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Research Article

ISOLATION AND SCREENING MARINE BACTERIAL FOR THE PRODUCTION OF AMYLASE AND PHOSPHATE SOLUBILIZATION

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ABSTRACT

Marine environment, which is unique in many ways harbors microorganisms with distinctive potentials. Researchers are now concentrating on products produced by microbes of special environment for various reasons. This work was carried out to screen and isolate marine bacteria with amylase production and phosphate solubilization capacities. A total of 12 bacterial strains were isolated from three sampling sites along the coast of Arabian Sea. Six of the isolated bacterial strains showed positive amylase activity. A considerable production was there at pH 4. The highest amylase producer is Bb1 and the organism produced the least is Bb3. Tb3 and Bb2, of whose production was low in acidic as well as neutral pH, increased manifold in alkaline pH. All bacterial strains invariably showed the greatest activity at alkaline pH, 9. In the case of the temperature of incubation, except Bb1 and Bb2, other strains showed maximum activity at 37°C. Of the six strains, Ab2 showed maximum activity (2.45 Units/mL) at 37°C. This was followed by Bb3 (2.15 Units/mL) and Ab1 (1.98 Units/mL). Strain Ab2, the maximum amylase producer, produced the least amount at 20°C. Except the isolate Bb1, all isolates showed the highest production of amylase at the third day of incubation. Of all the strains, Ab2 produces the maximum amount of enzyme (2.45 Units/mL). Out of 12 strains, seven bacterial strains showed clear zone around their colonies when cultured in media substituted with ticalcium phosphate, thus showing phosphate solubilization ability. Maximum clearance zone was given by isolates from near effluent discharge site (Tb3 and Tb4 with a zone size of 8 and 8.1cm respectively). Isolates Bb2 and Bb4 did not produce clearance zone and the other isolates from the same sampling site (near a harbor).

INTRODUCTION

Marine environment is unique in several respects. It is both ecologically and physiologically diverse. The living conditions are almost adverse because of rapid temperature, pH, salinity, pressure fluctuations, currents, precipitation system and wind pattern. Hence, the life in the ocean, particularly microorganisms are tailored to these conditions and as a result, they have many exceptional potentials in terms of enzyme production, (Jayadev *et al*., 2015) and novel bioactives (Annarita *et al*., 2010) that may not be produced by terrestrial counterparts. This property has attracted so many researches on capacities of marine microorganisms. Over the last decade more that 12,000 novel chemicals have been identified and isolated from marine life, (Faulkner, 2001). The bioactive compounds from the marine source are of high demand in industries. The most important compounds which are relayed on include enzymes, exopolysaccharides, biosurfactants, antibacterials, antiviral, anticancer compounds etc. Pathirana *et al*., (1992) and Trischman *et al*., (1994a, b) have isolated antibiotics, antitumor and anti-inflammatory compounds from bacteria of coastal sediments when grown under saline conditions. (Sreelekshmi *et al*., 2015). Marine actinomycetes were found to be good sources of industrially important enzymes by Lekshmi *et al*., (2014).

Amylase is one of the enzymes which is widely used in industrial applications. These enzymes have the ability to hydrolyse glucosidic linkages in polysaccharides. The enzyme has many sources including plants, animals, and microorganisms. But microbial amylase is considered advantageous over the other because they produce this as extracellular enzymes in bulk quantities. Also, microbes can be manipulated to obtain enzyme of desired characters, (Aiyer, 2005). Aswini *et al*., (2013) isolated and purified a marine eubacteria from Nicobal islands and evaluated the efficiency of isolated enzyme at different pH, temperature, substrate and nitrogen sources.

Phosphorus is an element which is seen in abundance in both terrestrial and marine environment but is in the status of limiting nutrient not much available to the plants as a nutrient. It is seen as phosphates in both organic and inorganic

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compounds. The inability of plants to solubilize phosphorus is counteracted by the efficiency of microorganisms in all kind of environments. Thus these microorganisms supply phosphorus to plants, (Thingstad and Rassoulzadegan, 1995). Devendran *et al*., (1974) reported that phosphate solubilizing bacteria isolated from sea sediments are capable of accelerating dissolution of apatite phosphate within the phosphorus cycle and interacting with the carbon cycle. Since marine environments are more diverse and extreme, the capacities of marine microbes are thought to be significantly higher than those of other environments.

Realizing the importance of marine microorganisms in the production of industrially important enzymes and in the restoration of ecological balance, the work was done to isolate and screen marine bacteria for amylase production and phosphate solubilization. The positive bacteria for amylase production were assayed and the activity was optimized in terms of temperature, pH and period of incubation.

MATERIALS AND METHODS

Collection of Sample

Marine water samples were collected from three locations along the coastline of the Arabian sea. The sampling sites are Sankhumugham, Veli and Vizhinjam. The first sampling site is a beach, the second site is near the effluent discharge point of an industry and the third is a fishing harbor where fishing boats are operated. Samples were collected in sterile conditions, maintained in cold chain and refrigerated till microorganisms were isolated.

