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Identification of urease producing *Virgibacillus* sp. UR1 from marine sediments

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Urease producing bacteria have been reported from various ecosystems including soil, water and host organisms such as humans and animals. This research describes the isolation and identification of ureolytic bacteria from calcium rich marine sediments of southern India. The potential strain was designated as UR1 and identified using polyphasic taxonomy. The results show that the strain belongs to the genus *Virgibacillus* and is closely related to the genus *Bacillus.* Further, the urease was extracted by 80% acetone precipitation method and the molecular weight was determined as ~80 kDA by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The high performance liquid chromatography (HPLC) analysis of the urease in comparison with standard indicates that the isolated enzyme is urease. The enzyme exhibited the maximum activity at the temperature of 45°C and pH of 9. Furthermore, the crude enzyme exhibited notable calcite precipitation signifying that the strain could be used for microbial induced calcite precipitation (MICP) and subsequently for biocementation process.

Keywords: Calcite; marine bacteria, *Virgibacillus*, urease.

Introduction

Urea gets hydrolysed by the reaction of the enzyme urease (EC 3.3.1.5) forming ammonia and carbamate which again undergoes spontaneous hydrolysis to form carbonic acid and a second molecule of ammonia. These products subsequently equilibrate in water to form bicarbonate, 2 molecules of ammonium, and 2 molecules of hydroxide ions. The release of hydroxide ions increases the pH, which in turn can shift the bicarbonate equilibrium, resulting in the formation of carbonate ions. These carbonate ions react in the presence of soluble calcium ions and gets precipitated as $CaCO_3$[1-2].

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad \ldots (i)$$
$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad \ldots (ii)$$
$$H_2CO_3 \rightarrow HCO_{3-} + H^+ \quad \ldots (iii)$$
$$2NH_3 + H_2O \rightarrow 2NH^{4+} + 2OH^- \quad \ldots (iv)$$
$$HCO_{3-} + H^+ + 2OH^- \rightarrow CO_3^{2-} + 2\,H_2O \quad \ldots (v)$$
$$CO_3^{2-} + Ca^{2+} \rightarrow CaCO_3 \downarrow \quad \ldots (vi)$$

Urease is a nickel containing metalloenzyme belonging to the superfamily of amidohydrolases and phosphotriesterases[3]. The reaction of urea breaking down into carbon dioxide and ammonia in presence of water is catalysed by this enzyme[4]. The urease obtained from jack beans was the first enzyme to be crystallised[5]. Urease is found as seed protein in various plant species like soybean (*Glycine max*), *Arabidopsis*[6] as well as in the vegetative tissues[7]. The presence of urease in different bacteria helps them to use urea as the sole nitrogen source[8]. It also plays a significant role for regulating the pathogenesis of different bacteria like *Proteus mirabilis*, *Staphylococcus saprophyticus*, *Yersinia enterocolitica*, and *Ureaplasma urealiticum*[9]. *Helicobactor pylori*, noted as a human pathogen for causing stomach ulcers, is a significant producer of urease enzyme. This urease plays a leading role in the pathogenesis of this organism[10]. Urease activity is also found in different fungal species and different invertebrates[11].

Along with different adverse effects, urease present in different ruminal and gastrointestinal microorganisms plays a beneficial role by recycling urea to nitrogen and release it in the environment. Microbial ureases help in bioremediation of different nitrogenous compounds and urea-based fertilizers present in the environment[12]. Urease obtained from different bacteria has been recorded for calcium carbonate precipitation and biocementation. Calcium carbonate ($CaCO_3$) precipitation occurs due to its production as a by-product of various metabolic

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processes like photosynthesis, sulphate reduction, and urea hydrolysis. Majority of the urease producing bacteria results in the improvement on concrete properties on various parameters like different pH and temperature conditions[13]. The process of $CaCO_3$ precipitation is carried out as noted below:

$$CO(NH_2)_2 + 2H_2O + Ca^{2+} \rightarrow 2NH^{4+} + CaCO_3 \downarrow \quad ...(vii)$$

Bacllus pasteurii is noted for its urease producing ability and $CaCO_3$ precipitation. $CaCO_3$ precipitation varies with different temperature which is the result of urea hydrolysis. This in turn gives a clear idea that the urease enzyme production is also affected with the temperature variations[14-15]. Various reports are noted for the $CaCO_3$ precipitation by different microbes in form of different $CaCO_3$ crystals. These reports in turn give the evidence for urease production in these organisms (Table 1).This study gives us a detailed idea about the presence of ureolytic bacteria in different marine samples collected along the southern coast of India. These bacteria are also noted for $CaCO_3$ precipitation and hence can be further used for different industrial applications like biocementation as well as bioremediation.

