Journal Homepage: -www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/22703
DOI URL: http://dx.doi.org/10.21474/IJAR01/22703

ISSN (O): 2320-5407
ISSN (P): 3107-4928

*RESEARCH ARTICLE*

# ISOLATION AND CHARACTERISATION OF HALOTOLERANT AND PHOSPHATE-SOLUBILIZING YEAST FROM SUBURBAN MUMBAI

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***Manuscript Info***

***Manuscript History***
Received: 16 November 2025
Final Accepted: 18 December 2025
Published: January 2026

***Key words:-***
Yeast, Phosphate-Solubilizing, Halotolerant, Biochemical Characterization

***Abstract***

Phosphate-solubilizingyeast is a type of organism that has not received extensive study regarding its contribution to the environment. The earth is made up of 70% of water in which the majority consists of saltwater. Halotolerance is one such characteristic that allows an organism to survive even in the saltiest of the waters. The aim of this study was to isolate and identify Phosphate-solubilizing, halotolerant yeasts and to study its characteristics. The given yeast was isolated from the waters of Bandstand in Bandra, Mumbai based on its ability to perform phosphate-solubilizing activity. The yeast showed better growth when it was incubated at 37°C. The salt tolerance of the isolate was studied against0 to 20% concentration of NaCl in which its survival was observed up to 6% NaCl. The isolate was then characterized with an array of biochemical tests such as the Alkaline Phosphatase test, Sugar Fermentation tests, Urease test, Citrate test, Germ tube test, and Amino acid test. Based on these tests, the isolated sample is speculated to be Pichiaor Groenewaldozyma.



## Introduction:-

Phosphorus is an essential macronutrient vital to existence, serving as a key element in various biological processes. It also acts as a limiting factor, constraining the overall productivity of these environments. Phosphorus existing in the form of insoluble minerals in the environment has driven numerous microorganisms, bacteria among them, have evolved specialized mechanisms to transform these inorganic phosphorus forms into soluble ones, thereby rendering them accessible to plants and other organisms. Among these microorganisms, phosphate-solubilizing bacteria (PSB) have garnered significant attention due to their capacity to augment nutrient uptake in plants and their contribution to sustainable agricultural practices. The phosphate scarcity, however, has presented an array of stressors in various ecosystems leading to constraints on the microbial communities. This has resulted in studies carried out to isolate and characterize phosphate-solubilizing organisms in an attempt to mitigate the problems faced by environments facing scarcity of the mineral. Previous studies on the same have yielded in isolation of phosphate solubilizing halotolerant microorganisms mostly of Bacillus spp having a strong capacity to dissolve calcium phosphate between 2.25 and 442 $mgL^{-1}$[9]. The phosphate solubilizing ability of halotolerant organisms has also been evaluated, mainly from rhizospheric soils [14][20].These organisms were mainly used for plant growth enhancement, improving soil

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biochemical properties, and salt-affected agriculture [8]. Furthermore, along with bacteria, there have been studies to isolate phosphate-solubilizing yeast. In one such study an extremely halotolerant yeast – Candida versatiliswas reported to tolerate up to 4 M NaCl[17] and thus could contribute to areas affected by extreme salinity. Studies carried out have isolated phosphate solubilizing yeasts from Teff [Eragrostistef (Zucc.) Trotter] rhizosphere soil which were then used as bio-fertilizers for increasing teff productivity [6]. Additionally, the isolation of Meyerozymaguilliermondii, a halotolerant yeast proficient in decolorizing and detoxifying azo dyes, offers promising solutions for treating azo-dye-containing wastewaters [5]. However, research regarding halotolerant phosphate solubilizers is focused largelyon bacteria. There is limited work carried out on halotolerant yeasts that are phosphate solubilizers. In this study, we delve into the less-explored territory of halotolerant phosphate solubilization. Our research shifts the spotlight from well-studied phosphate-solubilizing bacteria (PSB) to the relatively underrepresented halotolerant yeast community. The unique challenges presented by highly saline environments serve as the backdrop for our investigation. Unlike prior research primarily centered on halotolerant bacteria, we embark on an exploration of halotolerant yeasts as potential phosphate solubilizers. This rare focus paves the way for novel insights into their role in phosphorus cycling within extreme saline conditions. Our study will help in identification of potential halotolerant yeast which could contribute to areas affected by extreme salinity.

