Isolation and screening of actinomycetes from marine samples for enzyme production

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Abstract- Many of the ecological factors of the marine environment were helpful in developing a strategy for discovering useful bioactive agents from marine microorganisms. Among them actinomycetes have gained special importance as they play a major role in recycling of organic matter, production of novel pharmaceuticals, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. The present study was carried out to isolate actinomycetes, which have potential to produce enzymes from selected marine samples. A total of twenty actinomycetes were isolated and were characterized morphologically. Screening test was performed to determine cellulase, protease and lipase activity. Almost all the isolates showed maximum enzyme production.

Index Terms: Actinomycetes, Cellulase, Lipase, Marine, Protease,

INTRODUCTION

Incorporation of biotechnology and marine environment has opened up new horizons for finding novel organisms for trapping their potential resources. The industrial sector in India is developing fast for meeting the needs of food processing, pharmacy and textile industries. Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep-sea water. These distinctive characteristics have attracted many researchers to explore in depth since there is the potential of marine microorganisms used in industry [1]. Marine micro organisms are increasingly becoming an important source in the production of medical and industrially important enzymes. More than 4000 enzymes are known today, of which many are produced commercially. Majority of the industrial enzymes are microbial in origin. The need for microbial enzymes are increasing day by day due to their clean, ecofriendly and cost effective application in many of the biotechnological processes and also are becoming important for its technical and economical advantages. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased [2].Thus, it is very crucial for the isolation of new groups of actinomycetes from unexplored or under exploited habitats be pursued as sources of novel bioactive secondary metabolites. Marine environment is considered as a huge treasure-house of marine actinomycetes resources. Marine actinomycetes have gained special attention today as they are responsible for the production of about half of the discovered bioactive secondary metabolites [3],notably antibiotics [4], antitumor agents, immunosuppressive agents [5] and enzymes [6].

Marine actinomycetes have a diverse range of enzyme activities that are capable of catalyzing various biochemical reactions. Different commercial enzymes viz. L-glutaminase, α galactosidase, amylase, cellulase, protease, L-asparaginase, have also been obtained from the marine actinobacteria [7.] Cellulases (endo-1, 4-β-glucanase, EC 3.2.1.4) are a group of hydrolytic enzymes which hydrolyze the glucosidic bonds of cellulose and related cello-digosaccharide derivatives [8].In the current industrial processes, cellulolytic enzymes are employed in the color extraction from juices, detergents causing color brightening and softening, biostoning of jeans, pretreatment of biomass that contains cellulose to improve nutritional quality of forage, and pretreatment of industrial wastes [9].

Actinomycetes are one of the known cellulose producers has attracted considerable research interests [10].Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) has broad applications in the food, oleo chemical, pharmaceutical and detergent industries as well as in diagnostic settings. They have also been used in biotechnological industries for the synthesis of biopolymers and biodiesel [11]. Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity [12]. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer [13]. Lipase production from a variety of bacteria, fungi and actinomycetes has been reported by several workers [14]. Proteases are the most important group of the enzymes produced commercially and industrial purpose. The microbial protease represents 60% of the worldwide market of industrial enzyme. They find commercial application for toothpastes as antiplaque and antitartar, cosmetics and for the recovery of silver from used X-ray films [15].

In view to the significance of marine aquatic system which provide a rich source of novel actinomycetes and the increased industrial applications of the enzymes secreted by them, the present study is aimed to study the enzyme activity of certain actinomycetes isolated from marine samples.

MATERIALS AND METHODS

Sample collection

Marine water samples for the isolation of actinomycetes were collected from Sangumugham Vizhinjam and Veli coast, Thiruvananthapuram. The samples were collected in sterile bottles and were brought to laboratory maintaining a cold chain and refrigerated.

Enrichment and Isolation of Actinomycetes

For enrichment 1 ml of each selected sample was transferred to 100ml of starch casein broth supplemented with 25 mg/ml cycloheximide and 25 mg/ml nalidixic acid [16] and incubated at 300C for 7 days in shaker at 200rpm. Isolation of actinomycetes were done by the serial dilution and pour plate technique. A loopful of inoculum from the starch casein broth was streaked onto the starch casein agar (SCA) supplemented with 50 µg/ml fluconazole and incubated at 300C for 7 days [17]. Single separated colonies were selected and the subcultures were maintained on starch casein slants at 40C until further use.