Materials and Methods

Sample Collection

Water and sediment samples were obtained from different water bodies of Tamil Nadu and Kerala. Samples were collected from the coastal area of Velankanni (10.6819°N, 79.8437°E), Mahabalipuram (12°36′59″ N, 80°11′58″E) from Tamil Nadu and Puthuvypeen (9.977°N, 76.226°E), Valapad (10.398222°N, 76.091781°E), Chavara (8.9907°N, 76.5405°E), Snehatheeram (10.4330°N, 76.0768°E), Munambam (10.1667°N, 76.1833°E), from Kerala. The sample were maintained in the Marine Biotechnology and Bioproducts Laboratory, VIT at room temperature and processed for further analysis.

Table 1 — Different microorganisms producing urease and responsible for calcite precipitation

	Organisms	$CaCO_3$ precipitation
1	*Acinobacter* sp[16]	Vaterite
2	*Deleyahlophila*[17]	Aragonitic spherulites
3	*E. coli* HB101[18]	Calcite
4	*Myxococcus xanthus*[19]	Magnesium-calcite spherulites

Screening for Ureolytic Bacteria

Conventional bacterial isolation was carried out on modified urea agar (urea 20.0 g, NaCl 5.0 g, K_2HPO_4 2.0 g, glucose 1.0 g, phenol red 0.012 g, peptone 0.2 g, agar 15 g in 1000 ml distilled water, pH 6.8 ± 0.2) after serial dilution till 10^{-5}. Plates were incubated at 37°C for 48 hrs and single colonies were isolated. The colonies showed positive for urease activity by hydrolysis of urea to ammonia, thus changing the pH of the medium from 6.8 to 8. The change in pH is noted by the colour change of phenol red from light orange to pink. This change in pH does not affect the growth and metabolism of the ureolytic bacteria. The cultures were again sub-cultured in the same medium and maintained for further screening[20].

Morphological and Biochemical Characterization

The morphology of the isolated strains was identified by Gram staining technique[21]. The staining was performed to categorise the bacterial strains on the basis on their peptidoglycan cell composition and identified as Gram positive or Gram negative. The results were observed under microscope at 100X magnification (Weswox, India).

Samples for scanning electron microscopy (SEM) analysis were done according to the process as described by Folk and Lynch[22]. The pellet was collected and washed thrice with phosphate buffer saline. Glutaraldehyde solution (0.25% in water) was added and incubated at room temperature for 1 hour. The pellet was collected and again ethanol washed at 30%, 50%, 70%, 80%, and 90% with 10 min incubation. Finally, 100% ethanol was added and incubated for 1 hour. The pellet was dissolved in phosphate buffer solution and smear was prepared on cover slip.

Biochemical characterization was carried out according to the conventional protocol[21]. Indole, methyl-red, Voges-Proskauer and citrate utilization (IMViC) tests were carried out for the further identification of the bacterial strains. Presence of catalase and oxidase was also detected.

Extraction of Urease enzyme

The selected strains were subjected for mass culturing on Christensen's urea broth (urea 20.0 g, NaCl 5.0 g, KH_2PO_4 0.8 g, K_2HPO_4 1.2 g, glucose 1.0 g, phenol-red 0.012 g, peptone 1 g, in 1000 ml distilled water, pH 5 ± 0.2) and incubated for 5 days at 25°C. The media was filtered to separate and obtain a cell-free extract of urease enzyme in the filtrate.

This filtrate was further centrifuged and the supernatant was treated as the crude enzyme extract[23].

Enzyme Purification

The supernatant was subjected to osmotic separation using a dialysis membrane (Sigma–Aldrich) with a molecular weight cut-off of 20 K. Dialysis was carried out using 50 mM phosphate buffer (pH–7.4) for 24 hrs with intermediate replacement of the buffer. The solution obtained after dialysis was stored at 4^{o}C and further used for analysis[24].

Urease Assay

The crude extract of the mass production of ureolytic bacteria was used for assay according to the protocol as described by Solorzano[25]. To 10 ml of the extract, 0.4 ml of phenol reagent (10 g of phenol in 100 ml 95% ethyl alcohol) and 0.4 ml of nitroprusside solution was added followed by 1 ml of oxidizing reagent (mixture of 100 ml alkaline complexing reagent and 25 ml of sodium hypochlorite). The final mixture in the test tubes was shaken well and incubated for 2-3 hrs in dark. The standards were prepared by dissolving 4.7168 g of dry $(NH_4)_2SO_4$ and the final volume is made up to 1000 ml. Aliquots of this solution was taken as follows 1 ml, 750 µl, 500 µl, 200 µl, 100 µl, 50 µl and incubated with the above reaction mixture. The absorbance values were taken at 630 nm and the concentrations of the samples were calculated using standard curve.

