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Research Article

Bacteria from Coral Ecosystem of Kiltan Island, Lakshadweep: Resource for Hydrolytic Enzymes

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Abstract

Gelatinase, Protease, Amylase and Lipase are the important enzymes which are widely used in Chemical, medical, food industries and basic biological science. Enzymes reported till date is produced mostly by microorganisms of terrestrial origin. However, only a few number of reports reported from marine producers, their capability have not been explored in details. More over an enzyme from the marine origin may be an inimitable protein molecule not found in any terrestrial organism or it may be a known enzyme from a terrestrial source but with novel properties. Coral reefs are highly productive natural ecosystem and provide an excellent habitat for a vast array of marine organisms due to their structure, efficient biological recycling and high retention of nutrients. Considering all these, the present study made an attempt to screen the potential gelatinase, protease, amylase and lipase producing bacteria from coral ecosystem of Kiltan Island of Lakshadweep. Depending on the diameter of zone production in the respective substrate containing medium, two bacterial strains was selected for further studies. The isolated bacterium has showed varying enzymatic activity in different temperature and pH. The biochemical analysis and 16S rRNA gene analysis revealed that, the isolated bacteria closely related to B. fluxes and B. cereus.

Keywords: *Gelatinase; Protease; amylase; lipase; Halophilic; 16S rRNA*

1. Introduction

Microorganisms, animals and plants produce different type of enzymes. Because of the broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation Microorganisms signify the most common resource of enzymes (Bragger *et al*, 1989; Annison, 1992; Niehaus *et al*, 1999). Compared to enzymes from plants and animals, microbial enzymes were found to be more active and stable. Industries are looking for new microbial strains in order to produce different enzymes to complete the current enzyme requirements. Finding of new resource of novel and industrially valuable enzymes is a key research hunt in enzyme biotechnology. Hydrolytic enzymes are very important in industrial uses. Among them Amylase, protease, lipase and gelatinase has major role in many fields.

Amylases are a group of significant enzymes that breaks starch into sugar and it is mainly employed in the starch processing industries for the hydrolysis of polysaccharides like starch into simple sugars (Mitchell and Lonsane, 1990) and this enzyme accounts about 30% of the global enzyme production (Sivaramakrishnan *et al*, 2006). Amylases were used in bread making industry, and they can breakdown complex sugars such as starch into simple sugars such as glucose, maltose and dextrin.

Protease stands for more than 60% of all industrial enzyme sales in the world and this enzyme were used in the detergent industry, leather industry, and also for pharmaceutical applications, such as digestive drugs and anti-inflammatory drugs (Guerard *et al*, 2002; Franz *et al*, 1992). In 1960, Dane first isolated alkaline protease from *Bacillus licheniformis*. As far as this, it is still established that microorganisms are the most apt resources for protease production.

Lipases are omnipresent enzymes that catalyze the breakdown of fats and oils with consequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol. Lipases are also capable in various reactions such as esterification, transesterification and aminolysis (Babu *et al*, 2008). Many microbial lipases are available commercially. Majority of this enzyme were used in food flavoring, paper production, cosmetic production, detergents, organic synthesis *etc.* (Kobayashi *et al*, 2008; Seiichi *et al*, 1991).

Gelatinase is a protease enzyme, an extracellular metallo-endopeptidase or metallo protease which is able to hydrolyze gelatin and other compounds such as pheromone, collagen, casein and fibrinogen (Makinen *et al*, 1989). Gelatinase enzyme break down the gelatin into its sub- compounds (polypeptides, peptides and amino acids) that can cross the cell membrane and be

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used by the organism. Gelatinase enzyme is widely used in chemical and medical, food and basic biological science.

Enzymes reported till date is produced mostly by microorganisms of terrestrial origin. However, only a few number of reports reported from marine producers, their capability have not been explored in details. More over an enzyme from the marine origin may be an inimitable protein molecule not found in any terrestrial organism or it may be a known enzyme from a terrestrial source but with novel properties.

