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Production and Partial Purification of Amylase Enzyme from Marine Actinobacteria.

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Abstract
The marine environment represents largely untapped source for isolation of new microorganisms. Gram positive actinobacteria are of special interest, since they are known to produce enzymes with a wide range of biological activities. This research work aimed at isolation of actinobacteria from marine sediment samples, screened for amylase enzyme production and optimizes the most suitable medium with specific Temperature, pH, Carbon and Nitrogen source. Total of 8 different actinobacteria were isolated from marine sediments, collected from Konathur beach Chennai, Tamil Nadu India. The isolates were identified as actinobacteria by morphological studies. Amylolytic activities were exhibited by the strains 1, 2, 4, 5 and 8 and it was confirmed by formation of clear zones of hydrolysis around the colonies. Among the isolates maximum amylase activities were exhibited by 5th strain which was determined by DNS (3, 5-dinitrosalicylic acid) assay method. The optimum pH for strain 5 was found to be 7 and the optimum Temperature for strain 5 was found to be 40^{0}C . The best carbon source was found to be Maltose and the best Nitrogen was Ammonium Phosphate($(NH_4)_3HPO_4$).
Among the actinobacteria strains screened for amylase production, the 5th strain serves as the best for amylase production.

Keywords: Actinobacteria, Amylase Enzyme, Production and Purification

INTRODUCTION
The marine biosphere is one of the richest habitats of microorganisms. The oceans cover around 70% of the earth's surface and present themselves as an unexplored area of opportunity**.** Marine microorganisms are increasingly becoming important source in the production of medical and industrially important enzymes. Considering the fact that marine environment is saline in nature. It could provide rare and unique microbial products, particularly enzymes that could be safely used for human therapeutic purpose (Dhevagi & Porani, 2006).

The actinobacteria belong to a large group of aerobic, high G-C percentage gram positive bacteria that form branching filaments or hyphae and asexual spores. These bacteria closely resemble fungi in overall morphology. Presumably this resemblance results partly from adaptation to the same habitat. Studies of the fine structure of actinobacteria spores during germination have been confined to the genera *Streptomyces* (Kalakoutswl & Agre, 1973). The latter genus forms endospores which behave in a similar way to those of *Bacillus,* a new wall

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layer being synthesized inside the cortex of the spore and extending to form the germ-tube wall.
Das and Khan (2008) explained that actinobacteria are easily isolated from the marine environment, and other, often uncultured. Actinobacteria are detected using molecular techniques. This uncultured diversity has also shown increased success in being cultured. Actinobacteria are a pre-eminent source of enzymes, but in the hypothesis and process-driven field of marine microbial ecology they are not linked to the study of major ecological process nor often recognized as key species.

The marine actinobacterial diversity has been mainly investigated by culture-dependent methods, and at least 66 actinobacterial genera have been cultured from different marine environments (Goodfellow & Fiedler, 2010).Early study reported that the existence of actinobacteria in this environment was believed because of soil contamination, or to their presence on algal material floating on the surface of the sea, or to the fact that the samples of water were obtained near the docks.
Enzymes are biological catalysts which are indispensable component of biological reactions. The use of biological catalyst has been followed for a very long period of time. There are about 40,000 different enzymes in human cells, each controlling a different chemical reaction.

They increase the rate of reactions by a factor of between 10^{6} to 10^{12} times, allowing the chemical reactions that make life possible to take place at normal temperatures. They were discovered in fermenting yeast in 1900 by Buchner and the name enzyme means "in yeast" (Ajita & Thirupathihalli, 2014)
Amylase is a hydrolase enzyme that catalyzes the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metallo enzyme i.e. it depends on the presence of a metal co- factor for its activity (Oboh, 2005).

AIM:
The study aimed at Producing amylase enzyme from marine actinobacteria and Partially Purification of the enzyme

MATERIALS AND METHODS:
Sample collection:
The samples were collected in the month of January 2015 from Konathur beach Chennai, Tamil Nadu, India. The sample was collected from 10m deep soil where most of micrcrobial activities take place, and thus where most of the bacterial population is concentrated. Marine sediments (approx. 500g) were collected by using clean, dry and sterile polythene bags along with sterial spatula, marking pen, rubber band and other accessories.
Analyses of samples

Serial dilution of the isolated sample was carried out. Starch Casein Agar(SCA) media was prepared and was allowed to solidify, after which the media plates were labeled and the samples were inoculated with different serial dilutions (10^{-5},10^{-4},10^{-3}, 10^{-2},10^{-1},). 100µl of suspension of sample was transferred and plated over the surface of SCA medium (spread plate method). The plates were incubated at 28± 2◦C for 5 days. The plates where then observed for the identification of positive strain.

