EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 10
239-243
Year: 2016

OPTIMISATION AND DOWNSTREAM PROCESSING OF AMYLASE SYNTHESIZED FROM MARINE ACTINOMYCETES- *NOCARDIOPSIS ALBA* AND ITS DESTAINING PROPERTY.

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Article Received on 26/07/2016 **Article Revised on 16/08/2016** **Article Accepted on 06/09/2016**

ABSTRACT
Marine *actinomycetes* have been a well known source of many biological products. In the marine environment, marine microorganisms are the eminent source to discover new bioactive compounds. Among marine microorganisms, the *actinomycetes* are the major source for the production of new drugs which is proven by the various earlier researches. *Actinomycetes* are a group of prokaryotic gram positive filamentous bacteria which produce two types of branching mycelium, aerial mycelium and substrate mycelium. Some *actinomycetes* genera are slow growing microorganism. They belongs to the domain bacteria, phylum *actinobacteria*, subclass *actinobacteridae*, order *actinomycetales*, suborder *actinomycineae*, family *actinomycetaceae*. The member of this order contains high G+C content (>55 mol%) in their DNA. The present work is concerned about the economic and efficient production of amylase enzyme from *Nocardiopsis alba*. The sand sample was collected from Thiruvanmiyur Beach. The sand sample was used for screening and isolation of *Nocardiopsis alba*. The pure culture isolated was screened for its enzyme activity. The species showed positive results for Iodine test. The enzyme optimization using various parameters like pH ranging from 7.2 to 7.8, temperature- 4°C, 35°C, 37°C & 65°C with the media (ISP 1-7) was carried out. This was followed by the downstream processing of the enzyme and checked for the enzyme purity. The purified enzyme was then subjected to destaining activity as one of its applications.

KEYWORDS: Marine *Actinomycetes*, *Nocardiopsis alba*, Amylase, Optimisation, Downstream Process and Destaining property.

INTRODUCTION
About 70% of our planet Earth is covered by Oceans with 1370.323 million cubic km of water. Since, the ocean water is a rich medium with lot of unique properties; it supports 90% of world's living biomass. The marine environment is the exceptional reservoir of bioactive natural product, many of which exhibit structural or chemical features not found in terrestrial natural products.[1] India is one among the 12 Mega-Biodiversity countries & 25 hotspots of the richest and highly endangers eco-regions of world. Its marine environment has a coastline of about 8000km, Exclusive Economic Zone (EEZ) of the country has an area of 2.02 million km2 comprising 0.86 million km2 on west coast, 0.56 million km2 on east coast and 0.6 million km2 around the Andaman and Nicobar Islands.[2] The marine environment still contains many unexplored treasures in it, which opens a wide range of opportunities to researchers.

Actinobacteria are group of Gram positive bacteria found in ocean, widely distributed in different marine ecosystems. Their significance includes Mineralisation of organic matter, Immobilization of mineral nutrients, Fixation of nitrogen, Improvement of physical parameter[3], Environmental protection and so on. They are useful biological tools for the production enzymes[4], Antimicrobial activity secondary metabolites[5] involved as remedies for many diseases, Anticancer property[6] antibiotics etc.

Amylases
The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. Molecular weights of a-amylases vary from about 10 to 210 kDa.[7] α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its

activity. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries. Many metal cations, especially heavy metal ions, sulphydryl group reagents, N-bromosuccinimide, phydroxyl mercuribenzoic acid, iodoacetate, BSA, EDTA and EGTA inhibit α-amylases.[8]

