Indian Journal of Experimental Biology
Vol. 58, December 2020, pp. 853-860

Isolation, screening and identification of amylase and catalase producing bacterial strains from marine sediments

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Received 12 January 2020; *revised 29 October 2020*

Marine sediments are valuable source of industrially useful enzymes. Here, we attempted isolation, screening and identification of bacterial strains from marine sediments which produce industrially important enzymes amylase and catalase. Marine sediment samples were collected and cultured on zobell marine agar medium. After incubation, the isolates that showed amylase and catalase activity were selected for the assay. The strains AM01 and Ca07 showed the highest amylase and catalase activity, respectively. The selected strains were further sequenced for identification. Morphological studies indicated that the isolates were Gram –ve, rod shaped and non-motile organism. The phenotypic characterization and 16S rRNA of the strains AM01 and Ca07 revealed them to be *Klebsiella pneumoniae* and *K. quasipneumoniae*, respectively

Keywords: *Klebsiella* spp., Pour Plate method

Marine environment is the largest aquatic ecosystem on the planet with a high salt content and most important sources of biodiversity in the world[1,2]. Marine habitats offer diverse ecosystems and serves as an excellent source of natural bioactive molecules, novel compounds, secondary metabolites, and enzymes[3]. It houses a wide range of microbes such as archaea bacteria, fungi, viruses and protists. The enormous pool of biodiversity in marine ecosystems is a natural reservoir for acquiring an inventory of enzymes with potential novel biocatalysts for biotechnological application[4]. Marine habitat contain a wide range of bioactive compounds with various activities which include antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, anti-tuberculosis, antiviral, antitumor and antioxidant properties[5].

Marine microorganisms have unique properties and adopt to extreme marine environment conditions such as high temperature, alkaline, acidic water, high pressure and limited substrate in the deep sea water. These distinctive characteristics have attracted many researchers to explore the potential of marine microorganisms[6]. Marine microorganisms were proven already to have many beneficial bioactivities such as production of a large number of industrial enzymes. Marine microorganisms are increasingly being studied and becoming a hot spot in the search for industrially important biomolecules[7]. Marine enzymes are produced from many organisms including plants and animal with potential industrial applications[8]. Marine microorganisms are a valuable source of novel enzymes with ideal characteristics because of the halophilic nature of the marine bacteria[9].

Extracellular enzymes from bacterial sources are frequently used in industrial process. Among these enzymes are the starch degrading, amylolitic enzymes called amylases. There are various reports on starch degrading microorganisms from different sources and respective amylase activity[10-12]. There are about 3000 enzymes known today but only few are industrially exploited. These are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectin and cellulose. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability[13].

Catalases are metalloenzymes which catalyzes degradation of hydrogen peroxide (H_2O_2) into dioxygen and water, widely found in most plants, animals and microbes[14-17]. Catalase is used in a wide range of industrial applications like food industry, as a preservative, as an anticorrosive agent and a cleaning agent[18-21].

In the present study, we made an attempt to isolate novel source of amylase and catalase from the marine

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sediment samples. We screened the isolated samples for amylase and catalase activity, studied the morphological and biochemical characteristics and did quantitative assay. Further, gene sequencing and phylogenetic analysis were also done for the selected bacterial strain for each activity.

Materials and Methods

Sample collection

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

Isolation of marine bacteria

The collected marine sediment samples (1.0 mL) were serially diluted up to 10^{-9} with distilled water. Isolation of microbes were done by pour plate method[22,23]. The dilutions taken were 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}. The medium used for the growth of bacterial culture was zobell marine agar medium. The media was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 min. The plates were incubated at 37°C for 24 h. Isolated bacterial strains were streaked in zobell marine agar slant.

Screening for amylase activity (starch iodine test)

All isolated bacteria were tested for amylase production by starch hydrolysis. The pure culture colonies were picked up from each slant and streaked on starch agar plates. The plates were incubated at 37°C for 24 h. After incubation individual plates were flooded with Iodine solution (0.5%). Amylase positive strains were determined by the presence of a clear zone of starch hydrolysis around the colony on the starch agar plates[24]. Colonies having a clear zone around them were selected for further investigation.