Native PAGE

The partially purified enzyme was subjected to SDS-PAGE analysis with minor modifications according to the sample nature. The acrylamide percentage for the stacking gel was optimised to be 4.5% (w/v) whereas the concentration is increased to 5-6% (w/v) for resolving gel. Phenol red was used for the protein staining with variation in pH of the gel. After completion of electrophoresis, the gel was washed twice with 20 mM sodium acetate buffer (pH 5) for 30 min and later equilibrated for 5 min by a solution of pH 3 containing 1 mM EDTA, 0.1 mM $NiSO_4$, and 1 mM phenol red. The colour of the gel turns yellow due to the action of phenol red in acidic pH. The gel was then transferred to the staining solution and the areas having urease activity was indicated as dark red colour bands on the yellow gel[26-27].

HPLC Analysis

The sample was qualitatively analysed by using HPLC (Shimadzu, Japan) with UV/visible detector of 280 nm. Twenty mM phosphate buffer was used as a mobile phase with a flow rate of 0.5 ml/min. All the buffers required for HPLC was filtered by 0.45 µm membrane filter and stored at 4^{o}C. Twenty µl sample was injected and the run time was continued for 60 min[28].

Kinetic Studies

The crude enzyme extract was subjected to tests for its activity at varying conditions of pH temperature of incubation and the concentration of the substrate[24]. The enzyme activity and the pH profile were determined at varying pH acidic, neutral and alkaline i.e. 4, 7 and 9, respectively, along with varying temperatures of incubation 28^{o}C, 37^{o}C, 45^{o}C and also the varying concentrations of urea substrate 1M, 2M and 3M. The enzyme activity and effect of the temperature of incubation was determined at varying incubation temperatures 28^{o}C, 37^{o}C and 45^{o}C of the mixture of enzyme and urea, along with varying pH 4, 7, 9 and also varying molar concentration of the substrate 1M, 2M and 3M. The varying pH and temperatures were tested for different concentrations of the urea solution as substrate. The enzyme activity and effect of the substrate concentration was determined at varying molar concentrations as 1M (60.06 g/l), 2 M (120.12 g/l) and 3M (180.18 g/l).

Molecular Characterization of the Potent Strains

DNA extraction and quantification was carried out according to the procedure described by Sambrook *et al*[29] with minor modifications. The isolated DNA was subjected to PCR amplification based on the universal bacterial primers 27 forward (F) and 1492 reverse (R) according to Frank *et al*[30]. The single bands were observed at 1.5 kbps. These results were further analysed by Sanger sequencing methods (Eurofins, Bangalore). The obtained sequence was analysed by basic local alignment search tool (BLAST)[31] and the species showing the maximum similarity was noted. The sequences of the species showing 98-99% similarity was examined by multiple sequence alignment (MSA) using ClustalX[32] and the phylogenetic tree was constructed with the help of MEGA.6[33].

Calcium Carbonate ($CaCO_3$) Precipitation

$CaCO_3$ precipitation was carried out using the enzyme extract as described by Bachmeier *et al*. A

reaction mixture of 20 ml was prepared containing 25.2 mM $NaHCO_3$, 25.2 mM $CaCl_2$, and 66 mM urea, at 30°C with constant shaking at 130 rpm. The enzyme extract was added to the solution at various concentrations ranging from 0.5 to 5 U/ml. Sample was collected every 2 hrs to record the amount of insoluble Ca^{2+} present in the solution. The entire setup was repeated in triplicates and the mean amount is noted (Bachmeier *et al*., 2002).

Results and Discussion

Isolation of the Ureolytic Bacteria

Bacterial isolates from different marine samples were screened for its ureolytic activity. A total of 14 isolates were obtained from 6 different sample locations. Out of these 14, 6 strains were further screened by urea utilization test where the pink colour shows positive result (Table 2). Various ureolytic bacteria have been reported all over the world. *Marinobacter litoralis* isolated from the marine sponge *Xestospongia testudinaria* collected from the South China Sea have been studied for its urease production[34]. Seven novel strains of *Aspergillus niger* isolated from soil sample of Semnan, Iran is noted for its urease production[35]. Majority of the species have been isolated from different soil samples, hence, these new marine species possess distinct characteristics that are found to be different from its terrestrial counterparts.

Characterization of the Isolated Species

The microorganisms, when studied under 100X magnification, showed rod shaped structures, Gram positive in nature. These results were further confirmed by SEM images (Figs. 1a & 1b). From the structural orientation we can conclude that probably the organisms may belong to the genus *Bacillus.* Previously ureolytic bacteria like *Sporosarcina* sp., *Bacillus* sp.and *Brevundimonas* sp. were reported from samples collected from Beidaihe marine sediment, China[36]. Recent report also indicates the production of urease enzyme by *B. licheniformis* and its use for $CaCO_3$ precipitation[37]. Apart from *Bacillus*, different bacteria like *Klebseilla* sp., *Proteus* sp., *Lactobacillus* sp. and *Streptococcus* sp. have been previously isolated from marine sample of Porto Novo Coast, Tamil Nadu[38]. The biochemical characterizations of these strains are stated in Table 3, which gives an idea about their metabolic conditions. UR1 and UR3 showed results that are similar with *Bacillus* sp, which increases their probability of belonging to the same genus[39].

