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Original Article

ISOLATION, SCREENING AND DETERMINATION OF A-AMYLASE ACTIVITY FROM MARINE *STREPTOMYCES* SPECIES

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ABSTRACT

Objective: This study was aimed to isolate potent amylase producing *Streptomyces* from the marine source.

Methods: Soil samples were collected from less explored mangrove regions of Muthupet, Tamilnadu. Isolation of *Streptomyces* was performed by serial dilution plate technique using starch casein agar (SCA) (pH 7.2 and temp 28 °C). Morphological and biochemical characteristics were studied using Bergey's manual of systematic bacteriology. Preliminary screening and quantification of amylase activities were analysed in selected *Streptomyces* isolates by starch agar plate and dinitrosalicylic acid (DNS) method respectively.

Results: Totally 65 isolates were separated from the marine soil. Among them, 23 strains showed different morphological features. These strains were subjected to amylase activity. Eight *Streptomyces* isolates (S1-S8) exhibited positive for amylase activity. The zone of clearance was exhibited in the range of diameters between 4-20 mm. Fermentation was prompted with inorganic salt starch agar, international *Streptomyces* project (ISP-4) media at 28 °C and incubated in an orbital shaker at 250 rpm for 96 h (pH 7.5). The quantitative estimation of amylase activity was exhibited selected eight isolates in the range between 2.4±0.002-5.9±0.005 (U/ml). The *Streptomyces* species S4, S5 and S6 exhibited strong amylase activity in both qualitative and quantitative level.

Conclusion: This work motivating the amylase producing *Streptomyces* are originated in mangroves and it proved *Streptomyces* sp. S6 has a more efficient source of amylase production.

Keywords: *Streptomyces*, Isolation, Screening, Quantitative determination, Amylase

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INTRODUCTION

Amylase (EC: 3.2.1.1) plays a wide range of biotechnological applications in food industry, fermentation, textile and paper industries and having above 25% demand on a global scale [1-2]. Amylase has been used successfully in starch saccharification, brewing and distilling industries [3]. Presently, amylase production has reached up to 65% of the world market and continuously increasing the usage [4]. α-amylases have been derived from various sources such as plants, animals and microorganisms. Microbial amylase meet industrial demand [5] as there is a possibility of increasing the levels of microbial enzyme synthesized by classical genetic techniques, continuous culture selection, induction and optimization of growth conditions for the enzyme of interest [6].

Streptomyces are an economically important group of organisms among actinobacteria family. They are responsible for the production of about half of the discovered metabolites, notably antibiotics, antitumor agent, an immunosuppressive agent, enzymes and enzymes inhibitors. This insight has been a driving strength towards other drug discovery platforms including high throughput combinatorial production and rational drug design [7]. *Streptomyces* are known to produce the array of antimicrobial and antioxidant compounds [8]. Thus, it is crucial that new group of *Streptomyces* from unexplored or underexploited marine environment be pursued as a resource of novel compound [9].

The occurrence of amylase from *Streptomyces* and the genus considered as an active source of amylases [10]. Notably, *Streptomyces avermitilis*, *Streptomyces* sp. SLBA-08, *Streptomyces* strain A3, *Streptomyces rochei* BTSS 1001 are used for the production of amylase. Microbial amylase successfully used in medicinal research [11,12]. The most significant bacterial amylases are *Bacillus, Streptomyces, Micrococcus, Escherichia, Proteus* and *Serratia* [13,14].

Microbial enzyme technology has in recent years, grown to a multi-million dollar industry, exploration of microbial strains for discovering enzyme with novel properties has come close to the screening programs that multinational firms have been undertaking for the discovery of newer enzymes. The marine *Streptomyces* species are capable of producing a range of bioactive compounds, as well as enzymes [15]. *Streptomyces* adopted an incredible quantity of valuable products and are expected to the sources of many commercially important metabolites, including novel enzymes. Development in biology has to a great extent to improve the potential to make libraries of enzyme variants, but a vital challenge is to develop good screening tools that can identify the best performing strain [16].

Multi prospective application and demand pave the mode for growing native amylase production and searching for the more efficient process. Microbial bioprocess can meet more easily the current market demand for industrial enzymes [17]. Specifically, *Streptomyces* have been used to synthesize amylases [18,19]. Hence, the present study made an effort to screen and determine the amylase activity from marine *Streptomyces*.