Screening for catalase enzyme

The screening of catalase positive microorganism was done by bubble test (catalase test) using slide method[25]. Suspension of the organism was transferred from the slant cultures to a clean glass slide with a loop. About 3-4 drops of 3% hydrogen peroxide (H_2O_2) to the culture suspension was immediately added and the slides were observed for bubble formation, which showed the presence of catalase in it. If it doesn't show any bubble formation then our sample is catalase negative. The catalase positive colonies were screened for catalase activity.

Morphological and biochemical characteristics

Gram staining, motility, indole production, methyl red, vogues-proskauer's, citrate utilization, catalase, oxidase, nitrate reduction, urease, starch hydrolysis and casein hydrolysis were carried out. The potential bacterial strain was biochemically identified using Bergey's Manual of Determinative Bacteriology[26].

Enzyme production medium for amylase

For enzyme production, the media consists of peptone (0.5 g), yeast extract (0.3 g), NaCl (0.3 g), K_2HPO_4 (0.1 g), $MgSO_4$ (0.02 g) and soluble starch (1.0 g) were used for amylase. The medium was prepared by using mixture of 50% of sea water and 50% distilled water. The pH of the medium was maintained at 7. The medium (10 mL) was taken in a 100 mL conical flask and autoclaved at 121°C (15 lbs pressure) for 15 min. After cooling, the medium was inoculated with pure bacterial culture. The medium was placed in shaker incubator at 37°C for various incubation times 24 h (1st day), 48 h (2nd day), 72 h (3rd day), 96 h (4th day) and 120 h (5th day). At the end of each incubation period, the culture medium was centrifuged at 7000 rpm for 10 min. to obtain the crude extract, which serve as the enzyme source[27].

Enzyme Assay of amylase

Assay of amylase was evaluated using 3,5-dinitro salicylic acid (DNS) method[28]. Standard solutions of maltose (0-10 μmoles/L) are taken in the test tubes labeled as S1 to S4 and made up with 1.0 mL of distilled water and 0.2 mL of enzyme was taken as test. From this 0.8 mL soluble starch solution were added. All the tubes were incubated at 37°C for 15 min. The reaction was stopped by addition of 1.0 mL of DNS reagent and boiled for 15 min. The absorbance was read at 540 nm and obtained results of test were compared with standard of amylase. One unit of enzyme was defined as one micromoles of amylase released per mL of enzyme.

$$\text{Amylase activity (U/mL)} = \frac{\text{Milligram of maltose released} \times \text{Dilution Factor}}{\text{Incubation time} \times \text{Milli gram enzyme in reaction mixture}}$$

Enzyme production medium of catalase

For enzyme production, media consisting of one gram each of peptone, yeast extract NaCl were

used. The medium was prepared by using a mixture of 50 % sea water and 50 % distilled water. The pH of the medium was maintained at 7.2. The medium (10 mL) was taken in a 100 mL conical flask and was autoclaved at 121°C (15 lbs pressure) for 15 min. After cooling the medium was inoculated with pure bacterial culture. The medium was placed in incubated shaker at 37°C for various incubation times (24, 48, 72, 96 and 120 h). At the end of each incubation period, the culture medium was centrifuged at 7000 rpm for 10 min to obtain the crude extract, which served as the enzyme source[29].

Enzyme assay of catalase

Enzyme assay was performed to check the activity of catalase in the broth and was done by using hydrogen peroxide (100 mM), phosphate buffer (pH 7.0), potassium dichromate and enzyme mixture. The absorbance was read at 570 nm. A graph was constructed based on the standard curve of H_2O_2 using different concentrations of H_2O_2[30]. The activity of catalase samples were determined in terms of enzyme activity (U/mL) using the following formula

Enzyme Activity (U/mL) = (milligram of H_2O_2 released × dilution factor) / (time of incubation × milligram enzyme in mixture)

Estimation of total protein concentration

Protein concentration (mg/mL) in the crude enzyme was determined by Lowry's method[31] using Bovine Serum Albumin (BSA) as standard protein. The amount of the soluble protein was calculated from the standard curve.