## Material and Methods :-

**Sample and Isolation:**

One sea water and one wet sand samples from different coastal reagions of Mumbai were collected. The water sample was acquired from Bandstand Promenade, Bandra, Mumbai Suburban, Maharashtra, India (19.0427179°N 72.819131G°E), and a second wet sand sample was acquired from Juhu Beach, Mumbai Suburban, Maharashtra, India (19.10°N 72.83°E). The water sample was serially diluted in 0.89% sterile saline and the $10^{-4}$ and $10^{-5}$ dilutions were plated. The sand sample was suspended in 100ml of 0.89% sterile saline (1g in 100ml) and 100 µl was plated. Both the samples were plated initially on modifiedPikovskaya Agar containing 3% NaCl. The plates were then incubated at 25°C for 48 hours. Both the samples were also inoculated in Modified National Botanical Reseach Institute's Phosphate (NBRIP) broth with Bromophenol blue (BPB) indicator containing 3% NaCl. The broth tubes were then incubated at 25°C for 48 hours. Both the mediums utilized are selective mediums that serve for specifically screening probable phosphate solubilizing yeast. After sufficient growth purification of yeast isolate was achieved by the streak method and propagation was achieved by the growth of the isolated colonies from plates in YPD broths at 37°C. Long-time preservation was achieved by inoculating the yeast isolates on Nutrient Agar slants and YPD broth.

**Identification of Yeast Strain:**

**A. Morphological Characteristics:**

The yeast strains isolated were first checked for their morphological and growth aspects. Characteristics like textures (Mucoid, viscous); elevation (flat or raised); Colour (White, yellow, etc.); Surface (rough, dull, smooth); margin (entire, undulated, Filamentous, lobed) were identified. The actively growing culture was investigated morphologically by staining the cells and observing them under a microscope to determine the shape of the cells, and mode of cell division: budding or fission. The formation of germ tubes was tested by inoculating the 24 hrs actively growing culture in human blood serum at 37°C for approximately 1-2 hrs and then investigated under a microscope.

**B. Physiological and Biochemical Characteristics**

**Sugar Fermentation Test**

The ability of the isolated yeast to utilize, grow aerobically and ferment sugars as a sole carbon source was studied. Several carbon sources (D-Glucose, D-Maltose, D-Trehalose, D-Sorbose, D-Melezitose, D-Melibiose, D-Xylitol, D-Fructose, D-Raffinose, D-Sucrose, D-Inositol, D-Xylose, D-Arabinose, D-Cellobiose, D-Mannitol, L-Lactose) were studied. Briefly Sterile 1% Peptone broth with Andrade's indicator and 1% final concentration of the respective sugars were inoculated and incubated at 37 ºC for 24h. Growth was indicated by presence of turbidity and Sugar utilization was indicated by change in color of the medium to dark pink suggesting production of acid from the sugars. The test tubes contained an inverted Durham's tube to check for gas production. Anaerobic fermentation was studied by overlaying the inoculated tube with sterile paraffin oil prior to incubation.

**Amino Acid Utilization Test**
To check the ability of yeast to utilize nitrogen source as a sole source of energy. The yeast isolate was inoculated in L-lysine, L-arginine, and L-ornithine. The cultures were inoculated in the solutions to study both aerobic and anaerobic utilization of nitrogen. The anaerobic tubes were layered with mineral oil to prevent air contact. The color change was an indicator of decarboxylase and deaminase activity.

**Citrate Utilization Test:**
Certain yeast strains can utilize citrate as a carbon source. The yeast isolate was inoculated on Simmon's Citrate Agar slants containing bromothymol blue as an indicator. If the yeast isolates can utilize citrate as a carbon source a significant color change can be observed from green to blue.

**Phenylalanine Deaminase (PPA) Test:**
This test was performed to test the ability of the organism to produce a deaminase enzyme. Oxidative deamination of phenylalanine forms phenyl pyruvic acid which in reaction with ferric chloride ($FeCl_3$) shows a significant color change from orange to green. The yeast isolates were streaked on a PPA agar slant and incubated at 37°C for growth to occur.