MATERIALS AND METHODS

Chemicals and kits

Chemicals used in this study of starch casein agar media (SCA), Inorganic salt starch agar media (ISP-4), Iodine and maltose were purchased from Hi-Media (Mumbai-India). Dinitrosalicylic acid (DNS) purchased from Sigma Aldrich, (Mumbai, India). All chemicals were grade and all working reagents were prepared with deionized water.

Isolation and selection of *Streptomyces*

Soil samples were collected from Muthupet Mangrove forest (Lat.10° 20'N and Long.79 °35'E) in Tamilnadu during May 2012 situated on the south-east coast of India. Isolation of *Streptomyces* was performed by serial dilution plate technique using starch casein agar

(SCA) medium [20]. Based on the colony morphology as white coloured, powdery, dried, and rough, with irregular and regular margins were determined *Streptomyces* species.

Characterization of marine isolates

Marine isolates features were studied for morphological, physiological and biochemical characteristics according to the method [21]. Colony color, mycelium nature, texture, shape were complemented to Bergey's manual of systematic bacteriology [22]. Microscopic characterization was experimented by coverslip culture technique [23]. The mycelium structure and spore arrangement were observed through high power oil immersion (100 X) objective by light microscope (Olympus, CH20i). Biochemical characterization of isolates was performed by standard methods [24].

Screening of amylase

Isolated 23 *Streptomyces* were grown on starch agar medium and incubated at 28 °C for 72 h. After incubation, 3 ml of 1% iodine was flooded with each plate, and the development of stainless zone around the colonies indicated amylase production [25].

Flask scale fermentation

The spores suspensions (~10^6 spore ml^{-1}) of eight *Streptomyces* inoculums were aseptically transferred individually into 50 ml of medium. *Streptomyces* isolates were cultured in inorganic salt starch agar, international *Streptomyces* project (ISP-4) medium (50% sea water; pH 7.5). The cells were grown aerobically in 250 ml Erlenmeyer flasks containing 100 ml medium at 28 °C and incubated in an orbital shaker at 250 rpm for 96 h. After fermentation, the cells were centrifuged at 5000 × g for 15 min. The cell supernatant was separated and quantitatively studied amylase activity.

Determination of biomass

The cells were grown aerobically in 250 ml flask containing 100 ml medium at 28 °C with continuous shaking in an Orbital shaker (250 rpm). *Streptomyces* growth was monitored by measuring culture turbidity at $OD_{600\ nm}$ and estimated the biomass content. During the stationary growth phase, the biomass was collected by centrifugation at 15,000 × g for 10 min at 4 °C. The cell suspension was washed twice with distilled water and dried in hot air oven at 80 °C overnight. The cell dry weight was calculated 100 ml of culture broth ≥ 0.8 $OD_{600\ nm}$ corresponds to mg 100 ml^{-1} of cell dry biomass.

Enzyme assay

Amylase activity was estimated using the method of [26] with minor modifications. To prepare the reaction mixture 1 millilitre of supernatant was mixed with 1 ml of solubilized starch solution and then incubated at 60 °C for 10 min. The reaction was stopped by adding 2 ml of dinitrosalicylic acid (DNS) reagent. The mixture was cooled in an ice water bath for 10-15 min and then centrifuged 5000 × g for 5 min at 4 °C. The quantity of enzyme was measured at 540 nm using UV-Vis Spectrophotometer (Techcomp, 2310), with a blank sample as a reference. One unit of enzyme activity was defined as the amount of amylase needed to produce 1 µmol of maltose per min under the assay conditions. Amylase activity were performed in triplicates, values calculated by the mean values along with standard error mean.

RESULTS

Isolation and identification of *Streptomyces*

Totally, 65 isolates were selected from the marine sample. Most of the isolates appeared like *Streptomyces.* Morphologically versatile 23 isolates selected for amylase screening experiments. The isolates were grown at pH 7.2 and temperature 28 °C. Morphological characteristics of the *Streptomyces* isolates are given in table 1.