Table 2 — Bacteria isolated from different marine sediments and the final screening of the selected strains based on its urea utilization

Sample	Isolates	Screened Isolates	Identification Code
Puthuvypeen	P_1, P_2, P_3, P_4	P_2	UR1
Valapad	V_1, V_2, V_3	V_3	UR2
Mahabalipuram	M_1, M_2	M_2	UR3
Chowara	C_1, C_2	C_1	UR4
Munambam	MUN	MUN	UR5
Velankanni	VEL	VEL	UR6
Snehatheeram	SNE	-	-

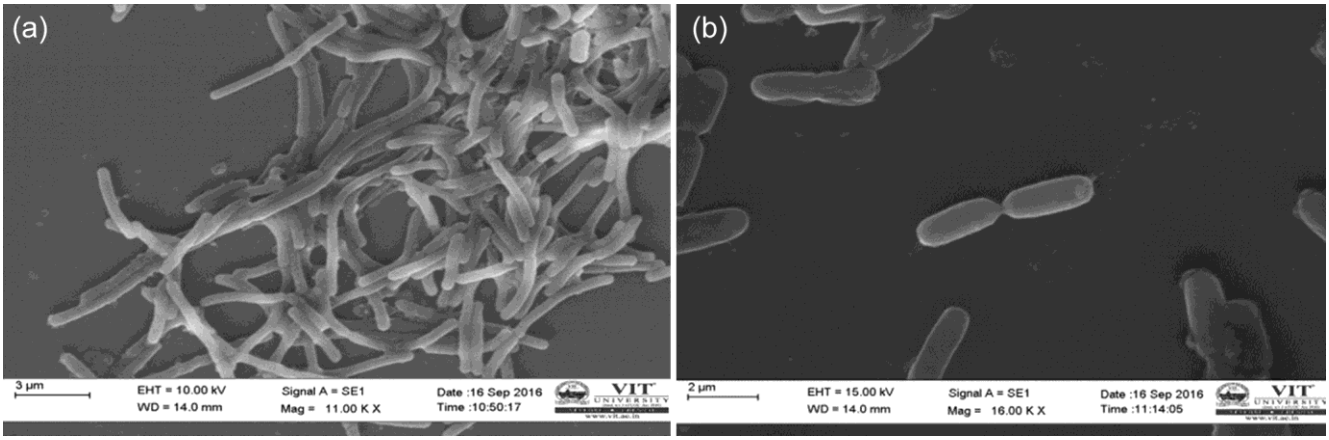

Fig. 1 — SEM images of the isolated organisms; a: UR1; b: UR3

Table 3 — Biochemical characterisation of the selected strains

Isolates	UR1	UR2	UR3	UR4	UR5	UR6
Citrate test	-	+	+	-	+	+
Indole test	-	-	+	-		
MR test	-	-	+	-	-	+
VP test	+	+	-	+	-	-
Catalase test	+	+	+	+	-	-
Gram staining	+	+	+	+	+	+

Extraction of Urease Enzyme

The production media for urease enzyme had initial pH 5 and inoculated with urea as substrate. The bacteria synthesize urease enzyme that liberates ammonia which makes the pH of the medium alkaline in nature. pH was measured for the collected samples at regular intervals. The increase in pH is directly proportional to the production of the enzyme. The elevation of pH for the cultures was examined in Figure 2. The maximum elevation of pH of 9.3 and 9.5 has been recorded for UR1 and UR3 respectively. The increasing pH of the medium indicates the production of enzyme increases with time which gradually reaches the maximum in 7-10 days. This implies the production the extracellular enzyme in the media which in turn increases the alkalinity of the medium[40]. Samples were collected and subjected to further enzymatic assay.

Urease Assay

The supernatant was analysed for urease production. The standard curve was prepared with ammonium sulphate and the unknown absorbance was plotted against it (Fig. 3). The enzyme produced has been estimated and used for further analysis. The enzyme estimation has been described in Figure 4. The production of urease was maximum for UR1 (250 U/ml) and UR3 (250 U/ml). The production for UR2 was also similar with these strains (247 U/ml). Other strains showed relatively less production (UR4 – 24 U/ml; UR5 – 33.81 U/ml; UR6 – 29.52 U/ml). Different bacteria belonging to the same genera have showed the production of urease enzyme. *B. megaterium* AP6 isolated from alkaline soil produced 553 U/ml of enzyme whereas *Bacillus* sp. CR2, isolated from mine tailing soil of Urumqi, China produced 432 U/ml of enzyme[41-42]. Previous reports on urease production from microbial source proved the presence of the enzyme within the cell free supernatant of *B. pasteurii*[43]. Similar organisms isolated from alkaline soil showed the production of intercellular urease enzyme[44] . *Sporosarcina globispora* (20 U/ml),