METHODS AND MATERIALS REQUIRED

Isolation of Actinomycetes

The soil sample was collected at maximum depth of 40 metres in a sterile zipped polythene bag[9] at the location Thiruvanmiyur beach, Chennai. Sand sample dried at room temperature for 3 days. Dried soil sample was treated with 0.1g of CaCo3 & allowed to dry for 3 days.[10] 1g of Pretreated soil was serially diluted (10-10 dilution).[11] The SCA media is used. Then add 100µl/0.1ml of Sample to solidified agar. Then incubated at room temperature. Growth seen after 3 days. The slant was prepared by Adding 5ml of media (SCA MEDIA-soluble starch 10 g, vitamin free casein 0.3 g, KNO3 2 g, NaCl 2 g, K2HPO4 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, agar 20 g, D.W 1000 ml, pH 7.0 ±0.2) culture[12] to all test tubes. Then continuous streaking is done and Incubated at room temperature in SCA media which was autoclaved at c for 20minutes. Incubated the plates at room temperature and observed the growth. The agar plates were investigated in terms of colony appearance, colony color and conidia type (spore configuration) after incubation. Then each selected colony was cultured separately and linearly in starch casein–agar to purify the isolates and selected isolates were further evaluated using Gram staining.[13]

Screening for amylase activity

The amylase activity of actinomycetes strains was screened by inoculating them starch plate (soluble starch 20 g, vitamin free casein 0.3 g, KNO3 2 g, NaCl 2 g, K2HPO4 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, agar 20 g, D.W 1000 ml, pH 7.0 ±0.2) The organisms were strea ed on the plate and incubated at c for hours Then the plate was flooded with Gram's iodine(Iodine 1g, Potassium Iodide 2g, Distilled water 50ml) and left for 5 mins. If the *actinomycetes* secretes amylase it can be evidently seen by zone of clearance or decolourization of iodine colour due to hydrolysis of starch.[14]

Screening for Cellulase activity

The cellulase activity of actinomycetes strains was screened by inoculating them in the carboxymethyl cellulose (CMC) agar plate (CMC 0.6g,soluble starch 10 g, vitamin free casein 0.3 g, KNO3 2 g, NaCl 2 g, K2HPO4 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, agar 20 g, D.W 1000 ml, pH 7.0 ±0.2) The organisms were strea ed on the plate and incubated at c for hours Then the plate was flooded with staining reagent (congo red 0.3g, distilled water 100ml) and left for 15 mins. Then it is discarded and the destaining reagent (1 N Nacl, distilled water 100ml) is added. If the *actinomycetes* starin secretes cellulose, it can be evidently seen by zone of clearance due to hydrolysis of CMC.[15]

Screening for Protease activity

The protease activity of actinomycetes strains was screened by inoculating them Skimmed milk (Skimmed milk o.6g, soluble starch 10 g, KNO3 2 g, NaCl 2 g, K2HPO4 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, agar 20 g, D.W 1000 ml, pH 7.0 ±0.2), Vitamin-Free Casein (Vitamin free casein 0.6 g, soluble starch 10 g, KNO3 2 g, NaCl 2 g, K2HPO4 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, agar 20 g, D.W 1000 ml, pH 7.0 ±0.2) and Gelatin plate(Gelatin 0.6g,soluble starch 10 g, vitamin free casein 0.3 g, KNO3 2 g, NaCl 2 g, K2HPO4 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, agar 20 g, D.W 1000 ml, pH 7.0 ±0.2) The organisms were strea ed on the plate and incubated at c for hours. Then the plate was flooded with mercuric chloride reagent (Mercuric Chloride 15g,Concentrated HCl 20ml, Distilled water 80ml) and left for 5 mins. If the *actinomycetes* strain secretes protease it can be evidently seen by zone of clearance formed due to hydrolysis of substrate.[16]

Table 1.Optimization of amylase activity

Parameters	Conditions
pH	**7.2** **7.4** **7.6** **7.8**
Temperature	
Media (International Streptomyces Project)	**ISP-1** **ISP-2** **ISP-3** **ISP-4** **ISP-5** **ISP-6** **ISP-7**

For optimization of starch activity different parameters were employed namely the pH **(**7.2, 7.4, 7.6, 7.8), temperature C C Cand C) and Media (ISP 1-7) the experiment was carried out in Erlenmeyer Flask containing the respective medium.[17] Then using agar well diffusion method the amylase activity was tabulated. Then in the plates well was cut using sterile Cork borer. Concentration range of 25µl, 50µl, 75µl, 100µl and allowed to diffuse for 24hours at room temperature. 3µl of distilled water was used as control. Following day the plate was flooded iodine solution and the zone of clearance was recorded.