Determination of specific activity of amylase

In order to determine the specific activity of amylase of the selected isolates, Lowry's method for total protein estimation was used.

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Extracellular protein concentration (mg/mL)}}$$

Determination of specific activity of catalase

The specific activity of catalase was determined by using the total protein content it was determined by using the following formula:

Specific activity (U/mg) = enzyme activity (U/mL) / total protein content (mg/ mL)

Phylogenetic analysis

At the molecular level, the rRNA genes are the most widely used markers for the identification of bacteria due to their conserved function and universal presence. Sequence analysis was performed using online tool BLAST of NCBI database and based on maximum identity score E value top most sequences was utilized for multiple sequence alignment (Clustal W2) and dendrogram was constructed. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The trimmed genetic sequences was then compared to different 16S rRNA gene of different bacteria in the reference RNA sequence (16S rRNA) database of NCBI Nucleotide BLAST using BLASTN 2.9.0+ program in order to identify the genus of the selected isolate. The query sequence was converted to FASTA format and was then used to create a phylogenetic tree.

Results and Discussion

Isolation and screening of amylase producing microorganisms

In the present study, marine sediment samples were collected and the samples were serially diluted, pour plated and incubated at 37°C for 24 h. About nine dominant morphologically distinct colonies were selected and streaked on the zobell marine agar slant. The bacterial strains were named as AMO1, AMO2, AMO3, AMO4, AMO5, AMO6, AMO7, AMO8, and AMO9.

The isolated marine bacterial strains were screened for the production of amylase activity using starch agar plate method. Out of nine isolates, seven bacterial strains showed positive result with the zone of clearance on starch agar media and two showed negative result (Table 1). AMO1, AMO3, & AMO9 showed the maximum zone of clearance on the starch agar medium. Hence these three strains were selected for quantitative assay of amylase.

Isolation and screening of catalase producing microorganisms

The marine sediment samples were serially diluted, pour plated and incubated at 37°C for 24 h (Fig. 1).

Table 1 — Amylase and Catalase activities of various bacterial strains

Bacterial Strains	Amylase Activity	Bacterial Strains	Catalase Activity
AM01	+ ve	Ca02	+ ve
AM02	+ ve	Ca03	+ ve
AM03	+ ve	Ca04	– ve
AM04	– ve	Ca05	+ ve
AM05	+ ve	Ca06	+ ve
AM06	– ve	Ca07	+ ve
AM07	+ ve	Ca08	+ ve
AM08	+ ve	Ca09	+ ve
AM09	+ ve		

About eight dominant morphologically distinct colonies were selected and pure cultured colonies were separately streaked on the zobell marine agar slant. The isolated bacterial strains were named as Ca02, Ca03, Ca04, Ca05, Ca06, Ca07, Ca08, and Ca09, and were screened for production of catalase activity by bubble test (catalase test) or using slide method. Out of eight isolates, seven bacterial strains showed the bubble formation with the positive result and among this one (Ca04) bacterial strain showed negative results (Table 1). The strains Ca03, Ca07, Ca08 showed the maximum catalase activity. Hence, these three strains were selected for quantitative assay of catalase.

Morphology and biochemical characteristics of amylase producing bacterial strains

Morphological and biochemical characterization of AM01 to AM09 were performed in accordance with Bergey's manual of determinative bacteriology. indole, methyl red (MR), vogues-proskauer (VP), citrate utilization, nitrate reduction, urease, catalase, oxidase, casein hydrolysis were performed. Morphology of the bacterial strains was identified by Gram staining. The results are shown in Table 2.