Table 1: Morphological characterization of *Streptomyces* isolates

Isolates	Appearance	Aerial/substrate mycelium	Size (mm)	Shape	Surface	Reverse pigment	Light microscopy
S1	White brown color, powdery	Ash with sandal white	6	Button	Smooth	Brown	Filamentous
S2	Dark brown with violet	Yellow with brown	8	Double ring	Smooth	Pink	Filamentous
S3	Powdery green with white	Ash with yellow	5	Round	Rough	Green	Rectus with filamentous
S4	Violet with yellow	White with sandal	9	Line with dots	Powder	Dark brown	Recti flexible
S5	Sandal with white colonies,	Grey with cream	8	Tiny round	Smooth	Light yellow	Spiral with filamentous
S6	Golden yellow with sandal	White with ash	4	Round	Smooth	Orange	Filamentous
S7	Yellow with Brown color	Brown with white	3	Feather with round	Rough	Pink with brown	Filamentous with spiral
S8	White with pink	Ash with white	5	Root with line	Smooth	Red with pink	Filamentous

S1-S8: *Streptomyces* species code; mm-millimeter.

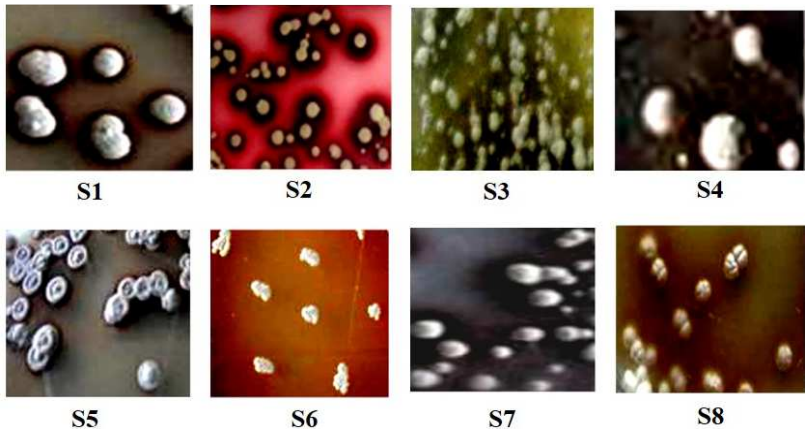

Fig. 1: Colony morphology of *Streptomyces* isolates on starch casein agar plates, representative isolates of amylase producers associated with mangrove soil; S-*Streptomyces* species Isolate code; S1, S2, S3, S4, S5, S6, S7 and S8

The isolates showed well developed aerial and substrate mycelia with sporulation on starch casein agar media. The colony morphology of *Streptomyces* isolates is given in fig. 1. Microscopic views of spore chains arrangements of *Streptomyces* appeared like filamentous (F) flexible-rectifexible (RF), and filamentous with spirals (FS) (table 1). Isolates emerged different shapes like round, tiny ring, and button shape colonies, and the texture appeared powdery, rough and smooth spores. Isolates synthesized numerous reverse color pigments like green, yellow, red, golden yellow, brown and violet color on starch casein agar plate (fig. 2).

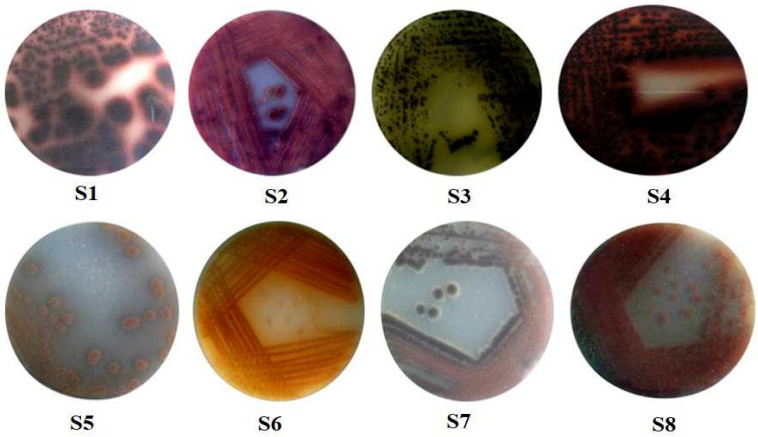

Fig. 2: Reverse side pigmentation on *Streptomyces* isolates, the pigmented isolates suitable for the identification of *Streptomyces* on genus level. S1-S8: *Streptomyces* species code

Biochemical characteristics of the isolates are given in table 2. Isolates are aerobic, G+ve, mesophilic, non-motile and non-endospore forming characteristics. Morphological and biochemical characteristics of marine isolates were appeared similar to the genus *Streptomyces*. This study provides genus level identification of *Streptomyces.*