The biodiversity of Lakshadweep is not well explored for any industrial application and the study on the industrially important enzyme producing bacteria is limited in Lakshadweep. A wide range biodiversity of bacteria, it mostly consisting corals, includes the largest range of habitats, hosting the most life-forms. Different Bacteria associated with corals and shows different activities by as mutual relationship, competition amongst microorganisms for space and nutrients in the marine environment is a powerful selective force, which has led to evolution. Coral reefs are highly productive natural ecosystem and provide an excellent habitat for a vast array of marine organisms due to their structure, efficient biological recycling and high retention of nutrients. Considering all these, the present study was aims to isolate potential Hydrolytic enzyme producing bacteria from coral ecosystem of Kiltan Island of Lakshadweep. The present study also made an attempt to analyse the effect of Temperature and pH on the enzyme activity. The genetic structure and phylogenetic relationship of the hydrolytic enzyme producing bacteria were also studied.

2. Materials and Methods

2.1 Study area

Kiltan Island is a coral island belonging to the Amindivi Subgroup of islands of the Lakshadweep archipelago in India.

Aerial view of Kiltan Island, Lakshadweep

The Kiltan Island lies 51 km north-east of Amini Island, between 11° 28' and 11° 30' N latitude and 72° 59' and 73° 01' E longitude, and has an area of 2.20 sq km. It has 3.4 km length and 0.6 km width at the broadest point. On the northern and southern ends of the island, there are high storm beaches. This island is 394 km (213 nautical miles) away from Kochi. Its lagoon area is 1.76 sq km.

The climate of Kiltan is similar to the climatic conditions of Kerala India. March to May is the hottest period of the year. The temperature ranges from 25^{o}C to 35^{o}C and humidity ranging from 70 -76 per cent for most of the year. The average rainfall received is 1600 mm a year. Monsoon prevails here from 15th May to 15th September. The monsoon period raises temperature to the mercury level between 27- 30 degrees. During the monsoon time, boats are not allowed outside the lagoon because of the violent sea. The presence of the reef maintains calm at the lagoon.

2.2 Sample Collection

Sea water collected from Costal ecosystem of Kiltan Island, Lakshadweep, west coast of India with the help of Scuba diver in sterile bottle. Water collected to the sterile bottle and sealed to prevent under water contact with air and possible oxidation and contamination, transported to the lab.

2.3. Bacterial Isolation

The sea water serially diluted with sterile distilled water. 100 micolitre of each dilution was spread homogeneously with the help of sterilised swab on LB media. LB media contains 10gm of tryptone, 5gm yeast extract, 30gm sodium chloride in 1000 ml distilled water, adjust slightly alkaline pH. The plates were incubated at 30°C for 24 hrs.

2.4 Detection of Enzymatic Activity

Bacteria were screened for various hydrolytic enzymes such as amylase, gelatinase, protease and lipase. Pure cultures were inoculated on plates supplemented with respective substrates and incubated at respective temperature for 24 hours and were detected enzymatic activity of bacteria.

2.4.1 Amylase

Starch media contains peptone 5gm, yeast 1.5gm, beef extract 1.5gm, starch 2gm, sodium chloride 5gm and 20 % agar in 1 litter. Isolated organisms spot on the media in different PH 7, 8, 9 and incubated at different temperature (room temperature, 30^{0}C and 37^{0}C) for 24 hrs. After incubation the plates were flooded with Grams iodine solution, presence of clear zone around the culture was taken as positive result. The diameter of the zone is measures that indicate the maximum activity of the enzyme.

2.4.2 Porotease

Casein incorporated in to nutrient agar was used as the media for detecting protease activity. Isolated micro organisms inoculated on plates supplemented with respective substrate in different PH 7, 8, 9 and incubated at different temperature (room temperature, 30^0C and 37^0C) for 24 hrs. Clear zone around the bacterial colony indicate the casein hydrolysis, which is the positive result.