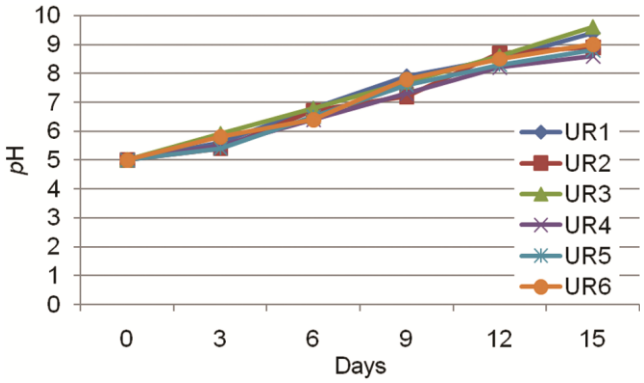

Fig. 2 — Change in pH of the medium indicates the production of urease enzyme by respective organisms.

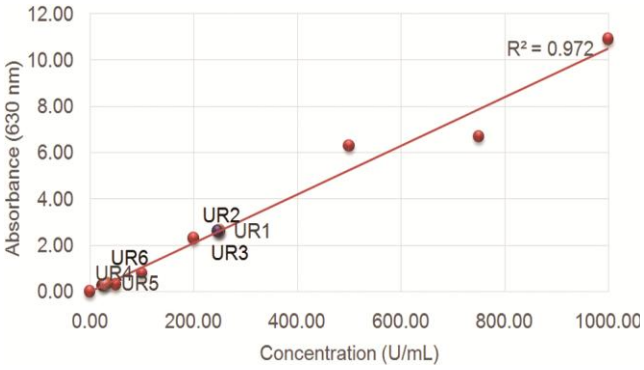

Fig. 3 — Unknown absorbance of the crude enzyme recorded against ammonium sulphate standard curve

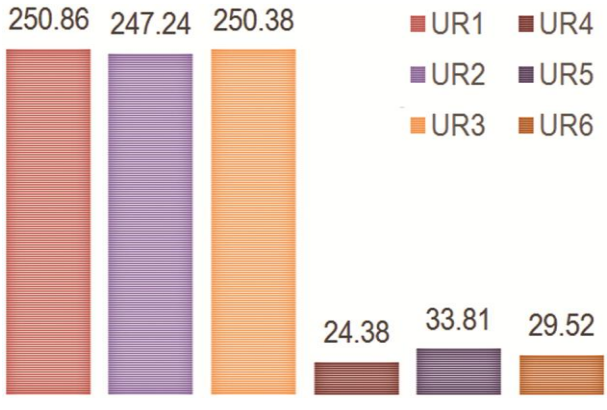

Fig. 4 — Urease production for different strains (UR1 – UR6)

Sporosarcina koreensis (15 U/ml), *Sporosarcina* sp. R-31323 (8 U/ml) and *B. lentus* (15.8 U/ml) was isolated from the terrestrial source in China[45]. Strains like *Sporosarcina pasteurii*, *B. pumilis* and *B. megaterium* was reported to produce ureases that helps in production of microbial carbonates, an ample alternative for cementing materials[46].

Native PAGE

The purified urease was used as standard with molecular weight 82 kDa. The native PAGE showed same bands for the standard and the crude enzymes of UR1 and UR3 (Fig. 5). This showed that the molecular weight of the obtained crude enzyme was nearly equal to 80 kDa. This also proves the presence of urease enzyme in the cell free supernatant. The ureases previously isolated from different microorganisms also have molecular weight similar to these values. Human pathogen *Helicobacter pylori* produced urease enzyme that has a molecular weight of 66 kDa[28]. Molecular weight of the subunits of the enzyme isolated from *L. fermentum* was recorded as 67 kDa, 16 kDa and 8.6 kDa, respectively while that of *L. reuteri* were estimated to be 68 kDa, 16.1 kDa and 8.8 kDa, respectively[47-48].

HPLC Studies

The standard was analysed along with the treated samples. The standard showed the retention time by 9.278 min where peaks were observed for UR1 and UR3 by 9.854 and 9.287, respectively (Fig. 6). These peaks also proved the presence of the urease enzyme in the crude extract. Very less data regarding previous HPLC reports were obtained due to the specificity of the parameters.

Kinetic Studies

The results show that in all the substrate concentrations (1M, 2M and 3M), maximum urease activity was shown at pH 9 and 45°C temperature of incubation by both the strains UR1 and UR3 (Fig. 7). and the least urease production was seen at pH 4 and 28°C temperature of incubation irrespective of the substrate concentrations (1M, 2M and 3M). The media was optimized for maximum production of the enzyme. The urease production was highest at 45°C for pH 9 for both the strains at 2M substrate concentration. The high pH and temperature did not affect the growth and metabolism of the bacteria. The media was saturated at 3M substrate concentration and the production of enzyme was nearly equal with that of 2M concentration. The enzyme production is explained in Table 4.