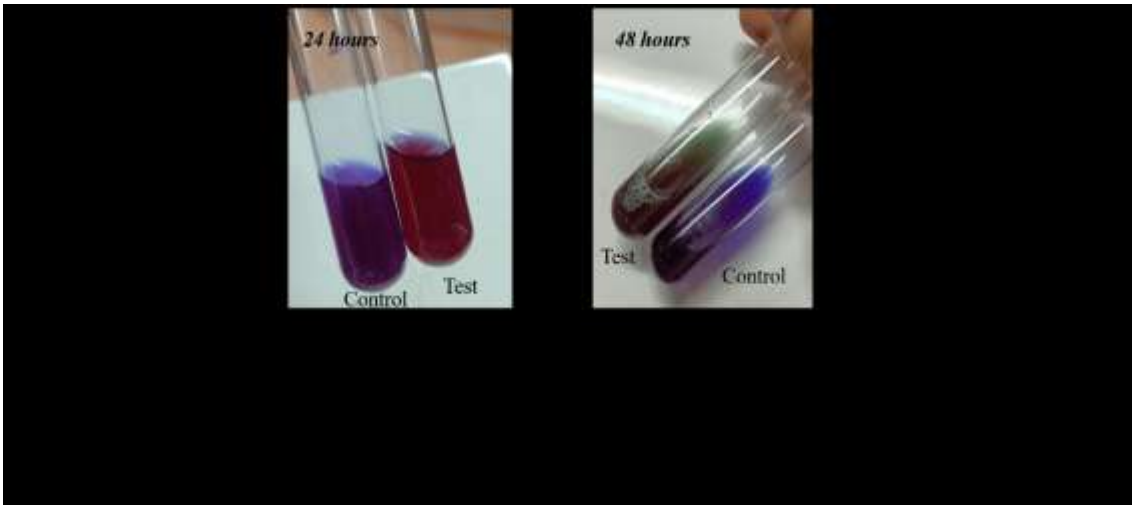
**Starch Utilization Test:**
The yeast isolates were streaked on a nutrient agar plate containing starch as a sole carbon source. Decolorization around the colonies is observed when iodine is poured on the agar plate. The starch utilization test is used to identify amylase activity in yeast.

**Urea Hydrolysis:**
Urea when hydrolysed produces ammonia ($NH_3$) and carbon dioxide ($CO_2$). Yeast isolates were streaked on Christensen's Agar to check for urea hydrolysis. On hydrolysis, Christensen's Agar displays light pink coloration.

**Alkaline Phosphatase Activity Assay (ALP):**
This test detects the presence of a phosphatase enzyme. Yeasts can produce both intracellular and extracellular phosphatase enzymes. The presence of phosphatase cleaves the phosphodiester bond in substrate: p-nitrophenyl phosphate releasing p-nitrophenol which is yellow in colour. The colour intensity is then measured using a colorimeter. Erba Manheim LIQUIXX-M ALKALINE PHOSPHATASE assay kit was utilised. The cell-free extract and cell extract were inoculated in the reagents and stored overnight at 37°C.