2.4.3 Gelatinase

Gelatine incorporated (4 gm) with LB agar in 1000ml of distilled water in different PH. Then the isolated micro organisms spotted on plates supplemented with respective substrate in different PH 7, 8, 9 and incubated for 24hrs at different temperature (Room temperature, 30^0C, 37^0C). After incubation gelatine incorporated LB agar flooded with 15% mercury chloride in concentrated hydrochloric acid, clear zone developed around the colony indicate the positive result other vice negative. The zone is measured.

2.4.4 Lipases

Tributyrin media was used for screening of lipase producers, which contains peptone 5gm, yeast 3gm, agar 15gm in 1 litter. The isolated microorganisms spotted on plates supplemented with respective substrate in different PH 7, 8, 9 and incubated for 24hrs at different temperature (Room temperature, 30^0C, 37^0C). After incubation clear zone developed around the colony indicate the positive result.

2.5 Microscopic Tests for the Identification of the Bacterium

2.5.1 Gram's staining

The bacterial cultures from 24 hour culture were taken with an inoculation loop and made a smear on a glass slide, then dried and heat fixed. Crystal violet was added to this, kept for 1 minute and was rinsed with water and then Gram's iodine was added and kept for 1 minute. The slides were then rinsed with water. Applied alcohol (decolouriser) for 10-20 seconds then rinsed with water and counter stain saffranin was added and kept for 30 seconds rinsed with water and dried and observed under oil immersion microscope.

2.6 Biochemical Characterisation of the Bacterium

2.6.1 Indole Test

The test organism is inoculated into tryptone broth, a rich source of the amino acid tryptophan. Indole positive bacteria produce tryptophanase, an enzyme that cleaves tryptophan, producing indole and other products. When Kovac's reagent is added to a broth with indole, a dark pink color develops.

2.6.2 Methyl Red and Voges-Proskauer Tests

The methyl red (MR) and Voges-Proskauer (VP) tests are read from a single inoculated tube of MR-VP broth. After 24 hours of incubation the MR-VP broth is split into two tubes. One tube is used for the MR test; the other is used for the VP test. MR-VP media contains glucose and peptone. All enteric organisms oxidize glucose for energy; however the end products vary depending on bacterial enzymes. When the pH indicator methyl red is added to acidic broth it will be cherry red (a positive MR test). In this neutral pH the growth of the bacteria is not inhibited. The bacteria thus begin to attack the peptone in the broth, causing the pH to rise above 6.2. At this pH, methyl red indicator is a yellow color (a negative MR test). The Voges-Proskauer test (VP) cultures are incubated at 30°C for 24-48 hours. Add 1 ml of Barritt Reagent B (R030 - 40% potassium hydroxide) and 3 ml of Barritt Reagent A (R029 - 5% a-naphthol in absolute ethanol) to 5 ml culture. Positive test is indicated by eosin pink colour within 2-5 minutes.

2.6.3 Citrate Utilization Test

The citrate test utilizes Simmon's citrate medium to determine if a bacterium can grow utilizing citrate as its sole carbon and energy source. Simmon's media contains bromthymol blue, a pH indicator with a range of 6.0 to 7.6. Bromthymol blue is yellow at acidic pH's (around 6), and gradually changes to blue at more alkaline pH's (around 7.6). Un inoculated Simmon's citrate agar has a pH of 6.9, so it is an intermediate green color. Growth of bacteria in the media leads to development of a Prussian blue color (positive citrate).

2.6.4 Triple Sugar Iron agar test

Triple Sugar Iron (TSI) Agar is used for the identification of gram-negative enteric bacilli on the basis of dextrose, lactose and sucrose fermentation and hydrogen sulphide production. Phenol red is the pH indicator.

2.7 Molecular Identification

Depending on the diameter of the zone formed in the different enzyme activity test, two bacterial samples were selected for the further analysis. Molecular identification of two selected bacteria was done by using 16S rRNA gene sequence. Genomic DNA isolation, PCR amplification and sequencing was done at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram as per following procedure.