Table 2 describes that all bacterial strains morphologically appears to be rod shaped bacterium. After staining the five isolates showed purple (gram positive) colour and other four isolates showed pink (Gram negative) colour. All isolates was capable of reducing nitrate to nitrite. The selected bacterial isolates (AM01) are Gram negative, rod shaped non-motile organism. This isolate was positive for VP, citrate, nitrate reduction, urease, catalase, and casein hydrolysis. While it was negative for indole, MR and oxidase test.

Morphological and biochemical characteristics of catalase producing bacterial strains

Morphological and biochemical characteristics of the selected strains Ca02, Ca03, Ca05, Ca07 and Ca08 were performed on Bergey's Manual of Determinative Bacteriology.

All bacterial strains morphologically appeared to be rod shaped bacterium and the selected bacterial isolate (Ca07) are gram negative, rod shaped, unmotile organism. This isolate was positive for methyl-red, voges proskaeur, citrate utilization, nitrate reduction, urease, catalase test while it was negative for indole and oxidase test (Table 3).

Amylase enzyme assay

The results of starch hydrolysis, revealed that the significant amount of amylase production was observed in three bacterial strains (AM01, AM03, AM09) compared to the other isolates (AM02, AM05, AM07 and AM08). Hence, the three isolates AM01, AM03, AM09 were selected for determining enzyme activity. Crude enzyme extract was studied for amylase activity and the estimation of amylase activity was carried out according to the dinitrosalicyclic acid

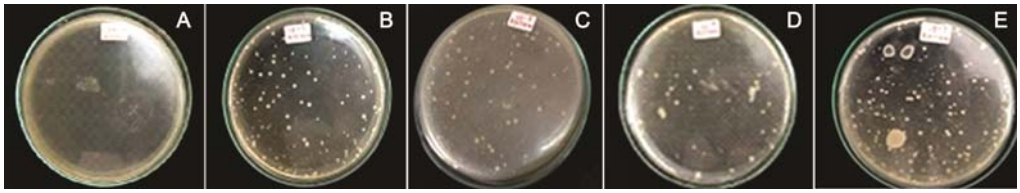

Fig. 1 — Isolation of catalase producing microorganisms by Pour Plate method

Table 2 — Morphological and biochemical characteristics of amylase producing isolate

Morphological characteristics	Bacterial strains								
	AM01	AM02	AM03	AM04	AM05	AM06	AM07	AM08	AM09
Gram Staining	–	+	+	–	+	–	–	+	+
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	–	+	+	–	+	–	–	+	+
Biochemical test									
Indole	–	–	–	+	–	–	–	–	–
Methyl red	–	+	+	+	+	+	+	+	+
Vogues-Proskauers	+	+	+	+	+	+	+	+	+
Citrate test	+	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	+	+	+	+	+	+	+
Urease test	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	–	+	+	+	+	+
Oxidase	–	–	–	–	+	+	–	–	–
Casein hydrolysis	+	–	+	+	–	–	–	–	–

Table 3 — Morphological and biochemical characteristics on catalase producing bacterial strains.

Morphological characteristics	Bacterial strains				
	Ca02	Ca03	Ca05	Ca07	Ca08
Gram Staining	+	+	+	–	–
Morphology	Rod	Rod	Rod	Rod	Rod
Motility	–	–	–	–	–
Biochemical Test					
Indole test	–	–	–	–	–
Methyl-red test	+	+	+	+	+
Vogusproskaeur test	+	+	+	+	+
Citrate utilization test	+	–	–	+	+
Nitrate reduction test	+	+	+	+	+
Urease production test	+	+	+	+	+
Catalase test	+	+	+	+	+
Oxidase test	–	–	–	–	–

(DNS) method. The results showed that 24 h was the optimum incubation period for maximum amylase production. It was observed that 120 h (5th day) the activity of amylase was decreased (Fig. 2A).

Fig. 2A illustrates that the amylase activity at various incubation time. The curve shows the decrease in enzyme activity from 24 h (1st day) to 120 h (5th day). This is because the cell may reach the decline phase and displayed low amylase synthesis. The bacterial strain showed maximum enzyme activity at 24 h. Amylase activity was relatively low at 120 h. From this, amylase activity was inversely proportional to time of incubation.