## Results:-

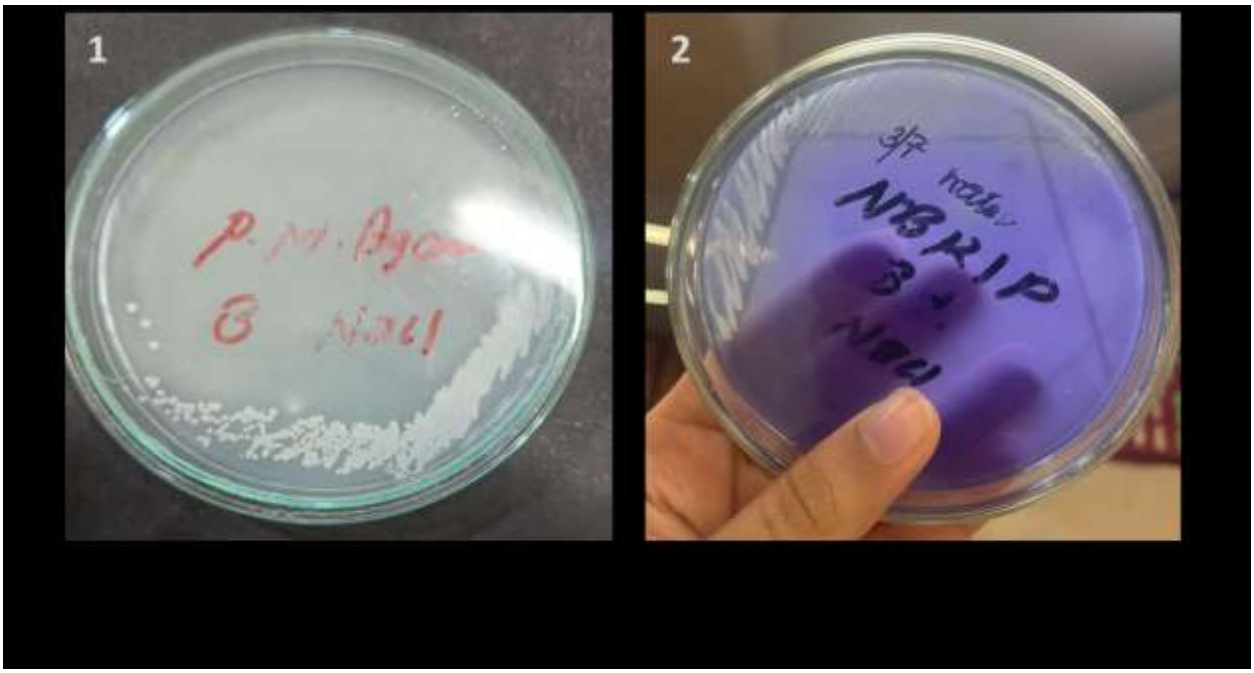
**NBRIP + BPB Broth**
The broth inoculated with the water sample showed a change in colouration from purple to red (Figure 1) and eventually to yellowish-green indicating the presence of a phosphate solubilizing organism in the sample. This could be observed due to the presence of an indicator called bromophenol blue in the medium which changes colour as the pH of the medium decreases. However, the broth inoculated with the soil sample showed no change in coloration indicating the absence of organisms with phosphate solubilizing activity.



**Growth on Plates:**

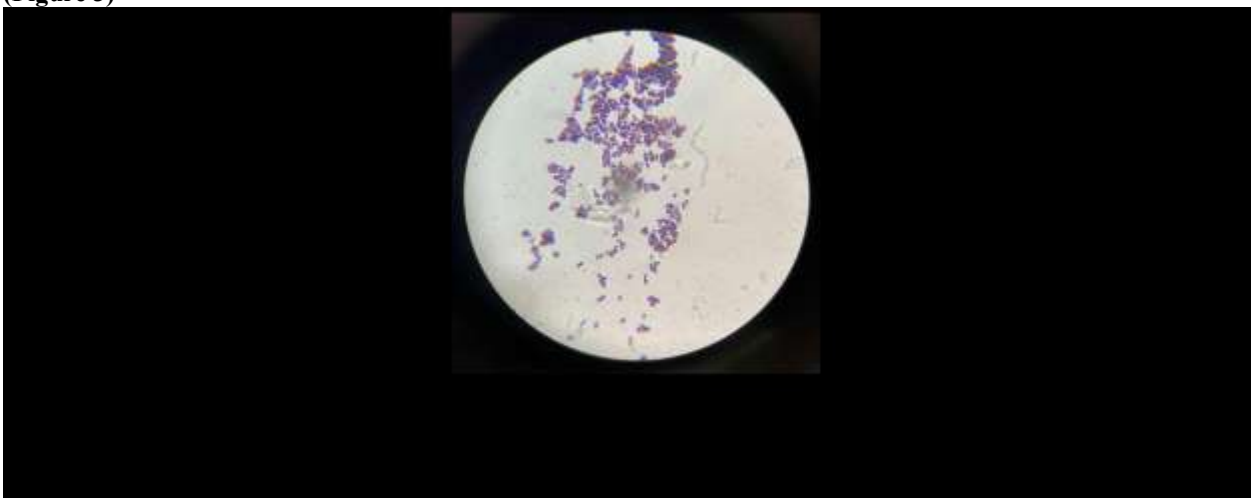
After streak plating both the samples on two separate modified Pikovskaya Agar + 3% NaCl plates (suitable medium for the growth of phosphate solubilizing organisms), white-coloured colonies were observed only on the plate which had the water sample while no growth was observed on the plates with the soil sample. (Figure 2-1)