Isolation of bacteria

Bacterial strains were isolated in marine Zobell broth. Pure cultures were prepared and maintained both as slants and as glycerol stock for further studies.

Screening bacterial isolates for amylase production

In primary screening, all the isolated strains were inoculated on starch agar media by streak inoculation method to screen amylase enzyme activity. The plates were incubated at 30°C for 4 days. After incubation, the plates were flooded with iodine. The colonies which produced a clear zone were selected as positive strains for the production of amylase.

Secondary Screening for amylase production

Those bacterial isolates which showed a positive activity for enzymes, were subjected to quantification of enzymatic activity at various temperature, pH and incubation times was done by using shake flask method.

Fifty mL of starch broth was prepared for each positive strain and inoculated with the strain. It was incubated for 48-72 hrs and then centrifuged at 5,000 rpm for 20 min in a refrigerated centrifuge. The cell-free supernatant (crude enzyme extract) was collected and the enzymatic assay was performed. 1mL of the starch substrate was added to 0.2mL of enzyme solution and made up to 3mL distilled water, and incubated for 20 min at 37˚C. The reaction was stopped by addition of 1mL of DNS reagent (1g of 3, 5- Dinitrosalicylic acid, 20mL of NaOH and 30g of sodium potassium tartarate in 100mL distilled water) (Bernfield *et al* .,1955). The absorbance was read at 540 nm. 1 unit (IU) is defined as the amount of enzyme that released 1µmol of maltose from starch per minute at pH 7.0 at 37˚C (Kavya *et al*, 2012).

Optimization of amylase production

In order to find out the effect of incubation period on enzyme production, the enzyme activity was checked for 3rd, 6th, 9th and 12th days of incubation. Enzyme production was also checked at various pH (4, 7 and 9) and temperature (20°C, 37°C and 50°C). The optimum conditions for maximum production of amylase were determined.

Screening for phosphate solubilization

For the screening procedure, the quarter strength of Zobell marine agar was prepared and 1% of tricalcium phosphate was added before autoclaving the medium. This resulted in a milky white medium. The medium was poured on petriplates and made to soldify. After this, the bacteria was patched in four corners of the plate and incubated for 7 days at room temperature (Mehta and Nautiyal, 2001). A clear zone around the bacterial patches indicates the ability of the bacterium to solubilize phosphate in agar medium. The positive cultures which showed phosphate solubilization is stored for optimization studies.

RESULTS AND DISCUSSION

Isolation of Bacteria

The isolated bacteria from the sample were compared morphologically and biochemically and a total of 12 bacterial morphologically distinct strains were selected for the study from the sampling sites. The distribution and percentage of isolates in each sampling site is shown in Fig. 2.

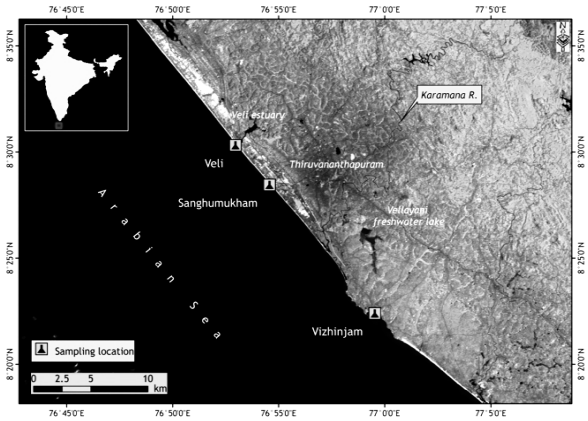

Fig 1 Sampling location

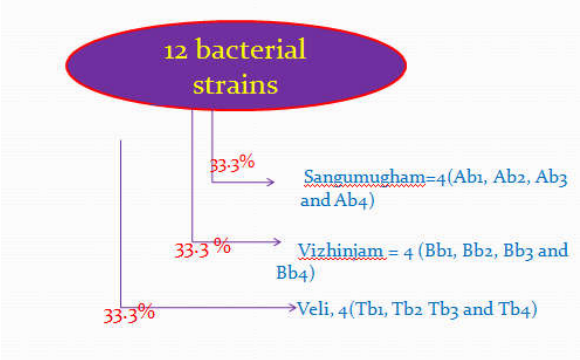

Fig 2 Distribution of bacterial strains isolated

Screening for amylase production

Six bacterial strains showed positive amylase activity. The strains were grown at various pH, temperature and duration of incubation to identify the conditions for optimum enzyme production.

pH

The positive cultures were cultured in three different pH (4, 7 and 9) corresponding to acidic, neutral and alkaline conditions to find the pH at which enzyme production is maximum. The results of this are given in table 1.

