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ISOLATION, SCREENING AND IDENTIFICATION OF UREASE-PRODUCING BACTERIAL STRAIN *BACILLUS SPECIES* FROM MARINE SEDIMENTS

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Marine sediment, Urease, Screening, pH, *Bacillus*

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ABSTRACT: Marine microorganisms are a valuable source of novel enzymes with ideal characteristics because of the halophilic nature of marine bacteria. The present study aims to isolate, screen, and identify the marine bacterial strains from marine sediment samples that produce an enzyme urease with clinical and industrial applications. Marine sediment samples were collected and cultured on zobell marine agar medium. After incubation seven bacterial strains were isolated from the culture. From this, five isolates were showed urease activity on the urea agar base medium. Morphological and biochemical test of urease-producing organisms was done. Urease-producing bacterial strains (UR S7B) were selected on the basis of their urease test. The selected strain UR S7B was further sequenced for identification. The phylogenetic characterization and 16S rRNA of the strain UR S7B revealed that bacterial cultures belong to *Bacillus sp*. The morphological studies indicated that the isolate was Gram -ve, rod-shaped and motile organism. The present study concludes that marine bacteria can be a urease source with important applications.

INTRODUCTION: Marine ecosystems are a rich chemical and biological diversity source. The actual biodiversity is still unknown to us; from a relatively small number of microbes, almost 12000 novel species are isolated [1]. Hence, the potential for diversity of novel molecules from yet-to-be-discovered marine organisms is very high [2-4]. Marine habitat offers diverse ecosystems and serves as an excellent source of natural bioactive molecules, novel compounds, secondary metabolites, and Enzymes [5]. Marine habitat products contain various bioactive compounds with various activities, including antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, antiviral, and antioxidant properties [6].

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Marine-derived products have a wide range of applications in pharmaceuticals as anti-tumor and antiviral, nutraceuticals as dietary supplements and food additives, agrochemicals with insecticidal, herbicidal, and fungicidal activities, and cosmetics as photoprotective and anti-aging compounds [7]. Marine bacteria constitute a large domain of prokaryotic microorganisms. Bacteria were among the first life forms to appear on Earth and are present in most of their habitats. Bacteria inhabit soil, water, acidic hot springs, radioactive waste, and the deep portions of the earth's crust [8]. Microbial enzymes are more stable and more diverse than other enzymes derived from plants and animals [9].

Urease catalyzes the hydrolysis of urea into carbon dioxide and carbamate, which spontaneously decomposes into ammonia and another carbon dioxide [10]. Functionally, ureases belong to the superfamily of amidohydrolases and phosphodiesterases [11]. The best-studied urease is that from jack bean, which was identified as the

first nickel metalloenzyme, and urease from jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized [12-15]. Ureases are found in numerous bacteria, fungi, plants and some invertebrates, and soil as soil enzymes. High soil urease activity rapidly hydrolyses applied urea to ammonia, contributing to soil nitrogen (N) losses and reducing the N use efficiency of crop plants. Urease activity tends to increase the pH of the environment by producing ammonia as a product [16-17]. The biological roles of ureases as enzymes and catalysts have been variously studied. The urease enzyme of microbial sources has a significant role in human pathogenicity, and the urease enzyme is used as a vaccine against microbial infection [18-20]. Urease can thus be applied to treat many health disorders like gastrointestinal infections and hypertension. Therefore, urease-producing soil microorganisms have received a lot of attention. In addition to internally generated urea, externally applied urea can also be utilized by plants. Urease is widely used as fertilizer because of its low costs, ease of handling and high nitrogen content [21]. So, urease have wide applications as anticancer agent to treat hypertension, and nitrogen metabolism of ruminants such as cattle, sheep, *etc*. in immunosorbent assay as vaccines against the infection of *H. pylori* [22-26]. In addition, urease has a significant role in the wine industry [27-28]. So, in the present study, we attempted to isolate the novel urease source from the marine sediment samples. We screened the isolated samples for urease activity and studied the morphological and biochemical characteristics. Further, gene sequencing and phylogenetic analysis were done for the selected bacterial strain.