After subculturing and streak plating the water sample on NBRIP + BPB + 3% NaCl plates (another suitablemedium for detecting phosphate solubilizing organisms) there was growth as well as small zones of clearance around the colonies on the plates indicating the presence of phosphate solubilizing organisms. (Figure 2-2)



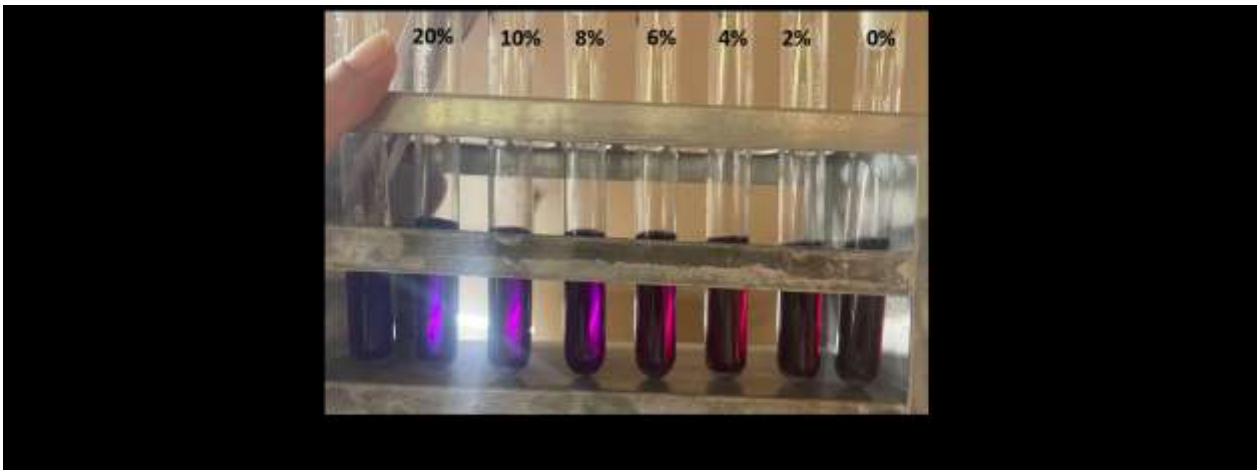

**Gram Stain for Identification of Organism**

**The cells appeared round to oval and had taken up the Crystal Violet stain and hence, appeared purple. Many cells had small buds appearing attached to them indicating the presence of budding yeast in the sample. (Figure 3)**



**NaCl Tolerance**

After inoculating the culture in NBRIP + BPB broths which had varying concentrations of NaCl (0%–20%) a change in colour from purple to red was observed in tubes with concentrations 0, 2%, 4% and 6% (Figure 4) indicating that the isolated organism is tolerant to a salt concentration of 6% NaCl.




**Germ Tube Test**

The isolate gave a negative test for Germ tube formation. On observing the inoculated sample post one hour of incubation in human serum, rapidly budding yeast were observed. However, post literature review it was concluded that for this test to be positive there should have been long tube like hyphae emerging from the surface of the body of the yeast.

**Figure 5: Yeast when incubated with human serum and observed under 100X magnification of compound light microscope. Germ tube development was not seen.**

**Biochemical Tests**

**Sugar Fermentation Tests:**

After inoculating the culture into 16 different sugar solutions, a colour change from light pink to dark pink was observed due to the fermentation of sugars leading to acid production by the organism in the samples of glucose, fructose, sucrose, maltose, raffinose and trehalose. In the case of glucose, fructose, sucrose and maltose the change in colouration was seen within twenty-four hours of incubation while it took more than twenty-four hours for the organism to ferment raffinose and trehalose indicating late fermentation. (Table 1)

| SUGAR | FERMENTATION |
|---|---|
| Glucose | ++ |
| Fructose | ++ |
| Sucrose | ++ |
| Maltose | ++ |
| Raffinose | + |
| Trehalose | + |
| Inositol | -- |
| Xylose | -- |
| Arabinose | -- |

| SUGAR | FERMENTATION |
|---|---|
| Melibiose | -- |
| Lactose | -- |
| Cellobiose | -- |
| Maltitol | -- |
| Sorbose | -- |
| Xylitol | -- |
| Melezitose | -- |

**Key: ++ fermentation observed, + later fermentation observed, -- fermentation not observed**
**Table 1: Fermentation of sugars leading to acid production by the isolate**

**Citrate Utilisation Test**
On streaking the culture on Simmon's citrate slant, a change in coloration from green to blue was observed due to the utilization of citrate as the sole source of carbon yielding pyruvate.