Fig.1. Bulk Production in optimized conditions

Bulk Production

The optimized media was prepared and the sample was inoculated in to it using inoculation loop and was kept in shaker for 7 to 8 days. Then the media was filtered using filterpaper and centrifuged at 8000 rpm for 15 mins and the supernatant was collected. Then the supernatant is used for further process.

Partial Purification of enzyme

Partial purification was carried out by Salt precipitation method Ammonium sulfate was added to supernatant, mixed well and it is stored in fridge at c ext day the mixture was centrifuged at 10,000 rpm for 20mins.The supernatant was collected separately and to the pellet 1ml of phosphate buffer was added and collected separately. The sample is dialyzed and the purified sample is used for SDS-PAGE Electrophoresis.[18]

Confirmation by SDS-PAGE

The purified sample is confirmed by using SDS-PAGE technique.[19]

Destaining Activity

Four pieces of cotton was taken of equal size. Then blood/tomato sauce was placed on the cloth and dried under sunlight.

Test Compostion

Control:- 20ml Distilled water

Positive:- 1ml of detergent solution (0.02g in 2ml of distilled water + 20ml distilled water)

Negative:- 20ml of Phosphate buffer

Test:- 1ml of detergent solution +2ml of enzyme solution + 20ml of distilled water.

The stained cloth incubated at c for mins After incubation, the solution was discarded and the cloth was rinsed in distilled water. Then the cloth is dried under sunlight and the results were visualized.

RESULTS

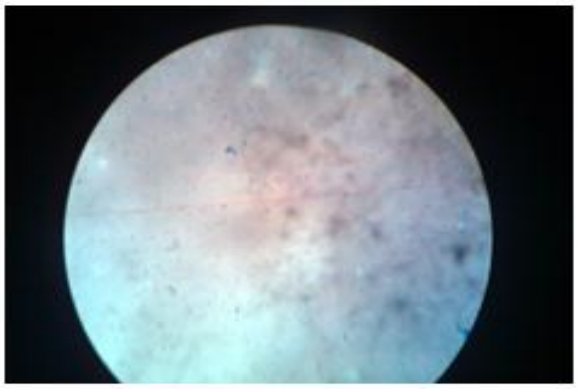

Fig.2. Gram's stained slide

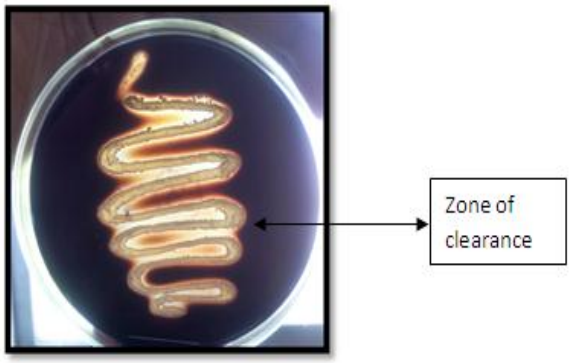

Fig.3. Species showing amylase activity

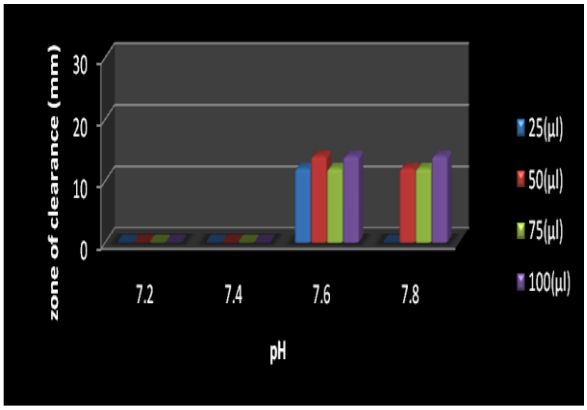

Fig.4. Graph illustrating the zone of clearances at various pH.

