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ORIGINAL RESEARCH ARTICLE

Screening and Quantification of Marine Actinomycetes Producing Industrial Enzymes Amylase, Cellulase and Lipase from South Coast of India

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ABSTRACT

Fifty six actinomycete strains were isolated from marine sediments of south Indian coastal region of India. The isolates were identified as actinomycete by morphological studies. Among these, nine isolates were selected on the basis of their amylolytic, cellulolytic, lipolytic activities by screening. Amylolytic and cellulolytic activities were exhibited by the strains 1, 3, 51 and 12, 29, 49 and it was confirmed by formation of clear zones of hydrolysis around the colonies. Lipolytic activity was exhibited by the strains 3, 10, 28 and it was indicated by formation of orange fluorescent halo around the colonies. Among the isolates maximum amylase, cellulase and lipase activities were exhibited by strains 3, 49 and 28 and it was determined by DNS (3, 5-dinitrosalicylic acid) assay method and titrimetric assay method. The enzyme activity of the strains 3, 49 and 28 was found to be 6.48, 8.93, and 700U/ml.

Key words: Amylase, Cellulase, Lipase, Marine actinomycetes, Screening.

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 [1]. Marine micro organisms are increasingly becoming an 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 [2].

Among the microorganisms, marine actinomycetes have been a great source of new compounds and their isolation all around the globe, from shallow coastal sediments to the deepest sediments [3]. Marine environment is a huge treasure trove of marine actinomycetes resources [4]. 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 [5].

α Amylase (endo-1- α-D-glucanohydrolases EC 3.2.1.1), starch degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries [10]. Bacteria, Actinomycetes and fungi secrete amylases to the outside of the cells to carryout extracellular digestion.

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 [11]. Actinomycetes are one of the known cellulose producers has attracted considerable research interests [12] [13]. With the advent of new frontiers in the field of biotechnology, the spectrum of cellulose has expanded into various industries, including food, textiles, laundry, pulp, paper, agriculture as well as in research and development [14].

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) has broad applications in the food, oleochemical, pharmaceutical and detergent industries as well as in diagnostic settings [6]. Furthermore, novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel [7]. So far, not many lipases with optimum activity under alkaline conditions have been studied [8]. Lipase production from a variety of bacteria, fungi and actinomycetes has been reported by several workers [9].

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The present study deals with screening of industrially important enzymes amylase, cellulase and lipase producing marine actinomycetes and assess their production.

MATERIALS AND METHODS

Sample collection from marine sediments;

The marine sediments were collected from different sites in Chennai, Tutucorin, Kerala and Pooombukar in south India. The samples were collected using alcohol rinsed Peterson grab and were transferred to new zip lock bags using sterile spatula [15]. The samples were transported to the laboratory for the isolation of actinomycetes.

Enrichment and Isolation of marine actinomycetes;

One gram of sediment was transferred to 100ml of starch casein broth supplemented with fluconazole and incubated at 30^{0}C for 7 days in shaker at 200rpm [16]. 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 30^{0}C for 7 days [17]. Single separated colonies were selected and the subcultures were maintained on starch casein slants at 4^{0}C until further use.

Identification of marine actinomycetes by coveslip method;

The isolated strains were confirmed as actinomycetes by observing their morphology under microscope. The SCA was poured on sterile slide and allow solidifying. Then the organisms were streaked on it and incubate at 37^{0}C 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 Amylase by Starch Iodine plate assay;

The screening of the actinomycete strains for amylase production was studied by inoculating them on starch plate [21]. The starch agar plates were prepared. The organisms were spot inoculated on to the media and incubate at 28^{0}C for 72hrs. After that gram's iodine stain was spread on the plate and left for 5 min. The organisms which secretes amylase, produced zone of clearance or decolorization against the blue color back ground.

Screening of Cellulase by Starch Iodine plate assay;

Cellulose agar plates were prepared using the method of [22]. The isolated actinomycetes were spot inoculated on to the media and incubate at 28^{0}C for 72hrs to express cellulose depolymerisation through cellulase production into its surrounding medium. After that, the plates were flooded with Iodine-Potassium iodide solution to detect clearing zones against a dark-brown back ground [23].

Screening of lipase by Rhodomine B agar plate assay;

The screening of the actinomycete strains for lipase production was studied by inoculating them on Rhodamine B medium [19]. The isolates were inoculated on media of the following composition (g/l) Nutrient broth (HiMedia), 8.0; sodium chloride, 4.0; agar, 10.0. The medium was adjusted to pH 7.0, autoclaved and cooled to 60ºC. Olive oil (31.25 ml) and 10 ml of Rhodamine B solution (0.001%, w/v) was added with vigorous stirring and emulsified by mixing for 1 min. The medium was allowed to stand for 10 min at 60ºC to reduce foaming. 20 ml of the medium was poured into sterile petriplates. Incubate it for 28^{0}C for 72hrs. [20]. Colonies which showed orange fluorescence under UV irradiation indicated true lipase activity and non-lipolytic bacteria formed pink colonies.