MATERIALS AND METHODS:

Sample Collection: Marine sediment samples were collected from Kovilthottam, the coastal area of Puthenthura (Chavara), located in Kollam district, Kerala, India. The place was located at 3 km distance from the Neendakara fishing harbour area (South) and at 4 km distance from KMML chemical factory (North). The sediment samples were collected in sterile bottles, brought to the laboratory, and stored at room temperature at 37°C until further analysis.

Isolation of Marine Bacteria: The collected marine sediment samples (1 ml) were serially diluted up to 10^{-9} with distilled water. Isolation of microbes was done by pour plate method [29-30]. The dilutions were 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}. The medium used to grow bacterial culture was zobell marine agar medium. The media was sterilized by autoclaving at 121 °C (15 lbs pressure) for 15 minutes. The plates were incubated at 37°C for 48 h. Isolated bacterial strains were streaked in zobell marine agar slant.

Screening for Urease Enzyme: The isolated bacterial strains were screened for the presence of the urease enzyme. For screening, bacterial cultures were streaked on a urea agar base medium. The plates were incubated at 37º C for 24 h. The pink colour change indicated positive urease activity [31-32]. Urea agar base contains urea and phenol red, which acts as a pH indicator. When the bacteria hydrolyze urea, ammonia accumulates in the medium, increasing the environment's pH, and making it alkaline [33].

Morphological and Biochemical Characteristics: Gram staining, Motility, Indole production, Methyl red, Voges- Proskauer, Citrate utilization, Nitrate reduction, Urease, Catalase, Oxidase carried out. The potential bacterial strains were biochemically identified using Bergey's Manual of Determinative Bacteriology [34].

16S rRNA Sequence Analysis: The bacterial strain UR S7B is selected for 16S rRNA sequence analysis. The partial sequence of the 16S rRNA gene was amplified by polymerase chain reaction. Here explored the possibility of 16S rRNA (27F and 1492R) forward and reverse primers for amplification. DNA was isolated from the culture. Electrophoresed the DNA in 1% agarose and visualized it under U.V. 16S region was PCR amplified with specific primers, and the amplicon was checked for appropriate size by agarose gel visualization. The amplicon was gel purified using a commercial column-based purification kit (Invitrogen, USA), and sequencing was performed with forward and reversed primers in ABI 3730 XL cycler sequencer.

Phylogenic Analysis: Sequence analysis was performed using the online tool BLAST of the NCBI database. Based on the maximum identity score E value, top most sequences were utilized for

multiple sequence alignment (Clustal W2), and a dendrogram was constructed. Forward and reverse sequences were assembled, and Contig was generated after trimming the low-quality bases. The trimmed genetic sequence was then compared to different 16S rRNA genes of different bacteria in the reference RNA sequence (16S rRNA) NCBI nucleotide BLAST website database using the BLASTIN 2.9.0+ program in order to identify the genus of the selected isolates. The query sequence was converted to FASTA format and then used to create a phylogenetic tree.

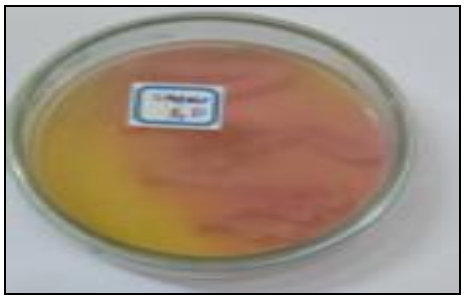
RESULTS AND DISCUSSION:

Isolation and Screening of Urease-Producing Microorganisms: In the present study, marine sediment samples were collected, and the samples were serially diluted, pour-plated, and incubated at 37^{o}C for 48 h. About seven dominant morphologically distinct colonies were selected and streaked on the zobell marine agar slant. The bacterial strains were named as UR S1D, UR S1B, UR S7B, UR S4A, UR S1C, UR S7C and URS7A. The isolated marine bacterial strains were screened for urease-producing ability on urea agar base medium. The pink colour change around the bacterial growth was identified as the positive urease producer **Fig. 1**.