Table 1 Effect of pH on enzyme production

Bacterial strains	Amylase Enzyme activity (Units/ml) pH		
	4	7	9
Ab1	3.95	1.98	7.76
Ab2	10.2	2.45	16.51
Bb1	12.07	1.01	17.32
Bb2	2.95	1.02	11.51
Bb3	3.26	2.15	3.57
Tb3	3.2	1.51	12.57

From the table, it can be seen that almost all the isolates showed a dip in amylase production at neutral pH after a good level of activity at acidic pH. This may be the indication that the organisms need some stress for the production of the enzyme. The highest amylase producer is Bb1 and the organism produced the least is Bb3. In the case of Tb3 and Bb2, though the production was low in acidic as well as neutral pH, the production increased manifold in alkaline pH. The microorganisms vary widely in their optimum conditions for growth and life activities. Of these Tb3 was isolated from Veli coast and the sampling site was near to the effluent discharge site of a major factory. This can be a possible explanation to the result.

It can be seen that all bacterial strains invariably showed the greatest activity at pH 9, that is an alkaline condition. This is in concordance with the finding of Aswini *et al*., 2012. They got maximum amylase secretion at pH 8, showing the strains they isolated required a slightly alkaline condition for amylase production. But after that, the activity was diminished. But in this study, all the bacterial strains isolated showed their requirement of a more alkaline pH for the production of amylase. A survey through the literature reveals that not much microbes are capable of producing amylase at a pH of 9. This shows the possible potential of the organism isolated in this study to be used for some specific industrial processes.

Temperature

In the case of the temperature of incubation, except Bb1 and Bb2, other strains showed maximum activity at 37°C. Of the six strains, Ab2 showed maximum activity (2.45 Units/mL) at 37°C. This was followed by Bb3 (2.15 Units/mL) and Ab1 (1.98 Units/mL). A notable finding is that the strain Ab2, the maximum amylase producer, produced the least amount at 20°C. The studies of Aswini *et al*., (2011) showed the maximum amylase production by microbe is 40°C. The results are represented as Fig 3.

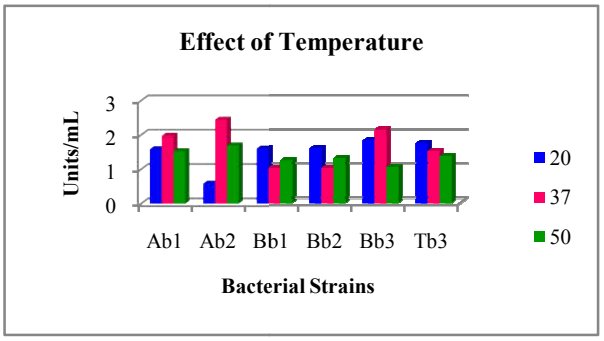

Fig 3 Effect of temperaure on amylase prodution

Period of Incubation

The effect of incubation time on amylase enzyme production is shown in Fig 4. All the strains Except Bb1 showed the highest production of amylase at the third day of incubation. Of all the strains, Ab2 produces the maximum amount of enzyme (2.45 Units/mL). Reports are there which shows the maximum enzyme production by bacteria will be in its logistic phase when they gets completely adapted to the growth conditions and starts metabolic activities at a high rate. Here in the experiment, 3rd day corresponds to the logistic phase of the isolates.

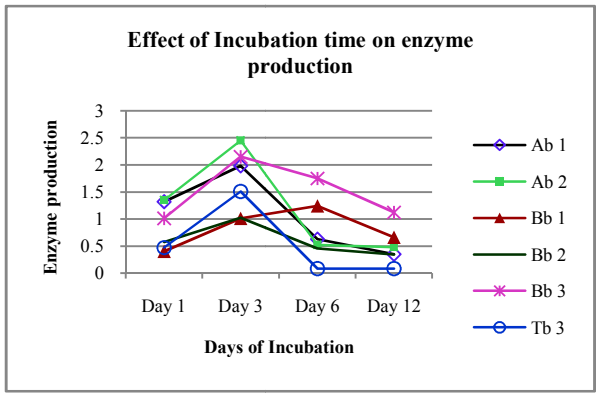

Fig 4 Effect of Incubation time on amylase production

Screening for Phosphate Solubilization

Out of 12 strains, seven bacterial strains showed clear zone around their colonies. Maximum zone of clearance is shown by bacterial isolates from near the industrial effluent discharge area. This shows the ability of the bacteria in the sampling site, which may be one of their surviving strategies. Strains isolated from beach area also show considerable phosphate solubilization.

Bacterial Strains	Phosphate solubilization Diameter (cm)
Ab1	7
Ab2	6
Ab3	6.2
Ab4	0
Bb1	3
Bb2	0
Bb3	2
Bb4	0
Tb1	6.8
Tb2	7.2
Tb3	8
Tb4	8.1

The lease activity is shown by bacterial isolates from the harbor. There can be some factors which cause the inhibition of the activity which need to be analyzed in detail. Table 2 shows the diameter of the clearance zone. Further quantification as well as characterization of positive bacteria will be done and is the future prospect of the project.

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