**Figure 6: Blue coloration of Simmons Citrate agar as the organism utilized citrate as the sole source of Carbon**

**Starch Utilization Test**
Clearance was observed around white bull colonies streaked on Nutrient agar plates containing starch when flooded with iodine solution, suggesting the ability of yeast to break down starch into simpler sugars.

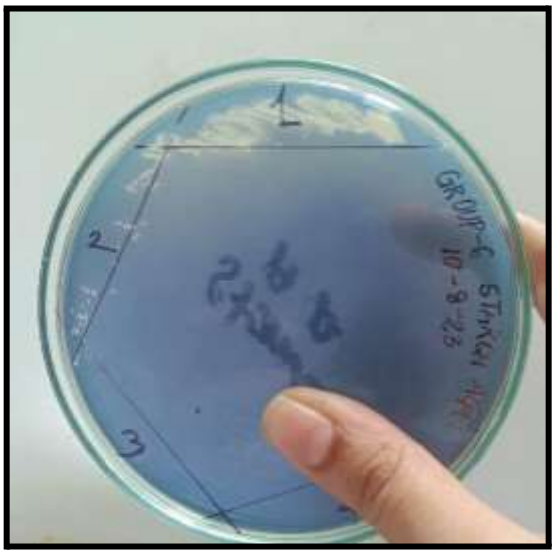


**Figure 7: Clearance observed around yeast colonies when the plate is treated with iodine suggesting starch breakdown.**

**Amino Acids Utilisation**

After inoculating the culture in 3 different amino acid solutions, a change in color from purple to yellow was observed. This was seen under aerobic conditions for Lysine and Arginine and under anaerobic conditions for Ornithine.

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| Amino acid | Aerobic conditions | Anaerobic conditions |
|---|---|---|
| Lysine | ++ | -- |
| Arginine | ++ | -- |
| Ornithine | -- | ++ |

**Key: ++ utilization observed, -- utilization not observed**

**Table 2: Utilization of Amino acids by the yeast**

**Urea Hydrolysis**

Significant color change was observed after 48 hours of incubation of the Christensen's agar slant indicating the ability of yeast to break down urea into ammonia and carbon dioxide.

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**Figure 8: Colourchange observed in Christensen's Agar Slant.**

**Alkaline Phosphatase Assay:**
Yellow colouration was observed after incubating the sample for 24 hrs. The absorbance values for the assay were found to be as follows: cell-free extract: 0.04 and cell extract: 0.09.

**Figure 9: Alkaline phosphatase assay color change observed.**

## Discussions:-

Isolation of yeast from natural sources can be proven to be one of the ideal methods to isolate yeast strains able to react to different exogenous compounds. The yeast isolated from different hypersaline environments were studied to test their halotolerance and phosphate solubilizing activity. It was found that the water sample collected from Bandstand showed good growth when provided required nourishing conditions in the medium. Whereas the sample from Juhu beach showed no growth, hence was discarded. The complex identfication of yeast requires about 60-90 different tests for correct identficationon the basis of physiological and biochemical assessment. Still, the reciprocality of the tests obtained needs to be verified for cofirmation. The biochemical and physical assessment of yeast isolate helped us narrow down to two possible genera: 1) Pichia sp. and 2) Groenewaldozyma sp. Some of the Pichia sp. have the ability to tolerate about 50-70 g/L of NaCl concentration[1].Pichia sp. yeast belongs to ascomycetes. The colonies usually appear as spherical, elliptical, or oblong cells. Pichia sp. Like Pichiasorbitophilais able to tolerate the high extracellular salt concentration thus maintaining the osmotic pressure through a glycerol/H+ symport.