Catalase enzyme assay

The catalase assay of the strains Ca03, Ca07 and Ca08 were determined. The result showed that 24 h was the optimum incubation period for maximum catalase production. Fig. 2B illustrates the activity of catalase on various incubation time. The bacterial strain Ca07 showed maximum activity at 24 h. After 24 h the catalase enzyme activity started to decrease. It shows that the cell reached the decline phase and hence displayed low catalase synthesis. Catalase activity was relatively low at 120 h.

Determination of specific activity of amylase and catalase

The specific activity of isolates AM01, AM03 and AM09 were determined (Fig. 3A). All isolates showed higher specific activity at 24 h and lower at 120 h. The results showed that the isolate AM01 is a better amylase producer than other isolates.

The specific activity of the catalase of the strains Ca03, Ca07 and Ca08 in different incubation period (24, 48, 72, 96 and 120 h) were determined using Lowry's method for the protein standard curve (Fig. 3B). The isolates (Ca03) showed higher specific activity at 24 h and the other two isolates

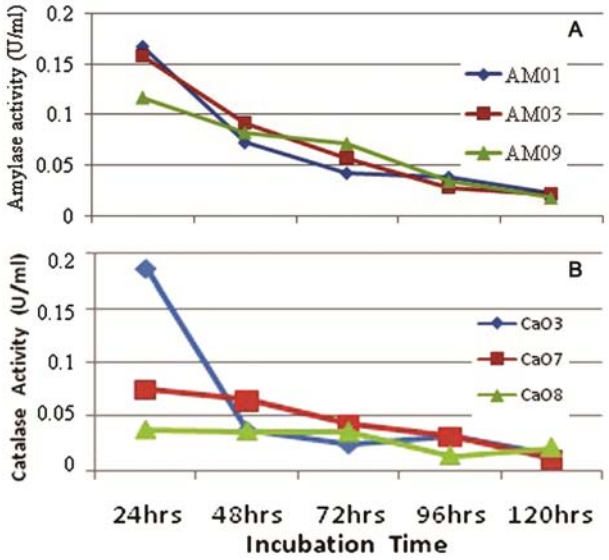

Fig. 2 — Estimation of (A) amylase; and (B) catalase activity on various incubation time

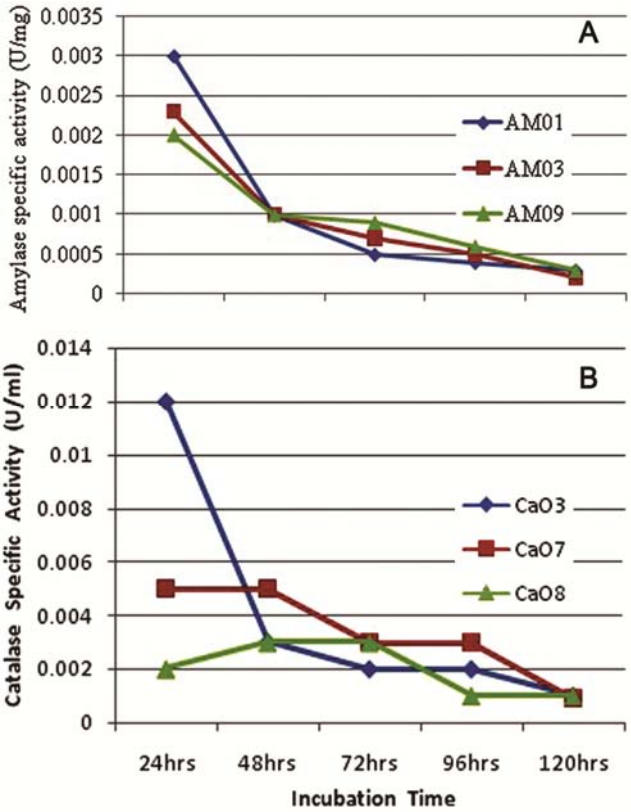

Fig. 3 — Determination of specific activity of (A_ amylase; and (B) catalase

(Ca07 & Ca08) showed higher activity at 48 h and lower at 120 h.

