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Research Article

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Characterization of α-amylase producing *Bacillus mycoides strains* from Bay of Bengal, Visakhapatnam

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ABSTRACT

Numerous marine microorganisms secrete enzymes which can provide new insights and understanding of enzymes. Bacteria have been regarded as treasure of many useful enzymes viz., amylases, proteases, lipases, hydrolases and reductases. Among them amylolytic enzymes have great biotechnological applications and economic exploitations. The bacterial genus Bacillus proved to be an important source of amylase in food, sewage treatment, textile and laundry industry. In the present study, α-amylase producing Bacilli were isolated from coastal waters of Bay of Bengal, Visakhapatnam, were characterized by employing various cultural, morphological and biochemical methods. Serially diluted samples were cultured on Starch agar plates and incubated for 24 h at 37^{0}C, and then the plates were flooded with Lugol's solution. The colonies showing large halo zone of starch hydrolysis were selected for further screening of Amylase activity. Three isolates of Bacillus mycoides; B. mycoides a1, B. mycoides a3 and B. mycoides F5 were selected and identified. The enzyme activity was estimated by DNS method for all isolates which were inoculated in nutrient broth and incubated at 37^{0}C for 24 hours. The Amylase activity of B. mycoides F5 was found to be maximum, 1023 µg/ml and three isolated B. mycoides a1, B. mycoides a3 and B. mycoides F5 showed within a range of 800 to 1100 µg/ml.

Keywords: *Bacillus mycoides,α-amylase, Cultural, Morphological, Biochemical characterization.*

INTRODUCTION

Microbial flora in marine environment forms an integral part of this unique ecosystem, but marine bacteria have remained unexplored[1]. Amylases have applications in fermentation, baking, brewing, detergent, textile, paper & distilling industries. However, amylases from bacterial sources have economically dominated applications in industrial sectors [2]. The *Bacillus sp.* is ubiquitous in terrestrial, fresh water and marine habitat[3]. *Bacillus* α-amylases isolated and characterized earlier in soil and marine waters[4-18]. Therefore**,** the present study was aimed to isolate and identify potent *Bacillus mycoides* strains showing high amylase activity from marine coastal waters of Bay of Bengal, Visakhapatnam.

MATERIALS AND METHODS

Collection of Samples

Marine water samples were collected from coastal areas of Visakhapatnam across the Bay of Bengal at two sites; Appugur (a) and Fishing harbor (F). Visakhapatnam, which was situated in the east coast of Bay of Bengal, Andhra Pradesh, India. The water samples collected in sterile BOD bottles were brought to the lab and stored in the refrigerator to carry out further work.

Primary Screening of α-Amylase producing Bacteria

The collected marine water samples were serially diluted 10^{-3} to 10^{-7} by Serial Dilution Technique. Hundred millileters of the diluted sample was spreaded with L-shaped glass rod on the starch agar plates by adopting Spread plate Technique. The discrete colonies growth was observed at 10^{-5} dilution on

incubated Starch agar plates for 24h at 37^0C. Then the plates were flooded with Lugol's solution (1% iodine in 2% potassium iodide w/v)[19]. Colonies forming large halo zones of starch hydrolysis were measured (mm) and isolated. The isolates cultured in nutrient broth were used to determine the enzyme activity by DNS Method [20]. One unit (U) of α-amylase activity was defined as the amount of µg of maltose equivalents liberated per min per ml of enzyme under the conditions of assay. The amount of maltose was determined from the maltose standard curve.

Identification of isolates

The identification of *Bacillus cereus* isolates was characterized by their Cultural, morphological and biochemical characters by adopting standard techniques from laboratory manual[21]. The isoles showing highest α-amylase activities were identified by referring Bergey's Manual of Determinative Bacteriology[29].

RESULTS AND DISCUSSION

Primary Screening of α-Amylase producing Bacteria

Isolation

Serially diluted water samples (10^{-5}) cultured on Starch Agar medium by spread plate technique. After incubation at 37^0C, discrete colonies were observed showing zone of Starch hydrolysis as indicated by Iodine staining. In the present study, 3 potent amylase producing isolates of *Bacillus* were selected based on the zone of starch hydrolysis showing more than 10mm. They were labeled according to the two different sites of coastal waters of Visakhapatnam and were designated. As Appugur (a1& a3) and Fishing harbor (F5)**.** The isolated three isolates were identified as *Bacillus* as per earlier reports, Isolation of bacteria by Spread plate technique[22, 23]. Enrichment technique[24, 25], and Serial dilution method[26]. Identification of isolates was based on Bergey's Manual [27-29].