2.7.1 Genomic DNA Isolation from Bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture is taken in a microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56 °C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

2.7.2 Agarose Gel Electrophoresis

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 1).

2.7.3 PCR Amplification

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

2.7.3.1 Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16SRSF	Forward	CAGGCCTAACACATGCAAG TC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

2.7.3.2 PCR amplification profile

95 °C - 5.00 min
95 °C - 30 sec
60 °C - 40 sec 35 cycles
72 °C - 60 sec
72 °C - 7.00 min
4 °C - ∞

2.7.3 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 2).

2.7.4 ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

2.7.5 Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The PCR mix consisted of the following components:
PCR Product (ExoSAP treated) 10-20 ng, Primer 3.2 pM (either Forward or Reverse), Sequencing Mix 0.28 µl, Reaction buffer 1.86 µl, Sterile distilled water make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

2.7.5 Post Sequencing PCR Clean up

Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed. 52 µl of master mix II was added to each reaction. Contents were mixed by inverting and incubated at room temperature for 30 minutes. Spun at 14,000 rpm for 30 minutes. Decanted the supernatant and added 100 µl of 70% ethanol. Spun at 14,000 rpm for 20 minutes. Decanted the supernatant and repeated 70% ethanol wash. Decanted the supernatant and air dried the pellet.

2.7.6 Sequencing

The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

2.7.7. *Sequence Analysis*

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6.

2.7.8 Identification and Phylogeny analysis

The final sequence was used for the analysis. The sequence similarity was searched by BLAST of NCBI. The phylogenetic analysis was done by MAGA7 software.

3. Results and Discussion

3.1 Isolation of Bacteria from Coral Ecosystem

About 16 different types of colonies were isolated from marine water collected from coral ecosystem Kiltan Island of Lakshadweep (Fig. 1). The morphological characters of isolated colonies were recorded as follows. 1. White, dull, irregular, large colony 2. Large white concentric irregular colony 3. Yellow, small mucoid, round colony 4. Mucoid, grey colony 5. Muciod white colony 6. Rhizoid ,rough ,slight pinkish colony 7. Rhizoid, white large, irregular, mucoid colony 8. Medium, round, Red color, mucoid colony 9. White, opaque, medium rhizoid colony 10. Concentric grey color, large, round colony 11. Small, yellow color colony 12. Smooth, circular, mucoid, convex, grey color colony 13. Red, medium size, mucoid colony, 14. concentric, irregular grey colour colony, 15.white smooth mucoid colony, 16. Pin point yellow colour, mucoid colony.

Rosenberg *et al* (2007) have depicted coral reefs as the prevalent structures finished by living creatures. Biogenic activities over millennia has lead reefs to evolve to become a complex, productive, yet fragile marine ecosystem with a biodiversity to rival that of a terrestrial rainforest (Rohwer et *al*, 2002; Allen and Steene). The presence of huge number of colonies in water collected from coral ecosystem may be due to its structure, efficient biological recycling and high retention of nutrients (Kleypas, 1997).

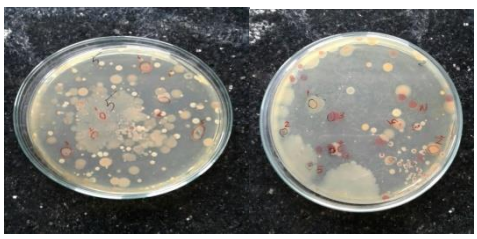

Fig.1 Plate showing different colonies of bacteria obtained in spread plate

3.2 Screening for Amylase Producers

The isolated bacterial colonies were screened for amylase production. Majority of the colonies (13 Nos.) showed the amylolytic activity (Fig. 2). The amylolytic activity of each bacterial colony was presented in diameter of zone formed in starch agar (Table 1).