Fig. 4A illustrates the specific activity of amylase on day 1 (24 h). When compared to other two strains, AM01 exhibit higher amylase

activity on 1st day of incubation. Hence, 24 h is the optimum incubation period for amylase production. Fig. 4B shows the specific activity of catalase (Ca03, Ca07, Ca08) on day 2 (48 h). When compared to other two strains, Ca07 exhibit higher catalase activity on 2nd day of incubation. Hence, 48 h is the optimum incubation period for catalase production of Ca07.

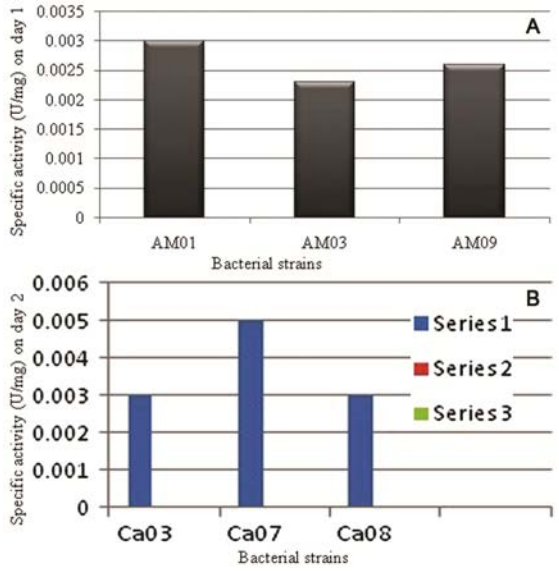

Fig. 4 — Specific activity of (A) amylase on 1st day of incubation; and (B)catalase on 2nd day of incubation

Phylogenetic analysis of AM01 and CA07

The phylogenetic tree based on a comparison of the 16S rRNA sequences of amylase producing bacterial isolates AM01 and some of their close phylogenetic relatives, the tree was created by the neighbor-joining method (Fig. 5A). Similarly, the phylogenetic tree based on a comparison of the 16S rRNA sequences of catalase producing bacterial isolates Ca07 and some of their close phylogenetic relatives, the tree was created by the neighbor joining method (Fig. 5B). It revealed that the strain Ca07 is *Klebsiella quasipneumoniae.*

In this study, we isolated bacterial strains from marine sediment samples that produce industrially useful enzymes amylase and catalase. Microbes are the preferred source of amylase and catalase for research and industrial applications because of their high activity levels, rapid cultivations and easy handling[32]. The maximum enzyme activity was observed in 24 h of incubation for the strain AM01 and 48 hours of incubation for the strain Ca07. The phylogenetic analysis and molecular characterization