The isolated strains were confirmed as actinobacteria by observing their morphology under microscope with 500X magnification. The culture of actinobacteria was inoculated at the intersection of the SCA medium and cover slip was buried in the solid SCA medium at an angle of 45◦C (Williams and Cross, 1971).

Screening For Amylase Production:

Screening of Amylase by Starch Iodine (Plate Assay)

The screening of the actinobacteria strains for amylase production was done by inoculating the strains on prepared SCA plate. The organisms were spot inoculated on to the media and incubated at 28^0C for 72hrs after which Gram's. Iodine stain was spread on the plate and left for 5 min. The organisms which secreted amylase, produced zone of clearance or depolarization against the iodine stain.

Optimization of some physicochemical parameters (Temperature, pH, Carbon Source and Nitrogen Source)

Temperature: Three conical flasks each containing 100ml of SCA were inoculated with the actinobacteria strains and one incubated at fridge temperature (0^0C), room temperature(30^0C) and incubator. (50^OC).

pH: Three flasks each 100ml of SCA containing were inoculated with the actinobacteria strains and the pH of the media varied as 7.0, 7.2 and 7.4.

Carbon source: Three flasks containing the SCA media were varied for carbon source as starch, sucrose and fructose. Actinobacteria strain was inoculated and incubated for 5days.

Nitrogen source: Three flasks containing the SCA media were varied for nitrogen source as tryptone, malt extract and$(NH_4)_2 HPO_4$. Actinobacteria strain is inoculated and incubated for 5 days

Spectrophotometric Assay of Amylase Enzymes:

The activity of amylase was assayed by incubating 100µl crude enzyme with 900µl soluble starch (1% w/v) and 0.5ml CMC (Carboxyl Methyl Cellulose - 1%, w/v) prepared in 0.1M sodium citrate buffer (pH 7.0). After incubation at 37^0C. The reaction was stopped by the addition of 2ml of 3-5-dinitrosalicylic acid reagent and absorbance was measured at 550nm in UV spectrophotometer.

Estimation of Protein Content:

The total protein was calculated using Bradford's method with BSA as standard. Coomassie brilliant Blue G-250 (100 mg) was dissolved in 50ml of ethanol. To this, 100ml of 85% phosphoric acid was added and made up to one litre. To 1.0ml of extract, 9ml of distil water and 5ml of CBB was added, mixed thoroughly. It was incubated for 5 mins and read at 595nm in a Beckman DU -50 spectrometer.

Partial Purification:

Ammonium precipitation:

100ml of the enzyme source was transfer to a beaker containing a stir bar and placed on magnetic stirrer. While the sample is stirring, 47.2g of ammonium sulphate was slowly added until desired saturation level. The ammonium sulphate was slowly added to ensure that the local concentration around the site of addition does not exceed the desired salt concentration. After the total amount of ammonium sulphate was added, the beaker was moved to 4^0C over night. Then, the sample was centrifuged at 85,000rpm for 15minutes. The supernatant was carefully removed and the pellet was dissolved in sodium phosphate buffer and stored in a refrigerator.

Dialysis:

The membrane was treated with sterile distilled water at 65^0C for 10 minutes to remove glycerol. Then, soaked in 10mM EDTA containing half pellet NaOH to remove heavy metals.

Then transferred to 2% sodium bicarbonate to remove the Sulphur molecules. Finally, it was washed with sterile distilled water.

RESULTS AND DISCUSSIONS

All positive stains were observed as milk colour after incubation of five days. The distinct colonies were taken using a sterile loop and sub-cultured on SCA slants. A total of 8 colonies were isolated and sub-cultured.

The inoculated sub-cultures were observed after incubation of 5 days. The following results were observed as recorded (figure1)

Figure 1.: Pure culture of marine actinomycetes.

Spore surface morphology was studied under simple microscope using coverslip culture technique (Fig: 2) the slides were prepared of 5 days incubation. The reading was taken at 500X magnification.