Fig.2 Amylolytic activity of bacterial colonies

Table 1 The amylolytic activity of Bacteria expressed in diameter of zone formed (cm)

	pH 7			pH 8			pH 9		
	RT	30°C	37°C	RT	30°C	37°C	RT	30°C	37°C
1	0.8	2.2	2.5	1.8	2.3	2	2.2	2.2	2.2
2	0.6	2.2	2.1	2.0	2.6	2	2.0	2.2	2.1
3	0.3	2.1	2.0	2.0	1.1	1.3	1.2	1.8	1.3
4	0.2	0.9	1.4	1.3	1.3	1.4	1.1	1.8	1.0
5	0.3	0.4	1.1	0.4	0.4	0.4	1	0.7	0.9
6	0.2	0.5	0.9	0.9	0.6	0.6	0.9	0.5	1
7	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
8	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
9	0.2	0.9	9.0	1.5	1.3	0.5	1	1.4	1.1
10	0.1	2.2	1.3	2.0	2	0.3	0.3	2.0	0.7
11	0.1	0.9	1.4	1.1	1.3	2	0.4	1.4	1.4
12	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
13	0.2	2.3	1	2.1	2	2.3	0.3	1.5	2
14	0.3	2	2.1	0.6	2.2	2	Nil	1.7	0.5
15	Nil	1.2	2	0.3	1	2	0.4	2	0.8
16	0.2	1.3	1.7	0.5	1.2	1.9	0.5	1.8	2

3.3 Screening for Protease Producers

Casein in-cooperated nutrient agar was used as the media for detecting protease activity. The zones produced due to protease activity were presented in Fig. 3 and proteolytic activity was measured by the diameter of zone produced (Table 2).

3.4 Screening for Gelatinase Producers

After incubation gelatin incorporated LB agar flooded with 15% mercury chloride in concentrated HCl, Clear zone around the bacterial colony indicate the casein hydrolysis, which is the positive results other ways negative.

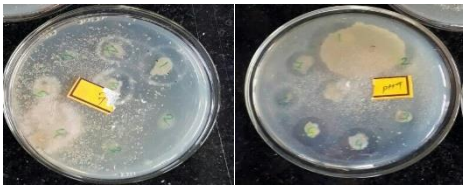

Fig.3 Proteolytic activity of bacterial colony

The selected bacteria were showed gelatinase activity in different ranges in varying temperature. The gelatinase activity were showed in Fig. 4 and Table 3.

Table 2 The proteolytic activity of bacteria expressed in diameter of zone formed (cm)

	pH 7			pH 8			pH 9		
	RT	**30°C**	**37°C**	**RT**	**30°C**	**37°C**	**RT**	**30°C**	**37°C**
1	Nil	1	0.3	Nil	1.2	2	0.2	2	0.6
2	Nil	0.8	0.9	Nil	1.7	0.9	0.1	1.6	0.6
3	Nil	Nil	Nil	0.6	1.1	Nil	0.4	0.6	0.9
4	Nil	Nil	Nil	0.3	1.3	0.5	0.2	1	0.8
5	Nil	0.6	0.8	0.5	1	0.4	0.4	1.4	0.9
6	Nil	0.1	0.4	0.7	1.5	1.1	0.2	1.6	0.6
7	Nil	0.6	1	0.8	1.9	2	0.3	2	1
8	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
9	Nil	0.8	1.1	0.8	1.3	1.1	0.7	1.2	0.9
10	Nil	0.6	1.3	0.5	1	1.3	0.1	1.1	1
11	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
12	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
13	Nil	0.3	0.6	0.2	1.4	1.9	0.2	1.4	1.3
14	Nil	0.2	0.8	1	0.2	0.8	0.9	1	1.3
15	Nil	0.6	0.4	0.6	1	1.2	0.3	1	1.6
16	Nil	0.5	0.8	0.3	0.9	1.4	0.2	1.7	2

