

action in releasing available Phosphorus (Chaykovskaya *et al.*, 2001). The solubilized phosphate was determined by Phosphate Molybdenum blue method (Fig. 5 & 6 and Table 1).

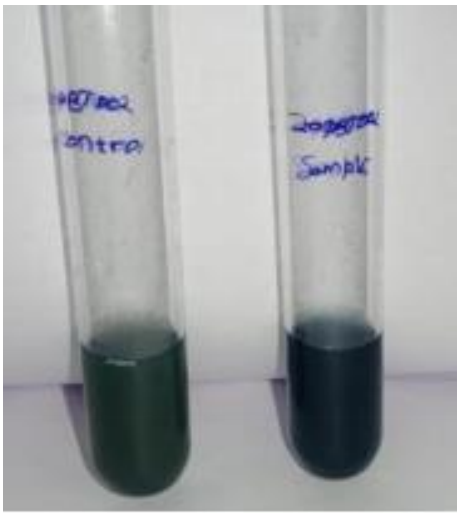

Fig. 5. Solubilized phosphate was determined by Phosphate Molybdenum blue method.

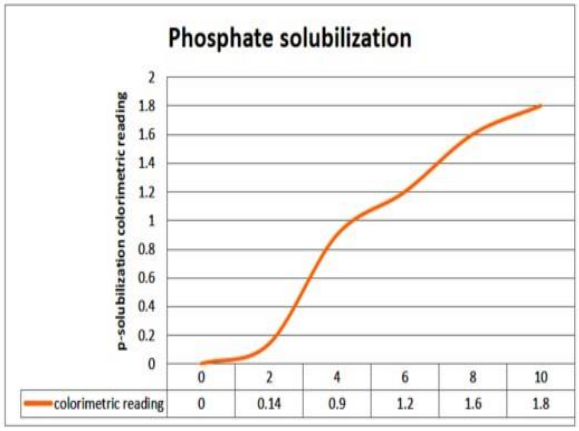

Fig. 6. Graphical representation of Phosphate solubilization.

Table 1: P-solubilization colorimetric reading.

Sr. No	Days	Control	Colorimetric reading
1	Day 2	0.00	0.14
2	Day 4	0.00	0.09
3	Day 6	0.00	1.2
4	Day 8	0.00	1.6
5	Day 10	0.00	1.8

Sorghum plant crops growth: After 15 days of treatment, the plant growth characteristics *viz.*, average root length, average shoot length and number of roots were analyzed, and average and standard deviation were calculated (Table 2 & Fig. 7). Solubilization of insoluble phosphorus by microorganisms was reported by Pikovskaya (1948). during the last two decades knowledge on phosphate solubilizing microorganisms increased significantly. Phosphate solubilization in Pikovskaya's broth medium for both isolates were analyzed quantitatively. Use of phosphate solubilizing yeast has been reported promising in reducing phosphate fixation and increasing the Phosphorus availability from soluble and insoluble phosphatic fertilizers. Beneficial effect of inoculation of phosphate solubilizers on the uptake of nutrients and on the yield of crops has been reported by many workers. There is increasing evidence that Phosphate yeast improves plant growth due to biosynthesis of plant growth substances rather than their action in releasing available Phosphorus. (Chaykovskaya *et al.*, 2001).

Fig. 7. Sorghum plant crops growth level.

Sorghum plant seeds germination: The seed was inoculated in the soil. After 5 days the seeds were germinated, and the size of plant was measured respectively 1 to 12 days (Table 3 & Fig. 8). Phosphorus deficiencies are widespread on soil throughout the world and one of the limiting factors for crop productivity. Phosphorus fertilizers represent a major cost for agricultural production. Many bacteria, fungi and a few actinomycetes are potential solubilizers of bound phosphates in soil thus playing an important role making it available to plants in the soluble form.

Fig. 8. Sorghum plant seeds germination growth level.

Table 2: Sorghum Plant growth measurement.

S. No	Days	Day1	S. D	Day2	S. D	Day3	S. D	Day4	S. D	Day5	S. D	Day6	S. D	Day7	S. D	Day8	S. D
1	Control (cm)	6.2	±0.2	5.9	±0.1	6.9	±0.1	7	±0.1	7	±0.1	7.9	±0.3	8.8	±0.3	10.2	±0.2
2	750gm Soil+5ml Yeast	5.8	±0.2	7	±0.1	7.5	±0.1	7.9	±0.1	9.3	±0.2	10.2	±0.2	10.3	±0.2	11.3	±0.1
3	750gm Soil+10ml Yeast	6.1	±0.1	7.2	±0.3	8.4	±0.2	10	±0.3	10.1	±0.1	10.4	±0.2	11.2	±0.2	12	±0.1
4	750gm Soil+15ml Yeast	6	±0.4	7.8	±0.1	9.1	±0.2	9.6	±0.1	10.2	±0.2	11.1	±0.1	11.9	±0.2	13.5	±0.2

Table 3: Sorghum Plant seeds germination growth measurement.

Sr. No.	Days	Control	1kg forming soil+5mlyeast culture	1kg forming soil + 10ml yeast culture
1	Day 1	-_	-_	-_
2	Day 2	-	-	-
3	Day 3	-_	-	-_
4	Day 4	-_	-_	-_
5	Day 5	-_	-_	-_
6	Day 6	0.5cm	1cm	1cm
7	Day 7	1cm	1cm	1.5cm
8	Day 8	2cm	2cm	2.5cm
9	Day 9	2cm	2.5cm	3cm
10	Day 10	3cm	3cm	4cm
11	Day 11	5cm	5.5cm	6cm
12	Day 12	6cm	7cm	8cm

CONCLUSION

Phosphorous is an essential macronutrient for plant growth and development. About 95- 99% present in soil insoluble form. Phosphate solubilizing microorganisms can increase soil phosphate solubility and availability. This study was aimed to identify and evaluate phosphate solubilizing yeast, yeast species were positive in phosphate solubilizing ability. Therefore, these species can be candidate and exploited after further evaluation as bio fertilizers. Utilization efficiency of crops for phosphate chemical fertilizer is around 30%, the remaining 70% exist in compound and bound form, such economic considerations and phosphate existence in compound form necessitate for an alternative less expensive and environmentally friendly bio fertilizer improving yield and quality.

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