Table 2: Biochemical characterization of *Streptomyces* isolates

Experiments	S1	S2	S3	S4	S5	S6	S7	S8
Gram's staining	+	+	+	+	+	+	+	+
Endospore	–	–	–	–	–	–	–	–
Starch hydrolysis	+++	+++	++	++	++	+++	+++	+
Citrate	+	+	–	–	+	+	+	–
Catalase	+	–	–	+	+	+	–	+
Urease	+	+	+	+	+	+	+	–
Nitrate reduction	+	–	–	+	+	+	–	+
CHO Fermentation Glucose	+	–	–	–	+	–	+	–

+= Positive, – = Negative. S1-S8: *Streptomyces* species code.

Screening of amylase activity

Primary screening of amylase production are given in fig. 3. Among 23 isolates, 8 *Streptomyces* isolates exhibited positive amylase activity in the starch medium. These potent isolates were displayed various diameters zone, ranging between 4-20 mm. *Streptomyces* S4, S5 and S6 exhibited higher activity than the other isolates.

Each *Streptomyces* produced the desirable zone of clearance. Especially, S6 exhibited higher amylase activity.

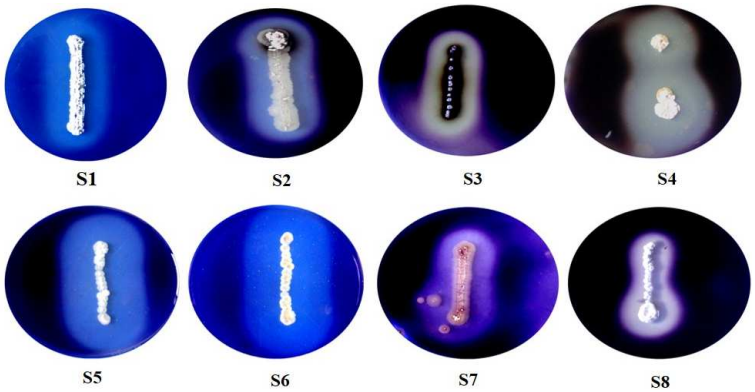

Fig. 3: Screening of amylase producing *Streptomyces* isolates from mangrove soil. The development of clear zone around the colonies was indicates positive for amylase activity. S1-S8: *Streptomyces* species code

Determination of biomass

The time course profile of eight Streptomyces species was monitored as optical density vs. incubation time (fig. 4). The maximum biomass production was recorded only in Streptomyces sp. S6, it has reached initial stationary phase at 96 h, and the maximum cell dry weight of 0.53 mg 100 ml-1. The S4, S3 and S8 isolates were reached in the range between 0.41-0.53 mg 100 ml-1.

Quantification of amylase activity

Amylase production was determined roughly (without optimization), the potent isolates produced desirable quantity. The results of extracellular amylase activity by dinitrosalicylic acid (DNS) method are given in table 3. The crude fraction of enzyme absorption was measured at 540 nm, the measurement provides the validation of amylase production. The crude fraction of amylase activity was observed in the range of 2.4±0.002-5.9±0.005 (U/ml).

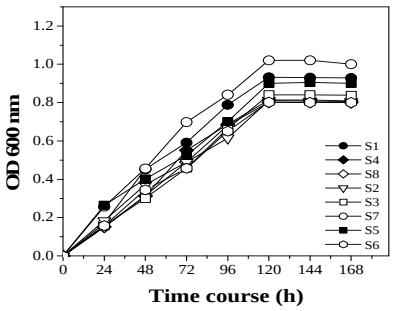

Fig. 4: Time course profile of amylase producing *streptomyces* isolates, the growth phase was determined with ISP-4 medium. The growth rate was observed regularly at 24 h time intervals and the concentration was measured at OD $_{600\,nm}$ during 24-168 h. The right portion of the curve was observed only in S4-S6, and high level of growth exhibited during 96 h

Table 3: Qualitative and quantitative determination of amylase activity by *Streptomyces*

Isolates	Clear zone (mm)	Cell dry biomass (mg 100 ml-1)	Enzyme activity (U/ml)
S1	4.0±0.81	0.23±0.003	2.4±0.002
S2	9.0±0.26	0.35±0.004	2.6±0.005
S3	7.0±0.31	0.24±0.003	3.5±0.001
S4	15±0.54	0.41±0.001	4.6±0.002
S5	18±0.98	0.51±0.004	5.6±0.005
S6	20±0.12	0.53±0.001	5.9±0.009
S7	12±0.81	0.41±0.005	4.1±0.002
S8	9.0±0.54	0.32±0.001	3.5±0.003

S1-S8: *Streptomyces* species code; mm-millimetre; mg-milligram; U/ml-Unit/ millilitre, Amylase screening and activity was performed in triplicates and the mean values along with standard error mean (mean±SEM., n=3).