Fig.4 Gelatinase activity of bacterial colony

Table 3 The gelatinase activity of bacteria expressed in diameter (cm) of zone formed

	pH 7			pH 8			pH 9		
	RT	**30°C**	**37°C**	**RT**	**30°C**	**37°C**	**RT**	**30°C**	**37°C**
1	0.5	3.3	1.8	0.2	2.9	3	0.8	2	3.2
2	0.3	1.5	1.2	0.3	2.6	2.8	0.3	2.8	2.5
3	0.3	0.7	0.8	0.3	1.5	2	0.6	0.4	0.9
4	0.2	0.9	1.2	0.2	2.7	3	0.4	1.5	2.4
5	0.4	1.2	2.4	0.6	2	2.4	0.8	1	1.5
6	0.6	1	2.4	0.3	1.6	2	Nil	0,8	1
7	0.1	1	1.2	0.1	0.6	1	0.7	2.2	3
8	0.3	1.2	2	Nil	1.5	2	0.9	2.6	3.5
9	0.3	1	3	0.3	1.9	1.6	0.4	1.6	2
10	0.2	0.6	3.2	0.1	1.2	1.4	0.1	0.8	0.9
11	0.1	0.4	0.6	0.1	1.4	1.5	0.2	0.8	1.1
12	0.2	0.5	0.9	Nil	1.9	2	1.8	2.3	3
13	0.4	1.2	2	0.1	2.5	3.5	1	2.5	3.5
14	0.3	0.8	1.1	0.3	2	2	0.5	1.7	1.9
15	0.5	1	1.6	0.4	1.5	1.9	o.8	2.2	2
16	0.2	0.9	0.7	0.2	1	1.4	0.3	1.7	2

3.5 Screening for Lipase Producers

Clear zone around the bacterial colony indicate the lipase hydrolysis, which is the positive results. Lipolytic activity of most of the bacteria was less compared to other enzymes. The result of lipase activity test was presented in Fig. 5 and Table4.

Fig.5 Lipolytic activity of bacterial colony

Table 4 The lipolytic activity of bacteria expressed in diameter zone formed (cm)

	pH 7			pH 8			pH 9		
	RT	**30° C**	**37°C**	**RT**	**30° C**	**37° C**	**RT**	**30°C**	**37° C**
1	0.2	1.7	2	0.5	1.4	1.9	0.3	2	2.2
2	Nil	0.2	1.9	0.3	2	2.3	0.4	2.5	2
3	0.1	0.9	1	0.2	1.5	1.9	0.3	1.5	1.8
4	0.1	1.7	1.2	0.3	1.6	2	0.4	1	0.8
5	Nil	0.4	0.6	Nil	2	1.2	0.5	0.6	Nil
6	0.2	1.1	1	0.5	1	1.4	0.1	1.2	2
7	Nil	0.2	0.4	0.3	0.6	1	o.3	1	1.2
8	0.1	0.5	0.5	0.1	1	1.8	0.3	1.4	2.4
9	0.4	1.5	0.6	0.5	1.6	2	0.5	2	2.2
10	Nil	0.3	0.6	0.2	1.7	1.9	0.3	1	1.1
11	0.1	1.2	1	0.1	1.2	1.4	0.3	1	1.4
12	Nil	Nil	2	0.5	1.1	1.5	0.2	1.6	2
13	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
14	0.3	1.4	1	0.5	1.2	1.4	0.2	1	1.3
15	0.5	1.4	1.7	0.2	0.8	1	0.3	0.9	1.1
16	Nil	0.5	0.8	Nil	0.4	0.9	Nil	0.9	1.2