Identification of marine actinomycetes by coveslip method

The isolated strains were confirmed as actinomycetes by observing their morphology under microscope. The starch casein agar was poured on sterile slide and allows solidifying. Then the organisms were streaked on it and incubate at 370C for 48hrs. After that added 2 drops of methylene blue dye and allow it for a minute. Then the slide was covered with coverslip and observed their morphology under microscope [18].

Screening of actinomycetes for enzyme production

The isolated actinomycetes strains were screened for the presence of different enzymes like cellulases, lipases and proteases. Two stages of enzymatic screening were done. All the isolated strains were subjected to primary screening while secondary scorning was done for those isolates which showed enzymatic activity in primary screening.

Primary enzymatic screening

In primary screening, all the isolated strains were inoculated on specific media by spot inoculation method to screen the selected enzyme activities (cellulase, lipase and protease). The plates were incubated at 300 C for 7 days. The media as criteria for enzyme activities are given in Table: 1

Table: 1 Primary enzymatic screening of isolated actinomycetes

Sl. No	Enzyme	Medium	Incubation days	Criteria for positive enzyme activity
1	Cellulase	Carboxymethyl cellulose media	7	Clear yellow zones after flooding with iodine
2	Lipase	Rhodamine B agar plate assay	7	Colonies showing fluorescence on UV irradiation
3	Protease	Skim milk agar	7	Clearing around the growth

Secondary enzymatic screening

A further study for enzymatic activity of the actinomycetes was done by using shake flask method. Those strains which showed positive enzyme activity were selected for secondary screening.

Cellulase assay

50 ml of ISP-4 broth was taken for each positive strain and inoculated with the strain. The strains were incubated for 48- 72hrs and then centrifuged in refrigerated centrifuge for 10min at 2,000 rpm. The cell free supernatant was collected and enzymatic assay was performed. 0.1ml of crude enzyme extract was added to 1 ml of carboxymethyl cellulose substrate and made up to 3ml with distilled water. The mixture was incubated at 60˚C for 20min and then reaction was stopped by addition of DNS reagent. The absorbance was measured at 540nm4. 1 unit (IU) is defined as the amount of enzyme that released 1µmole of glucose from carboxymethyl cellulose per minute at pH 7.0 at 60˚C[19].

Lipase assay

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis [20]. One ml of the culture supernatant was added to the reaction mixture containing 1ml of 0.1M Tris-HCl buffer (pH 8.0), 2.5 ml of deionised water and 3 ml of olive oil. The reaction mixture was mixed well and incubated at 37 °C for 30 min. Both test and blank were performed. After 30 minutes the test solution was transferred to a 50 ml Erlenmeyer flask. 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acid was titrated against 0.1M NaOH using phenolphthalein as an indicator. End point is an appearance of pink color [21]. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per minute under specified assay conditions. Enzyme activity was expressed as units per gram of dry substrate.

Protease assay

Proteolytic activity was assayed using casein as the substrate. A 0.5 ml aliquot of the enzyme extract was incubated with 1 ml of 2.0% casein solution in 0.1 M Tris HCl buffer, pH 7.0 at 37˚C for 10 min. The reaction was stopped by the addition of 5.0ml 5% trichloroacetic acid and incubated for 30 min. The mixture was filtered and 2.0ml of filtrate was added to 4.0ml of 0.1N NaOH and

0.5ml diluted Folin-Cocalteau reagent and incubated for 30 min and then the amount of tyrosine released into the filtrate was measured from its absorbance at 670 nm. Protein was estimated using BSA as the standard [19]. One unit of protease activity is expressed as the amount of enzyme which converts 1µg of tyrosine per 1min at 37˚C [22].

Effect of incubation period for the enzyme production

In order to find out the effect of incubation period on enzyme production each of the selected enzyme activity was checked for 3rd, 6th, 9th ad 12th days of incubation.

RESULTS

Isolation and identification of actinomycetes

A total of 20 actinomycetes strains were isolated from the selected marine samples. 10 (A1, A2, A3, A4, A5, A6, A7, A8, A9 and A10) from Sangumugham., 8 (B1, B2, B3, B4, B5, B6, B7 and B8) from Vizhinjam and 3(T1, T2 and T3) from Veli, coast. The morphological characters of the strains were identified usig cover slip method. The results are shown in Table.2