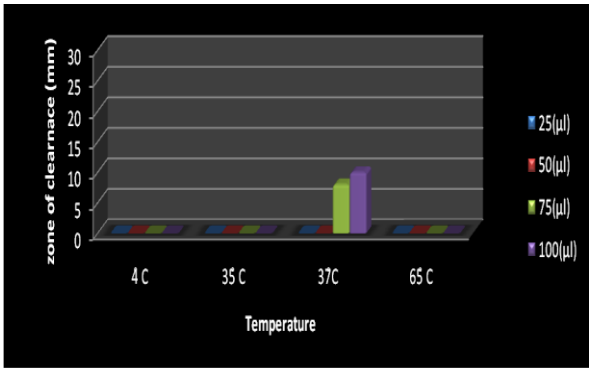

Fig.5. Graph illustrating the zone of clearances at various temperature conditions.

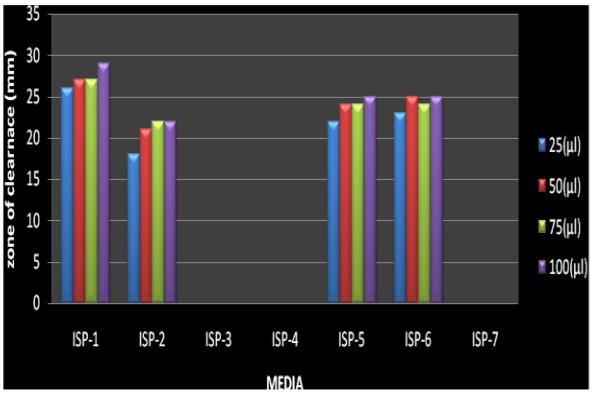

Fig.6. Graph illustrating the zone of clearances at various media compositions.

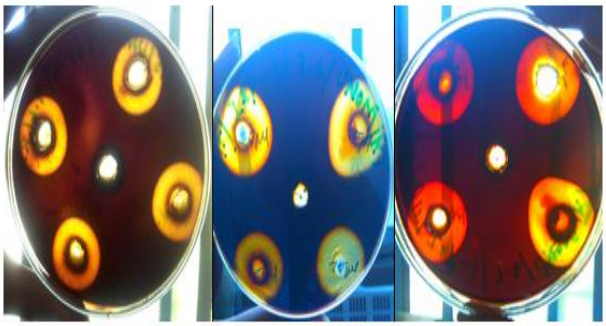

Fig.7. Optimized pH, temperature and Media

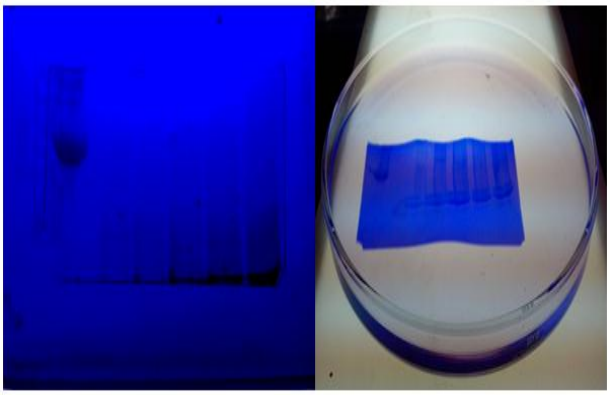

Fig.8. Polyacrylamide gel showing the protein bands.