The bacteria will induce to produce specific enzyme in the presence of large amount of substrates in the medium. To access these substrates, microorganisms must secrete enzymes capable of hydrolyzing these compounds (Marx *et al*, 2007). Most of the bacteria expressed the amylase activity comparatively in large quantity. The previous studies also reported the high amylase activity in marine bacteria (Shanmughapriya *et al*, 2009; Annie and Shanta, 2010). Since lipase utilize wide range of substrate, therefore it is considered as one of the most important enzyme (Clarke *et al*, 2005). Compared to other enzymes the production of lipase was less. Microbes of marine origin can be found as intracellular or extracellular symbionts, and their hosts are mostly marine animals (vertebrates or invertebrates). These symbiotic microorganisms must possess an arsenal of enzymes and pathways to fulfill the requirements of the host organisms (Vogel, 1977). In a previous study bacteria associated with the Caribbean demosponge *Ceratoporella nicholsoni* were able to consume substrates like DNA, gelatin and a range of fatty acids (Santavy and Colwell, 1990). The present study also confirms the production of gelatinase, protease, lipase and amylase production ability of microbes associated with coral ecosystem.

In the present study, different temperature such as RT, 30°C and 37°C were used to check the optimum temperature for the enzyme production by isolated

bacteria culture. Most of the bacterium showed maximum activity in 37°C and less activity in 30°C. The enzyme activity was studied in different pH also. Majority of the bacterium expressed maximum activity in pH 8 and 9.

A study by Larbi Daouadji *et al* depicted the effect of incubation time, medium pH, temperature, carbon source and nitrogen source for the lipase production (Larbi *et al*, 2014). In his study, he found that *B. licheniformis* showed were maximum at pH 8 and temperature 40°C. The variation in the enzyme activity in different organism in different temperature and pH may be related to the influence of physiological parameters on the secretion of extra-cellular enzymes by altering the physical properties of the cell membrane.

3.6 Morphological Characteristics of Bacteria

Gram's staining results clearly showed the basic morphological characters of selected bacteria. The results of Gram's staining were presented in Table 5.

3.7 Biochemical Characteristics of Selected Bacteria

The biochemical characters of selected bacteria were analysed. The result of IMViC and TSI were presented in Table 5.

Table 5 Biochemical and Gram's staining property

S. No.	Indole	MR	VP	Citrate	TSI		Gram's staining
					Slant	Butt	
1	-	-	+	+	No change	No change	Gram +ve bacilli
2	-	-	+	+	Yellow	No change	Gram +ve bacilli
3	-	+	+	+	No change	No change	Gram -ve cocci
4	-	+	+	+	No change	No change	Gram+ ve bacilli
5	-	+	-	+	Yellow	Yellow	Gram -ve bacilli
6	-	-	-	-	No change	No change	Gram +ve cocci
7	-	+	-	+	No change	No change	Gram +ve cocci
8	-	-	+	+	Yellow	Yellow	Gram –ve bacilli
9	-	-	+	+	Yellow	Yellow	Gram –ve cocci
10	-	+	+	+	Yellow	Yellow	Gram -ve, cocci
11	-	+	-	+	yellow	Yellow	Gram –ve cocci
12	-	-	-	-	yellow	Yellow	Gram -ve bacilli
13	-	+	+	-	Red	No change	Gram –ve cocci
14	-	+	+	-	yellow	No change	Gram -ve baclli
15	-	+	-	-	red	Yellow	Gram +ve cocci
16	-	-	-	+	red	No change	Gram +ve cocci

3.8 Molecular Identification

3.8.1 *Genomic DNA isolation*

The genomic DNA of two selected bacteria's was isolated and the quantity and quality was cheked by Agarose gel electrophoresis. The gel image of AGE is presented in Fig. 6.

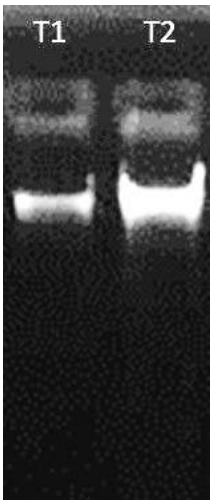

Fig.6 Gel image showing the genomic DNA isolated from bacteria

3.8.2 *PCR amplification and sequencing of 16S rRNA gene*

The 16S rRNA gene was amplified by using universal primers. The PCR amplified product was about 1300 bp in size. The PCR amplified product was showed in Fig. 7. The PCR products were sequenced using universal primers.