DISCUSSION

This study was aimed to isolate potent amylase producer from a marine source. *Streptomyces* produce the incredible source of intercellular and extracellular enzymes vital for industrial and economic use [27]. However, the distribution of *Streptomyces* in the marine is chiefly unexplored or underexploited habitats be pursued as sources of novel compounds for industrial usage [28]. Mangrove is a high moisture, high salinity and hypoxia to tolerant ecosystem [29] which breeds many kinds of novel microorganisms which are the main source of vital compounds [30]. Many researchers investigated the muthupet mangrove vegetation, isolation and characterization of bioactive compounds from *Streptomyces* [31-32]. A novel *Streptomyces* strain was isolated from mangroves soil at Tanjung Lumpur, Malaysia [33].

Morphological and biochemical features of isolated *Streptomyces* were similar to Bergey's manual of systematic bacteriology [22]. Furthermore, they are ubiquitous in nature and showed a higher diversity in color of colonies, secreted pigments, etc., compared to other bacteria. Strains can be readily differentiated by variation in colors of their aerial and substrate mycelium and based on physiological and biochemical characteristics [34]. The similar microscopic characterization of *Streptomyces* spore morphology and hyphae features was studied [35].

More studies involving isolation and screening of amylase by starch agar plate. Amylolytic activity was identified by *Bacillus subtilis* B19 [36]. Amylase production on the agar plate was proved by *Aspergillus versicolor* and *Penicillium* sp [37]. Amylase production has been done by numerous strains notably *S. gulbargensis*, *Streptomyces* strain A3, *S. avermitilis*, *S. rochei* BTSS 1001 [38-39]. This work coincided with the similar determination of previous analysis of amylase on starch agar.

Amylase is one of the most significant industrial enzymes. Every year several tons of amylase is used in the various industrial applications in Iran [40]. High level of extracellular amylase production was achieved newly isolated alkali-thermotolerant strain *Streptomyces gulbargensis* DAS 131[38]. Aquatic actinomycetes were exhibited amylase activity 62.97 U/ml [41]. Box-Behnken design method highly improved amylase activity 145.32 U/ml (pH 6, temp 35 ° C) [42]. *Aspergillus fumigatus* NTCC1222 showed highest amylase activity (164.1 U/ml) was studied [43]. α-Amylase was derived from *Bacillus* sp. BCC 01-50 [44]. Recently highest amylase production was achieved through optimization by *B. cereus*. The production was carried out under submerged state fermentation. The maximum production of amylase was 281.1±0.65 U/ml in wheat bran supplemented medium [45].

Analysis of amylase activity in the crude extract, the results provide a significant quantity of amylase. *Streptomyces* S4, S5 and S6 were exhibited (4.9-5.6 U/ml) activity. Few reports coincide with the present amylase activity. *Streptomyces cheonanensis* VUK–A was roughly produced 4.3 U/ml after optimization, reached 11.2 U/ml [46]. Amylase activity was explored during the short period, significant quantity achieved by *Streptomyces* sp S4, S5 and S6. The isolates S4, S5 and S6 are considered an efficient for amylase production to the technological application.

CONCLUSION

In the present study, the marine *Streptomyces* isolates had the significant ability to produce industrial amylases. *Streptomyces* are considered as a worthy source for amylases, identified from mangroves. It is enormous importance as secrete extracellular amylases. The results are more virtual and low-cost production of amylase by inexpensive single media without optimization. Further study is in progress to scale up the amylase production using optimization. Therefore, the future work deals with the optimization and improvement of amylase will be explored.

AUTHORS CONTRIBUTIONS

Sathya Rengasamy and Ushadevi Thangaprakasam were designed and executed the experimental analysis and written the manuscript.

CONFLICT OF INTERESTS

No conflict of interest declared

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