Table: 2 Colony morphology of the isolated actinomycetes strains

Name of the isolated Actinomycete strain	Colony Morphology
A1	Branching, Off white, slightly creamy, irregular
A2	Pink, Branching,Irregular,Dry
A3	Off white, Irregular, Powdery, Slightly creamy
A4	Grey, Powdery, Irregular, Dry, Early stage- off white
A5	Grey, Powdery, Irregular, Branching, Dry
A6	Slight grey, Powdery, Branching, Irregular
A7	Dry, Off white, Irregular, white margin, Dark cream dots at centre
A8	Off white, cottony, Irregular, Dry, yellow pigment producing
A9	Yellow, Creamy, opaque
A10	Greyish white, Branching, Irregular
B1	Slightly pink, branching
B2	Off white, Branching, Powdery, Irregular
B3	Off white, Waxy surface
B4	White, Dotted, Slimy, Non branching
B5	White, Become grey when mature, Branching, opaque, Dry, Irregular
B6	Off white, Grey at centre, Branching, opaque, dry, irregular, medium size
B7	Creamy, Branching, Irregular, opaque, Medium sized
T1	Yellow, Immature colonies-off white, Creamy, Dotted
T2	Brown pigment producing, Dry, cottony surface, irregular, opaque
T3	Off white, creamy, Irregular

Primary Enzymatic Screening

In primary screening, all the isolated strains were inoculated on specific media by spot inoculation method and the results are shown in the Table: 3Almost all the strains showed positive results for the selected enzyme activity. Out of the 20 strains, strain A2 showed none of the enzyme production. The strains which showed positive enzyme production were selected for further enzymatic assay.

Table: 3 Primary Enzymatic screening of isolated actinomycetes strains

Serial No.	Actinomycete Strains	Cellulase Activity	Protease Activity	Lipase Activity
1	A1	+	+	+
2	A2	-	-	-
3	A3	+	-	+
4	A4	+	+	-
5	A5	+	+	-
6	A6	+	+	-
7	A7	-	-	+
8	A8	-	+	+
9	A9	+	+	+
10	A10	+	-	+
11	B1	+	-	+
12	B2	+	+	+
13	B3	+	-	-
14	B4	-	+	+
15	B5	+	+	+
16	B6	+	+	+
17	B7	+	-	-
18	T1	+	+	+
19	T2	+	+	+
20	T3	-	+	+

Cellulase assay

In the case of cellulase enzyme activity almost all the strains showed maximum enzyme production. on the 6th day of incubation (Fig:1). Of all the strains, strain A3 showed maximum enzyme production followed by strain T2 (16.054 U/ml and 15.36 U/ml respectively). Strain B2, A6 and A10 showed almost equal rage of enzyme production (13.292, 13.119 and 13.810 U/ml respectively). Minimum enzyme production was shown by strain B7 (7.595 U/ml). All the other strains showed almost equal rage of enzyme activity (Fig: 1). All the strains showed a similar trend of enzyme production from 3rd to 9th day of incubation i.e. the enzyme production increased gradually from 3rd to 6th day of incubation and then decreased from 9th to 12 th day of incubation. (Fig: 1)

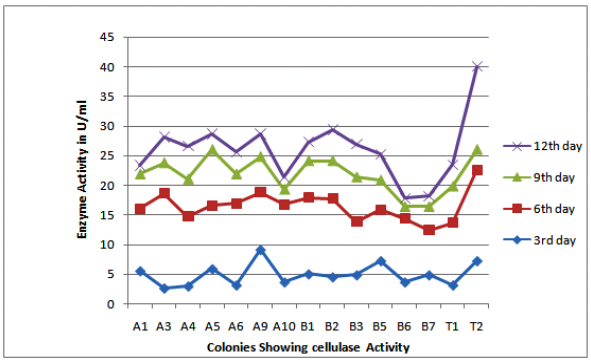

Fig: 1 Cellulase enzyme activity shown by the isolated strains

Lipase assay

In the case of lipase activity, almost all the strains showed good enzyme activity with slight variation (Fig:2). Out of the 14 strains, strain B1, B2, B6, T1 ad T3 showed maximum activity on 6th day of incubation where as all the other strains showed maximum enzyme production on 9th day (Fig: 2). Maximum enzyme production was shown by strain A3 on 9 th day of incubation (420U/ml). All the other strains showed almost equal range of enzyme production.