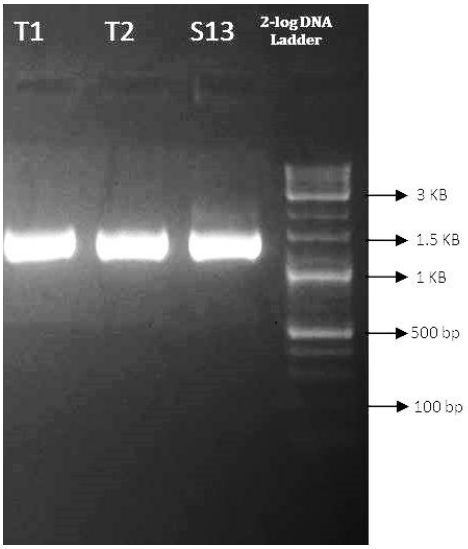

Fig.7 The gel image of PCR amplified product of 16S rRNA gene

3.8.3 *Sequence analysis and Phylogeny*

The final sequence was used for analysis. The PCR amplified 16S rRNA sequence of both bacteria showed variation with other group of bacteria and it showed maximum similarity with *Bacillus sp*. and *Bacillus flexus.* The evolutionary relationship of selected

bacteria was analysed by MEGA7 software. Sample No.1 showed close relation with *Bacillus flexus* and *Bacillus sp.* and sample No. 2 showed close relationship with *Bacillus cereus* (Fig. 8 and 9).

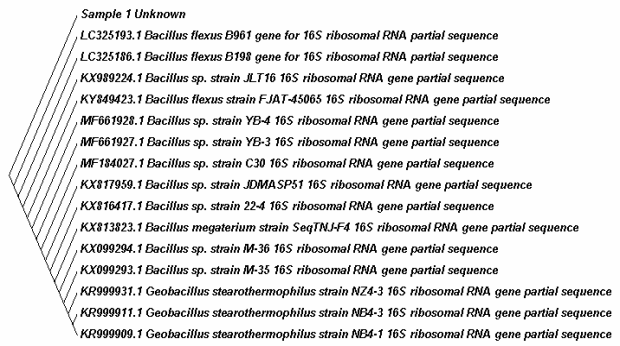

Fig.8 Evolutionary relationship of Unknown Bacteria (sample 1)

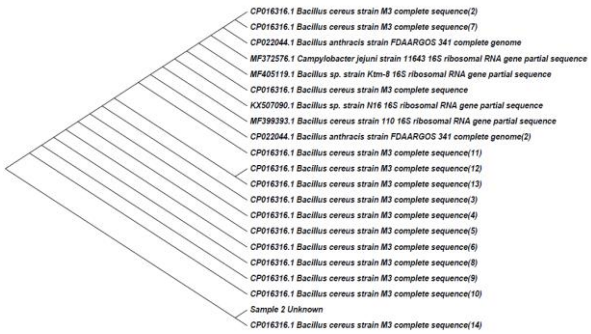

Fig.9 Evolutionary relationship of Unknown Bacteria (sample 2)

The most common housekeeping genetic marker 16S rRNA was using from far before for bacterial phylogeny and taxonomy due to following reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene(1,500 bp) is large enough for informatics purposes (Patel, 2001).

Conclusions

Based on the investigation this study has made following conclusions. The coral ecosystem is very good habitat for the marine microbes and the bacteria isolated from the coral ecosystem can able to produce major hydrolytic enzymes such as Amylase, Lipase, Protease and Gelatinase. There for these microbes has major role in Biogeochemical cycles in the coral ecosystem. Temperature and pH has influence on the production of enzymes and its activity. Phylogeneticaly sample No. 1 bacteria closely related to *B. fluxes* and Sample No. 2 bacteria closely related to *B. cereus*.

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