The Pichia sp. also doesn't assimilate or utilize lactose which agrees with our test results. Pichia myanmarensis, another strain of Pichia sp. Has the ability to tolerate other cationic salts like (LiCl). It also utilizes most of the sugars in a similar pattern as observed through our biochemical tests. It is observed that it can grow optimally in NaCl concentrations up to 10%. (Pichiamyanmarensissp.nov., a novel cation-tolerant yeast isolated from palm sugar in Myanmar). P.manshurica and all of the 8 diIerent strains isolated [12] showed NaCl tolerance up to 7.5% NaCl. Along with high salinity tolerance, some of the species have the ability to decolorize azo dyes, under high salinity conditions (30.0 g/L).Some Candida sp. have also shown high salt tolerance. C.halophila can tolerate salt concentrations up to maximum salt solubility [17]. C.halophila is said to have more than one ion transport system to extrude Na+ ions from the cells and also to prevent internal acidi1cation by H+ symporters. It is also observed that C.halophila is a slow-growing yeast in the absence of salt. Optimum growth was observed around 2M NaCl. It is said that it is able to maintain its cellular volume in high salt tolerance to various metabolic pathways, enzymes, and intracellular transporters. Groenewaldozyma genus is one of the most recently discovered genera in yeast. Three Candida strains were recently reclassfied into this genus: Candida auringiensis,Candida salmanticensisand Candida tartarivorans. The species utilizes sugars similarly to the yeast isolate. It is also observed that the Groenewaldozyma genus can tolerate salt concentrations up to 10%. This matches much with the biochemical characteristics of the yeast isolate though not much information is available about the genus thus can be considered as a potential match.

Halotolerant phosphate solubilizing yeasts have varied applications in food, brewery, environmental applications, and as biocatalysts. Pichia sp. has found wide applications in ethanol fermentation. They have extensive use in the winemaking industry and have the ability to release thiols and Roral and fruity esters [13]. Halophilic/halotolerant yeast are a great source for biotechnological applications. Genes from halotolerant yeasts were heterogeneously expressed in Saccharomyces cerivasiae for Na+/Li+ toxicity. They have also found extensive applications in the biofuel industry. Their osmotolerance capabilities make them excellent bio-remediators for salt toxicity and for the synthesis and accumulation of compatible solutes [16]. For example, a specific mutant for ligase AY30 in Pichia sp. can help in clearing out oil spills. For further identi1cation, we aim to conduct a Diazonium Blue B test to check for the possibility of Basidiomycetes species. We also plan to conduct the Nitrate Assimilation test to better understand the nitrate and nitrite utilization activity as an energy source. Through literature review, it was found that a few halotolerant yeasts need up to 2 hrs-2 weeks of time to start forming a germ tube. Thus conducting germ tube tests at different time intervals can help identify and confirm whether the yeast isolate is truly an Ascomycetes. Detection by wet KOH film can also help in confirming our results. The alkaline phosphatase assay conducted was a qualitative assessment. Our next step in identifying the activity includes mapping the enzyme activity in different conditions of halotolerance and comparing it with known species. Lastly identifying the species by genomic sequencing using ITS profiling and by rRNA sequencing.

## Conclusion:-

This study has reported the isolation and identfication of a halotolerant and phosphate-solubilizing yeast from the shore of Juhu Beach, Mumbai. The yeast isolated was characterised based on its ability to ferment specific carbohydrates and utilise other organic molecules. Germ tube test was conducted to observe the yeast's ability to develop a germ tube under explicit conditions. Based on the biochemical assay incorporated for characterisation of yeast, Barnett et al. (2000) and digitised version of Kurtzman et al. (2011) was referred to identify the genus corresponding to the test results obtained. The probable genera of the isolated yeast identified were Pichia and Groenewaldozyma. Further distinguishing of the genus as well as species can be facilitated through PCR- based 18S or 2GS rDNA sequencing via ITS1 or ITS4 primers respectively and comparing the obtained results with the various sequence databases [16]. The focus can then be drawn on isolation and characterisation of the phosphatase enzyme of the isolated yeast through puri1cation by a nity chromatography and subjected to Polycrylamide gel electrophoresis both in intact and denatured state [15] to understand the physico-molecular properties of the puri1ed enzyme. The puri1ed enzyme can also then be subjected to colorimetric analysis for the determination of Km, optimum pH and temperature of the enzymatic activity.

**Conflict of Interest**

Authors have declared there is no conRict of interest between them.

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