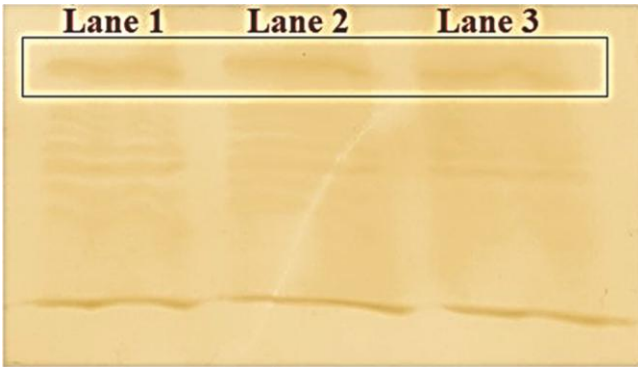

Fig. 5 — Native page for the standard and the crude enzyme; Lane 1 - Standard (urease); Lane 2 - Crude enzyme produced by UR1; Lane 3 - Crude enzyme produced by UR3.

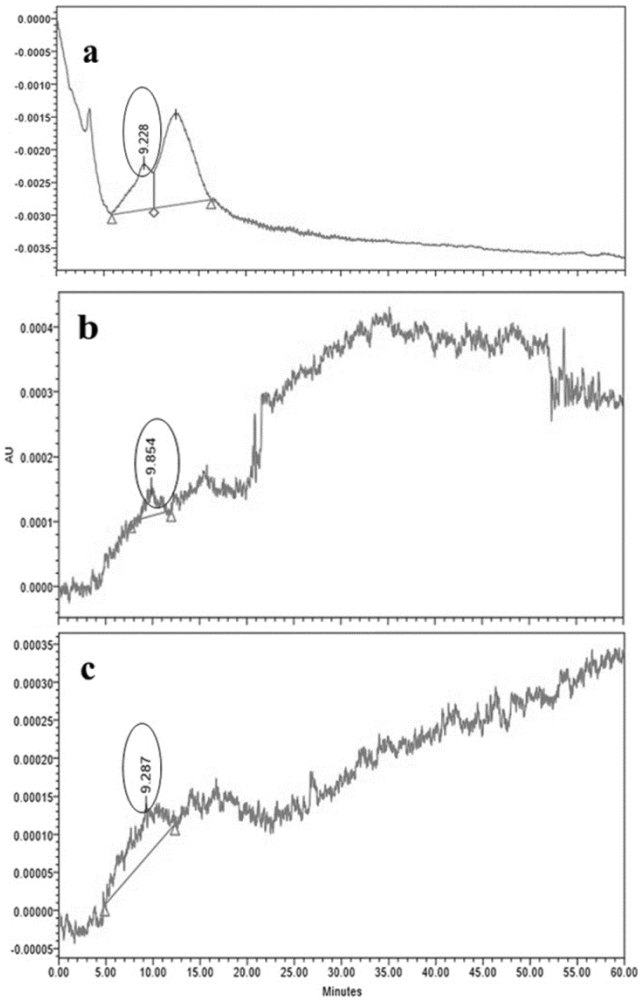

Fig. 6 — HPLC analysis for the standard and the crude enzyme; (a) Standard (urease); (b) crude enzyme produced by UR1; (c) crude enzyme produced by UR3.