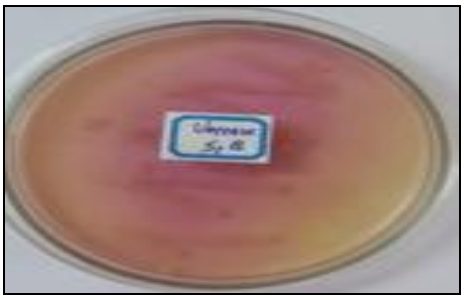
Among the seven isolates, five (UR S1D, UR S1B, UR S7B, UR S4A, UR S1C) showed maximum ureolytic activity. In contrast, the other two isolates (UR S7C and URS7A) showed poor ureolytic activity **Table 1**. Hence, the efficient urease-producing isolate UR S7B was selected for further identification.

TABLE 1: UREASE ACTIVITIES OF VARIOUS BACTERIAL STRAINS

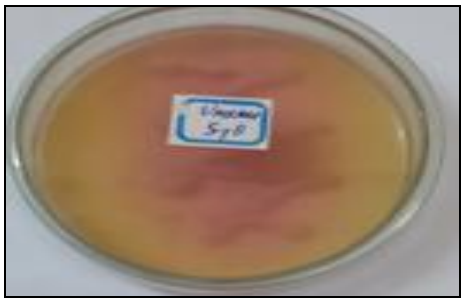
Sl. no.	Bacterial strains	Urease activity (Qualitative)
1	UR S1D	Positive
2	UR S1B	Positive
3	UR S7B	Positive
4	UR S4A	Positive
5	UR S1C	Positive
6	UR S7C	Negative
7	UR S7A	Negative



URS1D showing positive result

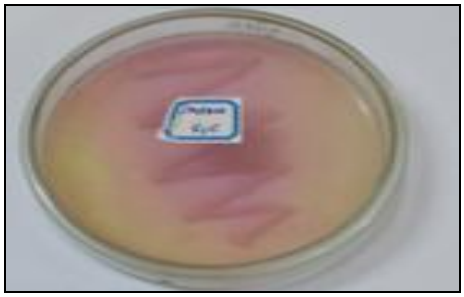


URS7B showing positive result

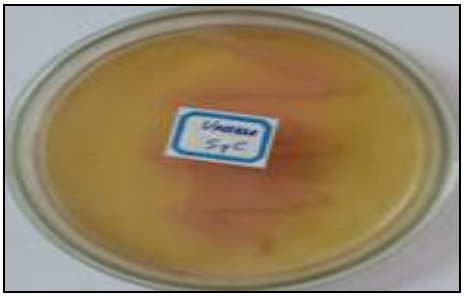


URS4A showing positive result

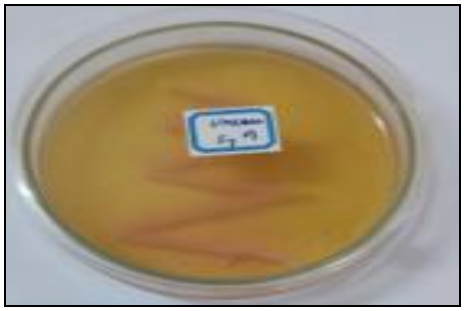
URS1B showing positive result



URS1C showing positive result



URS7C showing negative result



URS7Ashowing negative result

FIG 1: SCREENING OF MICROORGANISMS FOR UREASE ACTIVITY BY USING UREA AGAR BASE MEDIUM

Morphology and Biochemical:

Characteristics of Urease-Producing Bacterial Strains: Morphological and biochemical characterization of urease-producing bacterial strains(UR S1D, UR S1B, UR S7B, UR S4A, UR S1C) were performed by Bergy's manual of determinative bacteriology. Indole, methyl red, voges proskauer, citrate utilization, nitrate reduction, urease, catalase and oxidase test were performed. The morphology of bacterial strains were identified by Grams staining. The results are shown in **Table 2. Table 2** describes that all bacterial strains morphologically appear to be a rod-shaped bacterium. After staining three isolates showed purple (gram-positive) colour, and other two isolates showed pink (Gram-negative) colour. All isolates showed positive for Indole, methyl red, VP, citrate, nitrogen reduction, and urease. In contrast, it was negative for catalase and oxidase.