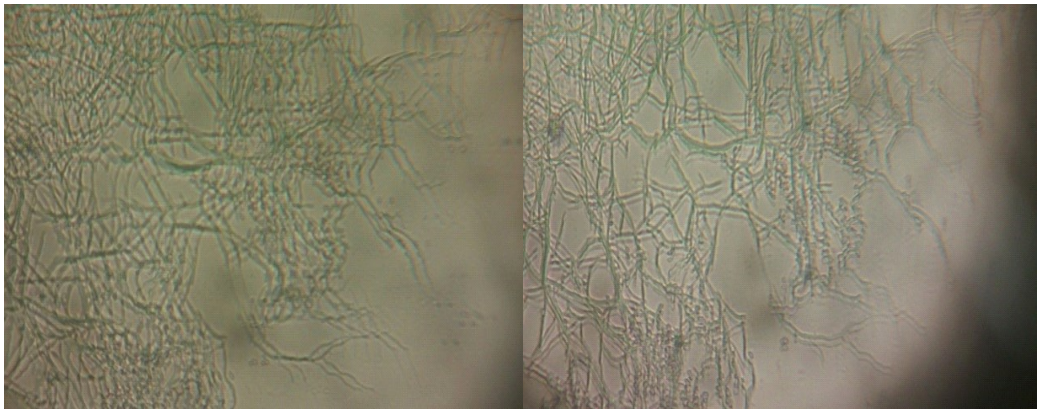

Figure 2: Branched free filament Actinobacteria

The actinobacteria strains tested on 1% starch and a clear zone around the colony was measured in millimeter after application of iodine solution and incubated for 5minutes . Strain number 5 indicating the highest zone when compared with other actinobacteria. Screening for amylase production was carried out by growing the actinobacteria on SCA plate and stain with iodine solution.

Table 1: Inhibition zones (cm) of different Bacterial Strains:

Strain No.	Zone of Inhibition (cm)
SBS1	1.8
SBS2	0.6
SBS3	-
SBS4	1.8
SB5	3
SBS6	-
SBS7	-
SBS8	2.5

Table 2: Enzyme activity

Strain	Concentration(µl)	Activity (EU/ml)
SBS5	100	1.77
Control	0	0.11

Table 3: Effects of Carbon and Nitrogen Sources on Amylase Enzyme Activity

	Carbon source			Nitrogen Source		
	Maltose	Sucrose	Fructose	Tryptone	Malt extract	$(NH_4)_2HPO_4$
Enzyme Activity (EU/ml	8.50	3.80	2.42	3.53	2.91	6.70

Table 4: Effects of Temperature and pH Sources on Amylase Enzyme Activity

	Temperature (^{0}C)				pH		
	0	30	40	50	7.0	7.2	7.4
Enzyme Activity (EU/ml)	2.4	5.0	10.0	2.5	9.5	5.4	3.6

Table 5: **Effect of Purification on enzyme activity:**

Sample	Protein estimation (mg/ml)	Enzyme assay (U/ml)
Crude sample	1.26	1.88
Precipitated sample	2.31	4.75
Dialyzed sample	3.36	5.23

Actinobacteria were isolated from marine source in this study. The research finding is in conformity with that of Das & Khan (2008) and Demirkan (2011), research which conclude that marine biosphere served as a rich source of actinobacteria all around the globe from shallow sediments to the deepest sediments.

Eight strains of the sediment isolates undergo morphological studies using SCA media in which the aerial mycelium, substrate mycelium and spore by cover slip method. This correlated with Gulve & Deshmukh,(2012) research in which the aerial mass color of almost all strains was whitish. Also a research conducted by Thippeswamy, Girigowda, & Mulimani, 2006 reported that white color series of actinobacteria they were the dominant forms. Color series were also recorded in soil, morphological observation of colonial characteristics such as amount and color of vegetative growth, and the presence and color of aerial mycelium and spores, and again the presence of diffusible pigments are recorded for each strain studied Colonial growth on agar plate.

The actinobacteria strains tested on 1% starch and a clear zone around the colony was measured in millimeter after application of iodine solution and incubated for 5 minutes .