Spectrophotometric assay of Amylase and Cellulase enzymes;

Amylase and Cellulase activity were measured by spectrophotometric assay. The activity of amylase and cellulase were assayed by incubating 0.5ml crude enzyme with 0.5ml soluble starch (1%, w/v) and 0.5ml CMC (Carboxy Methyl Cellulose - 1%, w/v) prepared in 0.1 M sodium phosphate buffer (pH 7.0). After incubation at 37^{0}C for 60 min the reaction was stopped by the addition of 2ml of 3-5-dinitrosalicylic acid reagent [26] [27] and absorbance was measured at 550nm in UV/Vis spectrophotometer. One unit (U) is defined as the amount of enzyme which releases 1µmol of reducing end groups of glucose per minute in 0.1 M sodium phosphate buffer (pH 7.0) with 0.5% (w/v) soluble starch and 0.5% (w/v) CMC as substrate at 37^{0}C.

$$\text{Enzyme activity (U/ml)} = \frac{(\mu\text{moles of glucose released})\ (1)}{(0.5)\ (30)}$$

1 = Total reaction mixture
0.5 = ml of enzyme in reaction mixture
30 = Incubation time (minutes)

Titrimetric assay of Lipase;

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis [24]. 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 [25]. 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.

$$\text{Enzyme activity (U/ml)} = \frac{\text{(NaOH) (Molarity of NaOH) (1000) (2) (df)}}{(1)}$$

(NaOH) = Volume of NaOH used for test - Volume of NaOH used for blank
1000 = Conversion factor from milliequivalent to microequivalent
2 = Time conversion factor from 30 minutes to 1hr.
df = Dilution factor
1 = Volume (in milliliter) of enzyme used

RESULTS AND DISCUSSION

Isolation and Identification of marine actinomycetes;

In the present study, fifty six marine actinomycetes were isolated from the marine sediments and the actinomycetes were identified morphologically by cover slip method. Amylase, Cellulase and Lipase enzyme producing actinomycetes were streaked on the starch agar plates (**Fig 1**).

Previous studies have shown that, actinomycetes are ubiquitous in nature including water and sediments of marine and estuarine environments. An enrichment of the sediment in Starch casein medium incorporated with flucaniozole yields high colony forming units of actinomycetes and it inhibits fungal contamination [36,37]. The use of cover slip method enables the visualization of the fragmented spores in the agar media without disruption and breakage [41].

Screening of Amylase and Cellulase by plate assay method;

Amylase and Cellulase screening were carried out on starch and cellulose plate assay method. Amylase and cellulase producing strains 1, 3, 51 and 12, 29, 49 were identified by formation of clear zones against dark blue and dark brown plates respectively (**plate 1 & 2**). The enzymatic hydrolysis of starch has been practiced on an industrial scale for many years and is gradually replacing the traditional acid hydrolysis process [39]. Hydrolysis zones visualized by iodine (KI/I_2) staining of cellulose-agar media after growth *of Trichoderma reesei QM6a, RUT C30, QM9136* (cellulase negative) or *Thielavia terrestris* [40].

Screening of lipase by Rhodomine B agar plate assay;

Screening of Lipase was carried out by Rhodomine B agar plate assay method. Lipase producing strains were clearly identified by formation of orange fluorescent halo around the colonies. Among the fifty six strains, strain 3, 10 and 28 showed orange fluorescent halo (**plate 3**). According to the previous report, screening of microorganisms for lipolytic activity was relatively easy and most frequently performed by employing agar plates containing trioleoylglycerol and the fluorescent dye Rhodamine B [38].

Amylase and Cellulase activity;

Spectrophotometric assay of amylase and cellulase production were carried out for strains 1,3, 51 and 12,29,49 and their activity was found to be 2.40, 6.48, 2.04 and 3.11, 3.24, 8.93 U/ml. From the above results, strain 3 and 49 showed maximum amylase and cellulase activity respectively and the further studies will be carried out on these isolates (**Table 1**).

The fungus *Penicillium fellutanum* isolated from mangrove rhizosphere soil and its production level of amylase was 1.38 U/ml [31]. In the previous report, production of amylase by *Aspergillus niger* in submerged cultivation on two wastes from food industries were carried out and the enzyme activity was 6.0 U/ml [32]. According to the previous report, the actinomycete *Streptomyces transformant* T3-1 showed optimum cellulase production and it was 2.6 U/ml [33]. The optimum production of cellulase was achieved by sponge associated *Marinobacter Sp., MS 1032* the activity was 29.52 U/ml [34]. In the previous report, spectrophotometric assay for enzyme cellulase were performed by the actinomycete *Microbispora bispora.* The activity was found to be 6.2 U/ml [35].