The amylase production was estimated by DNS method after incubation in nutrient broth, pH 7, at 37^0C for 24 hours from all isolates of *Bacillus* (Table 1). Out of three isolates tested, *B. mycoides* F5 showed maximum production (1023 µg/ml) whereas two isolates *B. mycoides a1, B. mycoides* a3 showed 800 to 1100 µg/ml production. Niziolek[30] had studied 41 strains of the genus *Bacillus*, and found that 19 strains were low-productive and 12 were medium-productive strains (10-25 U/ml). *Bacillus subtilis* AS-1-108, *Bacillus subtilis* NCIB 8159 and *Bacillus licheniformis* NCIB 7198 strains were included among the higher-productive as they produced about 370, 170 and 40 U/ml of alpha amylase respectively. Similar work with fungi was done by Tokhadze *et al* [31.] They isolated 86 strains of the *Aspergillus* producing acid stable alpha-amylase.

Identification of isolates

All the 3 isolates of *Bacilli* were further classified at genus and species level by referring Bergey's manual of determinative bacteriology and identified as *B. mycoides* a1, *B. mycoides* a3 *and B. mycoides* F5 (Table I, II and III). Pretorius et al [25] reported 134 alpha amylase strains of *Bacillus*, divided into 12 groups by their biochemical and morphological characterizations.

Bacillus mycoides

B. mycoides a1 showed small, rough, viscous, lobate, raised, irregular, translucent colonies, pellicle growth on surface of nutrient broth and arborescent growth on NA slant (Figure1a).They were gram-positive (Figure1b), *Streptobacilli*, 1.826±0.018 µm x 0.860±0.028 µm in size (Figure 1c), capsulated, non-sporulating, and non-motile. They fermented lactose, dextrose, and sucrose without gas production. Produce amylase (Figure1d). Beta-hydrolysis on blood agar, catalase positive, oxidase positive, utilize citrate, VP positive, reduces nitrate to nitrite and resistant to bile salts.

B. mycoides a3 showed moderate, slimy, butter like, white, wavy indentations, slightly elevated, indented peripheral edge, opaque colonies, Sediment of growth at the bottom of nutrient broth and filiform growth on NA slant (Figure 2a). Gram positive (Figure 2b) *Bacilli*, 1.471±0.024 µm x 0.814±0.015 µm (bulge) in size (Figure 2c), bipolar spore forming cells. Produced amylase (Figure 2d) and protease. Alpha hydrolysis on blood agar.

B. mycoides F5 colonies showed small, dull, dry, light yellowish, tooth-like appearance, raised, irregular, opaque colonies, flocculent growth in the nutrient broth and effuse growth on NA slant (Figure 3a).They

were Gram positive (Figure 3b), stout *Bacilli*, 1.668±0.090 µm x 0.692±0.017 µm in size, (Figure 3c), bipolar spore forming and mannitol fermentation. Produce amylase (Figure 3d) and oxidase negative.

Table I. Colony characterization of *Bacillus mycoides*

Character	*B. mycoides* a1	*B. mycoides* a3	*B. mycoides* F5
Size	Small	Moderate	Small
Surface texture	Rough	Mucoid	Rough
Consistency	Viscous	Butyrous	Dry
Chromogen pigmentation	Water insoluble	White	light yellowish
Margin	Lobate	Undulate	Serrate
Elevation	Raised	Raised	Raised
Form	Irregular	Irregular	Irregular
Optical character	Translucent	Opaque	Opaque
Nutrient broth culture	Pellicle	Sediment	Flocculent
Growth form on slant	Arborescent	Filiform	Effuse

Table II. Morphological characterization of *Bacillus mycoides*

Character	*B. mycoides* a1	*B. mycoides* a3	*B. mycoides* F5
Morphology:Shape Size : Length (µl) width(µl)	*Streptobacilli* 1.826±0.018 0.860±0.028	*Bacilli* 1.471±0.024 0.814±0.015	Stout bacilli 1.668±0.090 0.692±0.017
Gram staining	Gram +ve	Gram +ve	Gram +ve
Spore staining	-ve	Bipolar spore	Bipolar spore
Acid fast staining	-ve	-ve	-ve
Capsule staining	+ve	+ve	+ve
Motility	-ve	-ve	-ve

Table III. Biochemical characterization of *Bacillus mycoides*

S.No.	Test	Observation	*B.mycoides* a1	*B.mycoides* a3	*B.mycoides* F5
01	Amylase activity	zone of hydrolysis	+ve	+ve	+ve
02	Protease activity	zone of hydrolysis	-ve	+ve	-ve
03	Lipase activity	zone of hydrolysis	-ve	-ve	-ve
04	Blood agar	hydrolysis	β	α	β
05	Chocolate agar	Mucoid grey colonies	+ve	+ve	+ve
06	Cetrimide agar	No color change	-ve	-ve	-ve
07	Macconkey agar	Pink or red color colonies	+ve	+ve	-ve
08	Mannitol salt agar	No color zone	-ve	-ve	+ve
09	Eosin methylene blue agar	Yellow zone	-ve	-ve	-ve
10	Bile esculine agar	Colorless colonies	+ve	+ve	+ve
11	Indole test	No ring formation	-ve	-ve	-ve
12	Methyl red test	Yellow	-ve	-ve	-ve
13	Voges-proskauer test	Pink/red color	+ve	+ve	+ve
14	Cirtate utilization test	Green slant	+ve	-ve	+ve
15	H_2S test	Black ppt.	-ve	-ve	-ve
16	Urease test	Yellow	-ve	-ve	-ve
17	Catalase test	Bubbles	+ve	+ve	+ve
18	Oxidase test	Purple color	+ve	-ve	-ve
19	Carbohydrate Fermentation				
a)	Lactose	Gas pH Turbidity	Nil Yellow(alkaline) +ve	Nil Yellow(alkaline) +ve	Nil Yellow(alkaline) +ve
b)	Dextrose	Gas pH Turbidity	Nil Yellow(alkaline) -ve	Nil Yellow(alkaline) -ve	Nil Yellow(alkaline) +ve
c)	Sucrose	Gas pH Turbidity	Nil Yellow(alkaline) -ve	Nil Yellow(alkaline) +ve	Nil Yellow(alkaline) +ve