Strain number 5 indicating the highest zone when compared with other actinobacteria. Screening for amylase production was carried out by growing the actinobacteria on SCA plate and stain with iodine solution. A clear zone was seen by amylase producing actinobacteria similar results were reported by Ajita & Thirupathihall, (2014) which says that amylase decolorized iodine due to its strong affinity to degrade starch. Also Salvam (2011) in his research screen Actinobacteria for marine production using plate assay method by SCA medium and found that amylase producing actinobacteria produce a clear zone against iodine staining.

Enzyme activity of two strains was checked using spectrophotometric assay using DNS the OD is measured at 540nm and SBS5 shows highest enzyme activity. Same method is describe by Oboh, (2005), Babu& Styanarayana (1995) which explained that actinobacteria producing the higher amylase shows enzymatic activity when subjected to DNS test compared to other strains.

To study the effects of pH and temperature change on enzyme production and growth , the organisms were grown in the SCA media at different temperature of 0, 30 ,40 and 50◦C and then at pH 7.0,7.2 7.4 and 7.6 .Optimum temperature was found to be 40◦C and pH for maximum enzyme production pH 7.This is in conformity with many researches carried out for optimization .Earlier reports also witness the research findings by Krishna and Chan ,(1996) Kundu and Das(1970) obtained optimum PH 7.0 for *Streptomyces spp* .Also 7.0 was observed in recent studies by Gulve and Deshmukh (2012).For temperature similar research findings by Babu and Styanarayana,1995 for Bacillus spp.an optimum temperature of 40◦C was reported for *Streptomyces gulbargensis* by Gupta et al.,,(2013). Santos *et al.*,(2012) observed optimum temperature of 45◦C for amylase by *Streptomyces cavife.* (Mouna et al., 2015). (Derakhti, Shojaosadati, Hashemi, & Khajeh, 2012) also explained that optimal temperature of 50^0C was observed when temperature was varied within a range of 30-90°C for production of α-Amylase by *Aspergillus oryzae* when solid state fermentation was carried out for production of the enzyme by *Penicilliumjanthinellum* in a range of 30-55°C, the optimum incubation temperature was found to be 45°C .The enzyme production increases with increase in temperature till it reaches the optimum. With further increase in temperature the enzyme production decreases. This may be due to the loss of moisture in the substrate which adversely affects the metabolic activities of the microbes leading to reduced growth and decline in enzyme production. Thippeswamy *(*2006) also reported in their research that *Bacillus amyloliquefaciens* produces the enzyme with an optimum pH of 7.0. *Halomonasmeridiana* was studied for optimization of α-Amylase production. The study revealed that the amylase exhibited maximal activity at pH 7.0, being relatively stable in alkaline conditions.
In order to investigate the effect of carbon source on amylase activity was assayed in the presence of different carbon concentrations. As seen in table specific activity was observed with maltose having the highest enzyme activity. This is similar to Sivaramakrishnan *et al.*,(2006)research which they explained that *A. tamarii* actively synthesizes α-Amylase when cultured on maltose, starch and glycogen under static conditions. This yield was a fourfold increase when compared to shaking cultures.

While purifying the -amylase from actinobacteria by precipitation using ammonium salt and dialysis 3.36 mg of total protein was obtained where the protein content of the initial sample was 1.26 mg and the total activity recovered was 4.75 U/ml while the initial activity was 1.88 U/ml with the specific activity of 5.23U/ml. This is similar to Demirkan (2011) study of purification and characterization of an extracellular α-Amylase from *Clostridium perfringens* Type A, crude enzyme concentrate was prepared by precipitation with polyethylene glycol. Purification of α-Amylase produced by a mutant *Bacillus subtilis* strain was done in a series of

steps employing precipitation with 80% ammonium sulfate dialysis and SP Sepharose column chromatography

CONCLUSION

The actinobacteria were isolated, characterized, amylase was produced and purified .it was revealed that optimum temperature of activity was and optimum pH was 7.0. Maximum enzyme activity was seen in maltose as carbon source and $(NH4)_2$ HPO_4 as nitrogen source.

Recommendations:

- Further research should be carry out on Actinobacteria to check the production of other enzymes
- Molecular analysis on the on production of Amylase enzyme from Actinobacteria should be carry out
- Molecular studies such transcriptomic analysis to study the pathway of amylase enzyme.
- Genetics analysis like QTL mapping should be also carried out to determine the different QTLs for the Candidate and other genes responsible of the production of amylase

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