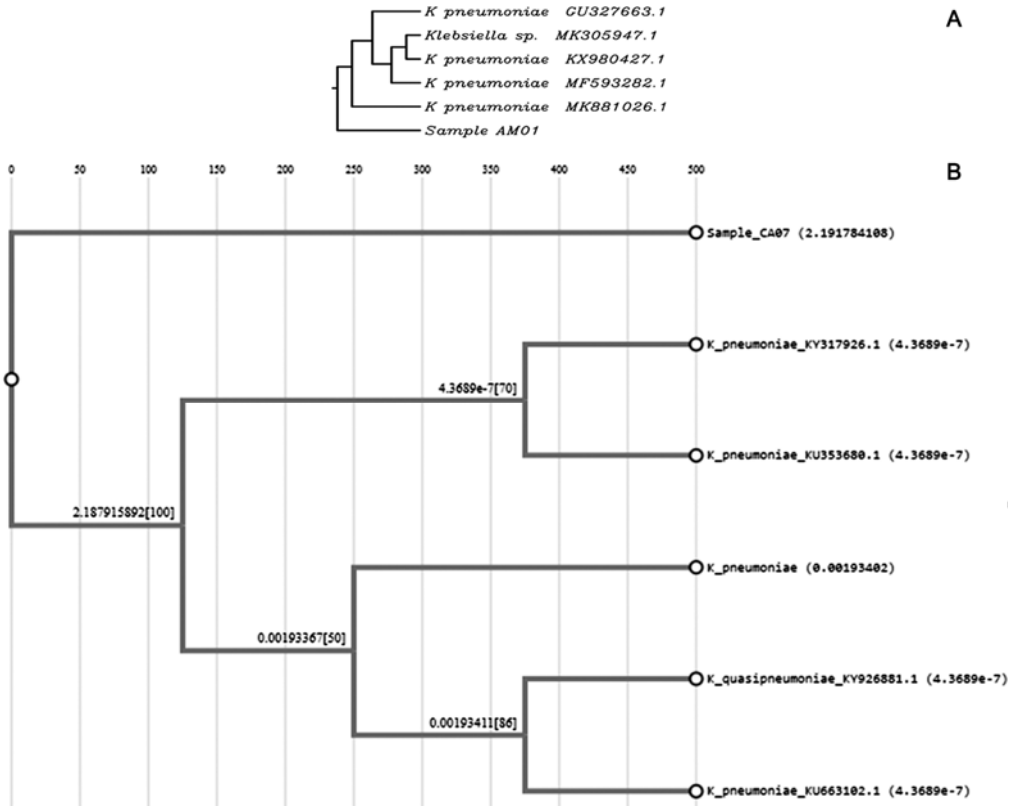

Fig. 6 — Phylogenetic tree of bacterial strain (A) AM01; and (B) Ca07

of the strain AM01 and CA07 revealed that the isolated strains are *Klebsiella pneumoniae* and *K. quasipnuemoniae*, respectively. The location of the marine sediment samples collected for the study showed the significance of the industrial area which is nearer to KMML factory and also Neendakara harbour. Reports showed that human activities such as human settlements, aquaculture farming, anthropogenic pollution leads to the contamination of marine habitat with various potential pathogenic bacteria including *E coli*, *K.pneumonia*, *Serratia marcescens* and *Enterobactor clocae*[33].

The *Klebsiella* genus is responsible for a variety of diseases in animals and humans[34]. Out of the four disease causing *Klebsiella* species, *K. pneumonia* is clinically the most important species as compared to *K*. oxytoca, *K*. ozaenae and *K. rhinsocleromatis. K. pneumonia* has both clinical and non–clinical habitats[35]. Surface water, drinking water, soil, plants, sewage and industrial effluent are the environmental reservoirs of *K. pneumonia*[36]. It can also behave as opportunistic human pathogen and can fix nitrogen in anaerobic conditions. This study also reveals the diversity of *Klebsiella* species and their amylase and catalase producing potential from the marine sediment samples. Isolation of this amylase and catalase producing bacterial strain would also provide scope to assess their biotechnological potential.

Conclusion

In this study, we identified, isolated and genetically characterized a new amylase and catalase producing bacterial strain *Klebsiella pneumoniae* and *Klebsiella quasipnuemoniae*, respectively from the marine sediment samples. Specific activities of amylase isolates were determined and the strain AM01 (*K. pneumoniae*) showed the higher amylase yielding organism. Hence, it was selected for gene sequencing and further identification. Similarly, determining the catalase production of the isolates by bubble test (catalase test), the high yielding isolate Ca07 at 48 h (*K. quasipneumoniae*) was selected for identification. Morphological and biochemical characteristics indicated that the isolate was Gram negative, rod shaped and non-motile organism. 16S rRNA gene fragments were sequenced and phylogenic tree was constructed. The results confirmed the isolate AM01 to be *Klebsiella pneumoniae* and CA07, *Klebsiella quasipneumoniae*. This preliminary screening of *Klebsiella* species and their enzymes producing potential from marine sediment revealed a high taxonomic diversity among these isolated *Klebsiella*.

Conflict of Interest

The authors declare no conflict of interests

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