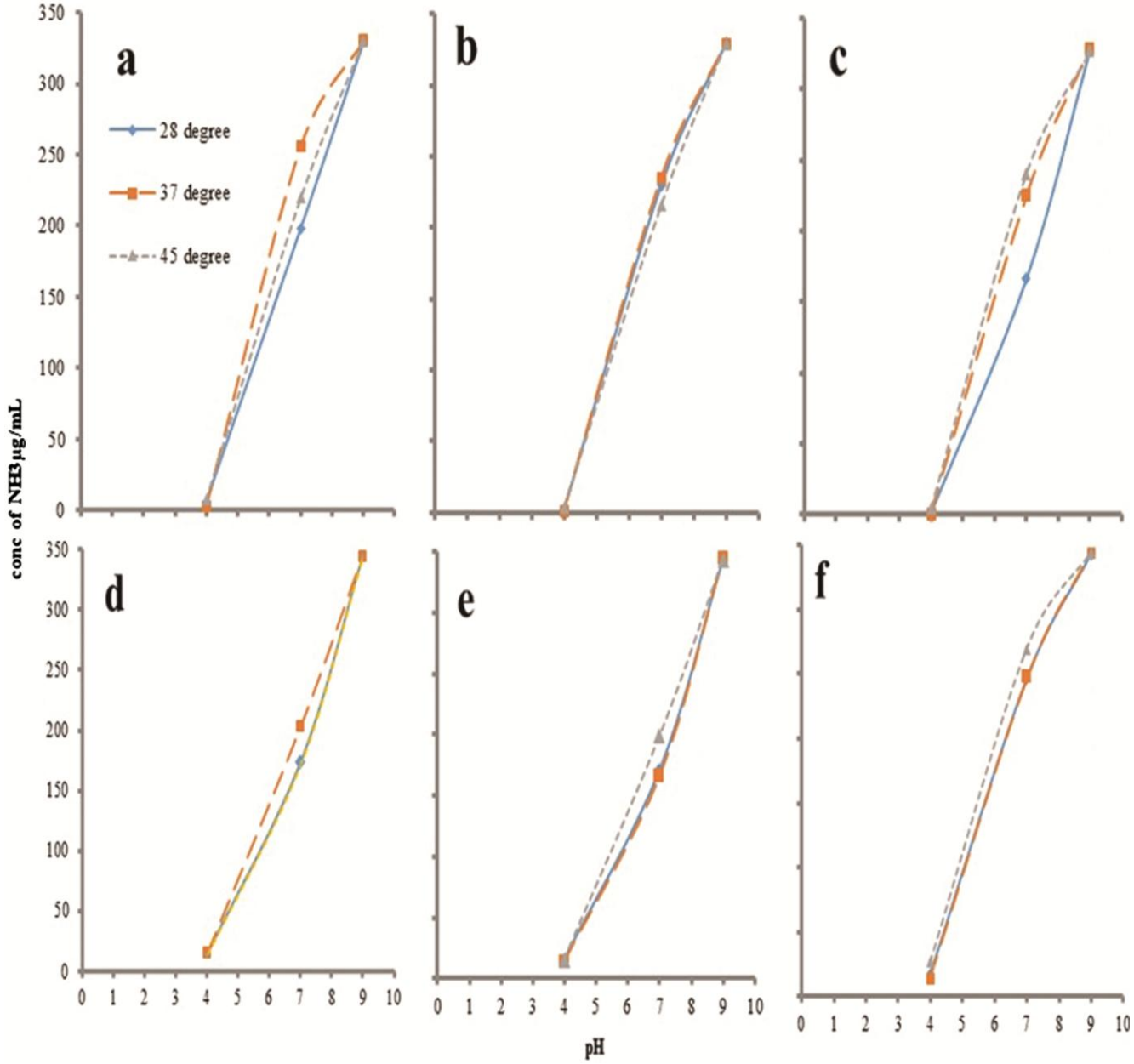

Fig. 7 — Kinetic studies of UR1 and UR3 with different pH (4, 7 and 9), different temperature (28˚C, 37˚C and 45˚C) and different substrate concentrations (1M, 2M and 3M); (a) UR1 with 1M substrate; (b) UR1 with 2M substrate; (c) UR1 with 3M substrate; (d) UR3 with 1M substrate; (e) UR3 with 2M substrate; (f) UR3 with 3M substrate.

Table 4 — Enzyme production by strain UR1 and UR3 by different media conditions

Substrate conc.		1M			2M			3M		
pH		4	7	9	4	7	9	4	7	9
UR1	28˚C	5.4	1.98.1	329.7	1.6	229.6	329.5	1	166.4	328.4
	37˚C	2.3	256	328.8	1.2	234.8	329.2	0.5	225.5	329.5
	45˚C	7.2	219.3	329.7	3.3	215.1	330.4	3.5	240.8	326.9
UR3	28˚C	15.3	173.8	344.9	16.2	171.1	343.8	17	246	342.9
	37˚C	14.9	203.3	343.4	13.9	166.6	344.9	13.9	247.8	344.2
	45˚C	13.2	171.6	344.1	15.4	198.5	345.5	26.4	269.5	344.1

Taxonomical Studies

From the results of the previous experiments, it can be concluded that the strain UR1 belongs to the genus *Virgibacillus*. The molecular characterization was carried out and the phylogenetic tree was constructed. According to the phylogram, the strain was designated as *Virgibacillus* sp. UR1. The morphological and physiological characteristics also showed similarity with this genus. The phylogenetic characterization is shown in Figure 8.

$CaCO_3$ Precipitation

$CaCO_3$ has been successfully precipitated by the use of the crude extract of UR1. The precipitate was analysed by FT-IR analysis. The standard of $CaCO_3$ showed similar results with that of the crude extracts. The standard $CaCO_3$ showed peaks at 1402.25, 871.82, 711.73 and 443.63 nm. These peaks were coincided with that of microbe induced calcite precipitation (MICP) which showed peaks at 1438.9, 1409.96, 1085.92, 871.82, 746.45, 711.73 and 420.48 nm. This data was again verified from NIST standard reference database (https://webbook.nist.gov).

Various reports are available for the production of microbial calcite. These organisms are generally used for biocementation. MICP have been previous reported by Stocks-Fisher *et al* from the *B. pasteurii* (isolate from alkalophilic soil). Bachmeier *et al* reported the plasmid pBU11 that induces the production of urease enzyme in *B. pasteurii* has been successfully transformed into *E. coli* HB101 and the recombinant organism was successful in calcite precipitation. The production of calcite by *Sporosarcina pasteurii* (formally known as *B. pasteurii*) was supported by Wiffin[49]. Calcite precipitated by *S. pasteurii* was also used for soil enrichment[50]. Microorganisms isolated from Beidaihe marine sediment, China were also reported by Wei *et al* for calcite precipitation. *B. lentus* CP28, *B. diminuta* CP16 and *S. soli* CP23 was successful in calcite precipitation. Recent studies showed that ureolytic bacteria and MICP is also responsible for bioremediation of toxic metals from the environment.