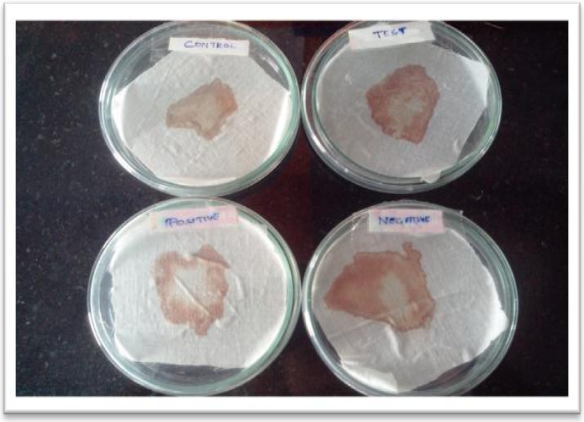

Fig.9. Test Setup

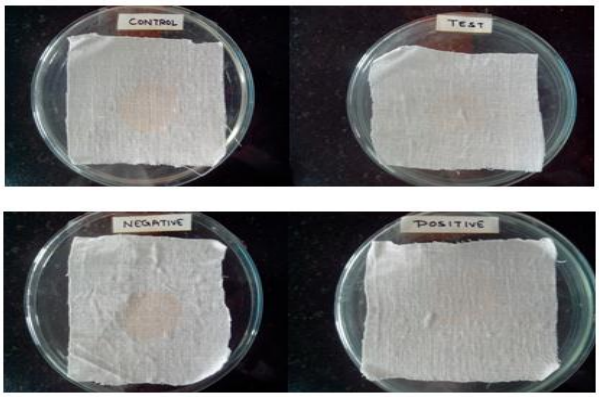

Fig.10. Results of De-staining

DISCUSSION

Fig.2 shows the result of Grams staining, appearance of purplish-blue colour colonies suggest that the species-*Nocardiopsis alba* isolated is a Gram positive bacteria. This is produced because the cell walls of gram positive organisms has a thick layer of protein-sugar complexes called peptidoglycans. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. At the end of the gram staining procedure, gram positive cells will be stained a purplish-blue color. Fig.3 illustrates the amylase activity exhibited by the isolated species of *Nocardiopsis alba*, when the gel surface was flooded with iodine reagent and after approx. 10 mins at room temperature, the iodine solution is poured off and the diameter of each clear zone measured.[20]

The amylose (unbranched/lin portion of starch) forms helices, which allow iodine molecules to assemble, forming a dark blue/black color. The amylopectin (branched portion of starch) forms much shorter helices due to the branching present and iodine molecules are unable to assemble, leading the color to be of red brown or red violet. As starch is broken down or hydrolyzed by the amylase enzyme the blue-black color is not produced. Hence a clear zone around the wells can be seen.[21] The enzyme was optimized at various parameters as listed in Table.1. The experimental results of optimal activity was observed at **pH 7.6**, the results are graphically illustrated in Fig.4 respectively similar results were observed.[22] The optimal activity of the enzyme was observed at the temperature which is graphically indicated in Fig.5 respectively similar results were shown by.[23] The optimal activity of the enzyme was seen in **ISP-1 medium** when compared with other parameters, which is graphically indicated in Fig.6 respectively similar results were produced in.[24] Using the optimized conditions bulk production of the amylase enzyme was carried out and purified using Downstream processing. Fig.8 represents the result of SDS-PAGE, where 1st well shows BSA (standard) and other wells shows the bands of amylase loaded in increasing volumes (10µl-50µl) repectively. The amylase is said to have excellent strain removal from the textiles[25], this was tested. The destaining property of the amylase was successfully

examined which are depicted in Fig.9 & Fig.10. From the images we can confirm that the test (1ml of detergent solution +2ml of enzyme solution + 20ml of distilled water) showed better stain removal property when compared to the positive91ml of detergent solution), whereas negative (20ml of Phosphate buffer) and control (20ml Distilled water) didn't show much significant changes.

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