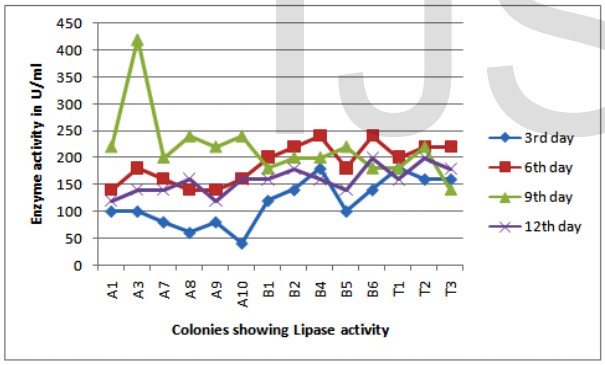

Fig: 2 Lipase enzyme activity shown by the isolated strains

Protease assay

In the case of protease production, except strains A1 and A4, all the other strains showed maximum enzyme production on the 6 th day of incubation where as A1 and A4 showed maximum enzyme production on the 9 th day (Fig: 3). Of all the strains strain B6 showed maximum protease activity (111.25 U/ml) followed by T1 and T2 (96.25 U/ml). All the other strains showed almost similar range of enzyme production. (Fig: 3)

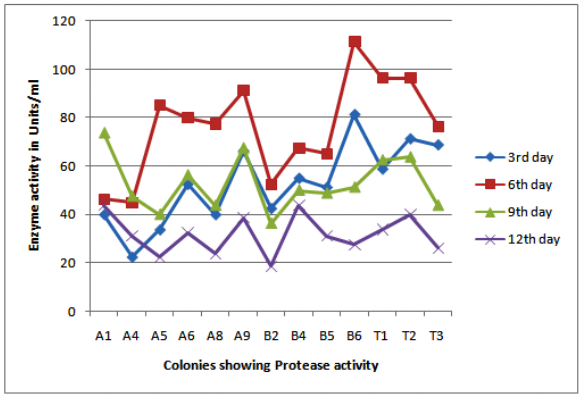

Fig: 3 Protease enzyme activity shown by the isolated strains

DISCUSSION

Marine microorganisms were proven already to have many beneficial bioactivities such as production of industrial enzymes [23],[24], plant growth promotion potentials such as production of phytohormones, antibacterial and probiotic activity [25]. Of that marine actinomycetes are potential producers of a variety of biologically active enzymes. Prevoious studies show that actinomycetes have gained enormous importance since they possess a capacity to produce and secrete a variety of extracellular hydrolytic enzymes [26], [27]. Studies on marine actinomycetes are very limited. In the preset study marine samples were collected from Sangumugham Vizhinjam and Veli coast, Thiruvananthapuram. The selective screening of these marine samples resulted in the isolation of 20 marine actinomycetes. All the cultures grow well in the Starch Casein Agar medium. Marine actinomycetes's physiological, biochemical and molecular characteristics such as 16SrRNA and terrestrial actinomycetes a great difference, followed by metabolic pathway is also different from terrestrial actinomycetes, which produced a variety of biologically active enzymes [28]. In the present study, all the isolated strains were tested for their cellulase, lipase and protease activities. Enzymatic activities of the isolated strains revealed that out of 20 actinomycetes 15(75%), 13(65%), and 14(70%) were possessing cellulase, protease and lipase activity respectively. In the present study, when comparing the enzyme activity, cellulase producing enzymes are more. Previous reports also revealed that actinomycetes are one of the known cellulose producers [29].

The secondary screening of the isolated strains showed that strain A3 showed superior activity in the case of lipase and cellulase production. Previous studies on enzymatic activity of the active isolates shows that approximately 90 % of isolates produced one or more

enzyme activity [30]. In this study almost all the strains possess more number of enzyme activities. In the present study the isolates showed a potential to produce a wide range of enzymes which may be due to the result of natural selection of microorganisms in order to survive in the competing environment [31]. Earlier studies have also proved that marine bacteria including marine actinomycetes are exhibiting diverse pattern in secreting extracellular enzymes [32], [33]. It was found that Strain A2 lacks the selected enzyme production. The enzyme activity of the 20 actinomycetes varied from isolate to isolate depending upon the growth and physical condition of the isolates. This difference may be due to the nature of the sample from which they isolated. Mukesh Sharma [34] also reported that biological functions of actinomycetes mainly depend on the sources from which the bacteria are isolated.

CONCLUSION

From this study, it is clearly indicated that marine water system can be considered as an important resource for screening useful enzyme producing actinomycetes. The selected actinomycetes in the present study showed excellent activity of cellulase, protease and lipase enzymes. These isolates may be effectively used in large scale production for commercial, industrial and pharmaceutical applications in the coming future. Further investigations are required to make use of the full potential of these organisms for enzyme production by modern protein engineering technology.

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