Fig. 9 — FT-IR Results comparing commercial standard $CaCO_3$ and microbial induced $CaCO_3$ precipitation (MICP).

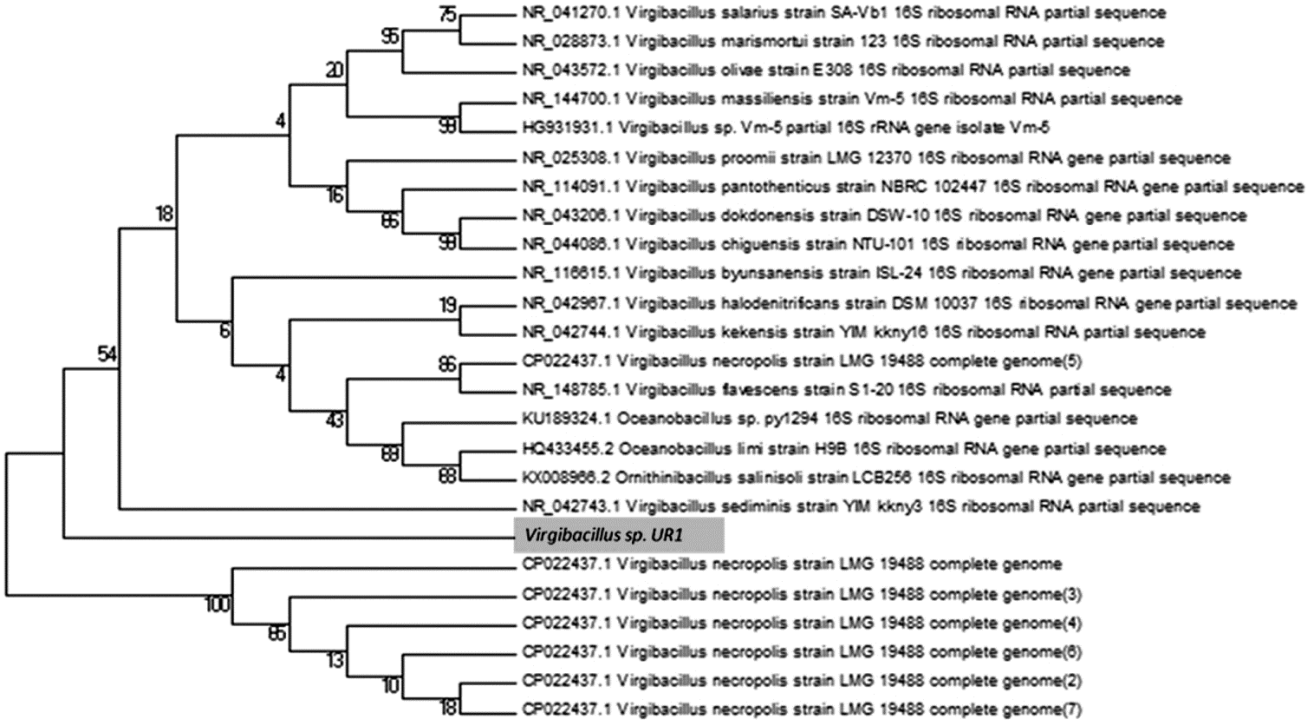

Fig. 8 — Construction of phylogenetic tree of the isolated bacterial strain UR1 and the other closely related bacteria

The advantage of using ureolytic organisms for bioremediating metal pollution in soil is their ability to immobilize toxic metals efficiently by precipitation or co-precipitation, independent of metal valence state, toxicity and its redox potential[51].

Conclusion

Ureolytic bacteria were screened from marine soil samples and crude enzyme was extracted from six potential strains namely UR1, UR2, UR3, UR4, UR5 and UR6. The kinetic study of the enzymes was carried out with two most potential strains (UR1 and UR3) in three different substrate concentrations (1M, 2M and 3M). The maximum urease production was at 45^{o}C for pH 9 for both the strains at 2M substrate concentration. The high pH and temperature did not affect the growth and metabolism of the bacteria. The molecular weight of the isolated urease was compared with the standard by SDS PAGE and recorded to be nearly ~80 kDa. MICP was successfully observed and analysed with the standard structure. This can be further implemented for biocementation and bioremediation purposes. Thus, these marine ureolytic bacterial strains UR1 and UR3 could be a potent source of urease enzyme production and can be used for further commercialization on a large scale. The $CaCO_3$ production by the organism was also of immense importance in different industrial aspects.

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