TABLE 2: MORPHOLOGICAL BIOCHEMICAL CHARACTERISTICS OF UREASE PRODUCING ISOLATES

Morphological characterestics	Bacterial strain				
	UR S1D	UR S1B	UR S7B	UR S4A	UR S1C
Gram's staining	-	-	+	+	+
Morphology	Rod	Rod	Rod	Rod	Rod
Motility	Non motile	Non motile	Motile	Motile	Non motile
		Biochemical test			
Indole (I)	Positive	Positive	Positive	Positive	Positive
Methyl red(MR)	Positive	Positive	Positive	Positive	Positive
Vogues Proskauer's (VP)	Positive	Positive	Positive	Positive	Positive
Citrate utilization	Positive	Positive	Positive	Positive	Positive
Nitrogen reduction	Positive	Positive	Positive	Positive	Positive
Urease test	Positive	Positive	Positive	Positive	Positive
Catalase	Negative	Negative	Negative	Negative	Negative
Oxidase	Negative	Negative	Negative	Negative	Negative

Phylogenetic Analysis of UR S7B: The phylogenetic tree based on a comparison of the 16SrRNA sequences of urease-producing bacterial isolates UR S7B and some of their close phylogenetic relatives, the tree was treated by the neighbor-joining method. It revealed that the strain UR S7B is *Bacillus sp*. **Fig. 2.**

In this study, we isolated bacterial strains from marine sediment samples that produce industrially useful enzyme urease. Urase has wide applications such as urea content analysis in blood, urine, alcoholic beverages, natural water, and environmental waste waters; analysis of heavy metal content in natural waters, waste waters, and soil; determination of creatinine, arginine, and IgG; urea removal from artificial kidney dialyzates, alcohol beverages and fertilizer wastewaters; wastewater reclamation for life support systems in space; pH control or shift for multi-enzyme reaction system; and urea hydrolysis as sources of ammonia or carbon dioxide in special cases [35].

The location of the marine sediment samples collected for the study showed the significance of the industrial area nearer to KMML factory and Neendakara harbour. *Bacillus* sp. produce a variety of compounds involved in the biocontrol of plant pathogens and promotion of plant growth, which makes them potential candidates for most agricultural and biotechnological applications [36].

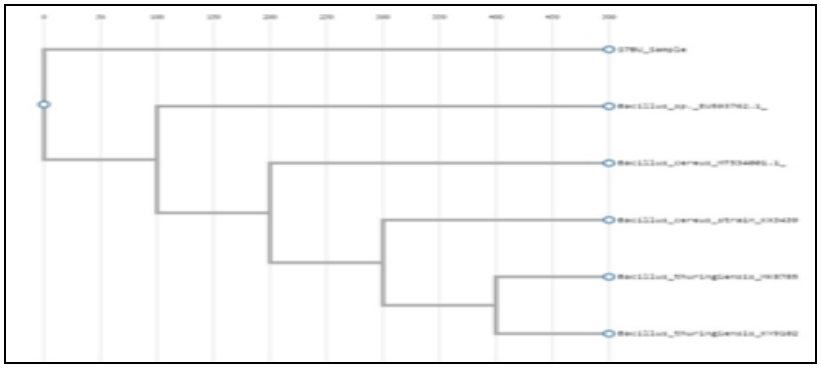


FIG. 2: PHYLOGENETIC TREE OF BACTERIAL STRAIN UR S7B

CONCLUSION: In this study, we identified, isolated, and genetically characterized a urease-producing bacterial strain *Bacillus sp*. from marine sediment samples. The efficient urease-producing isolate UR S7B was selected for gene sequencing and further identification. Morphological and biochemical characteristics indicated that the isolate was a gram-positive, rod-shaped, motile organism. 16S rRNA sequence homology was compared, and a phylogenetic tree was constructed. This result confirmed that isolate URS7B, was *Bacillus sp*. The present study disclosed that this urease-producing strain could also be helpful for industrial and clinical applications. The present study is a preliminary screening report of the diversity of *Bacillus sp* and their enzymes producing potential from marine sediments and also revealed a high taxonomic diversity among these isolated *Bacillus*. Isolation of bacterial strains from marine sediment samples would also provide extensive scope to assess their biotechnological potential.

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