Lipase enzyme activity;

Lipase enzyme activity was carried out by titrimetric assay method. Strains 3, 10 and 28 exposed maximum lipase activity and it was found to be 580, 560 and 700 U/ml. Based on maximum enzyme activity, strain 28 was selected for further studies (Table 1). The previous study investigated in the lipase producing bacteria from a Kenyan alkaline soda lakes and the activity was found to be 104.66 U/ml [28]. According to the previous report [29], extracellular lipase from *Alternaria brassicicola*'s was found to be 1526.2 U/ml. The bacteria *Sarratia marcescens* showed maximum lipase activity by adding tween 20 as a substrate and the activity was 250 U/ml [30].

From the above results, strains 3, 49 and 28 quantified as an effective producer of an industrially important enzymes amylase, cellulase and lipase and further studies will be carried out by those strains.

Strain 01 03 51

Amylase enzyme producing strains

Strain 12 29 49

Cellulase enzyme producing strains

Strain 03 10 28

Lipase enzyme producing strains

Fig 1: Amylase, Cellulase and Lipase producing marine actinomycetes

Control Test

Plate 1: Amylase screening

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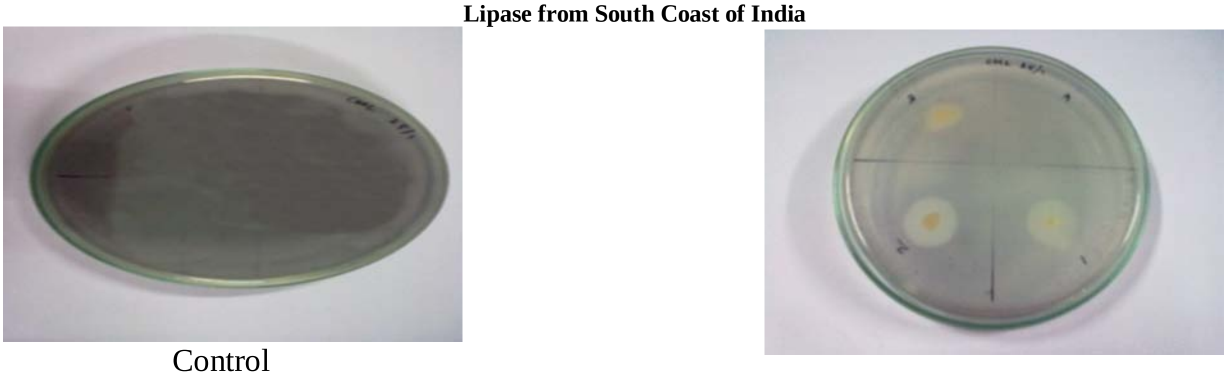
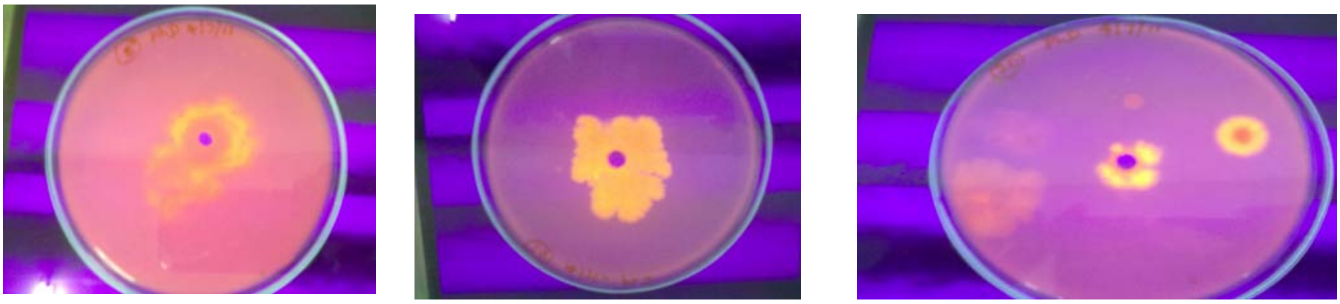


Control
Test

Plate 2: Cellulase screening

Control



Test

Plate 3: Lipase screening

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Table 1: Amylase, Cellulase and Lipase producing marine actinomycetes

Strains	Enzyme activity (U/ml)
	Amylase
1	2.40
3	6.48
51	2.04
	Cellulase
12	3.11
29	3.24
49	8.93
	Lipase
3	580
10	560
28	700

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