20	Nitrate reduction	Cherry red (a+b) (nitrate to nitrite):	+ve	+ve	-ve
21	Gelatin liquefaction	No liquefaction	-ve	-ve	-ve
22	Triple sugar iron agar test				
a)		Slant	Red (alkaline)	Yellow (acid)	Red(alkaline)
b)		Butt	Yellow (acid)	Yellow (acid)	Yellow (acid)
c)		H_2S	-ve	-ve	-ve
23	Coagulase test	Serum liquidifies	-ve	-ve	-ve
24	Hektoen enteric agar	No green color	+ve	-ve	-ve
25	Thiosulphate citrate bile salt sucrose agar	Green color colonies	+ve	-ve	-ve
26	Deoxycholate agar	Pink color colonies	+ve	-ve	-ve
27	Pheylalanine deaminase test	Yellow color	-ve	-ve	-ve

Fig. 1: *Bacillus mycoides* a1: a, Colony characterization cultured on nutrient agar; b, Gram staining (2000x); c, Morphology and d, Zone of starch hydrolysis

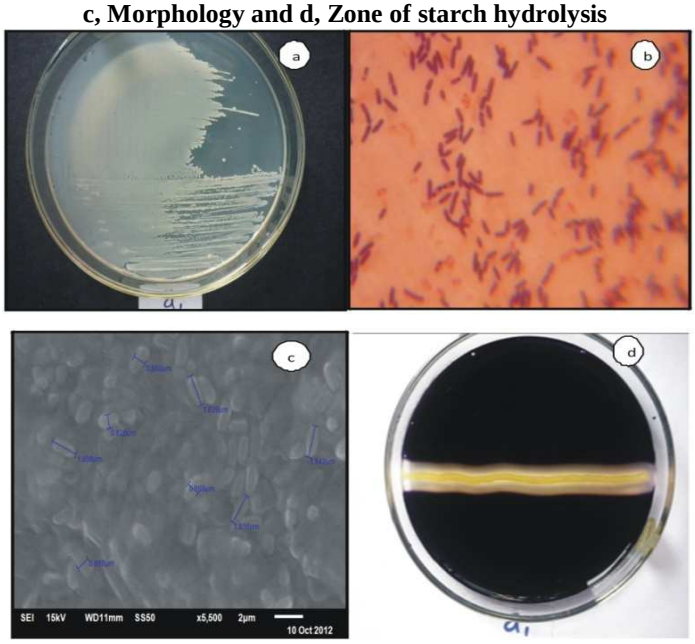


Fig. 2: *Bacillus mycoides* a3*:* a, Colony characterization cultured on nutrient agar; b, Gram staining (2000x); c, Morphology and d, Zone of starch hydrolysis

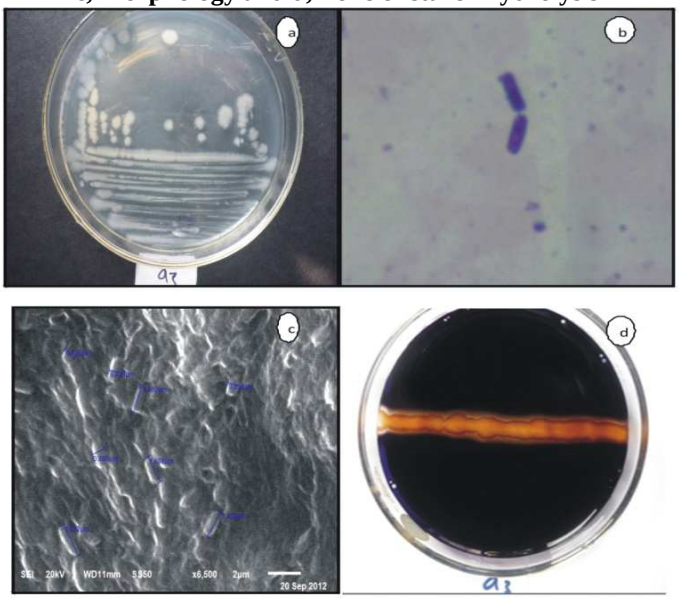
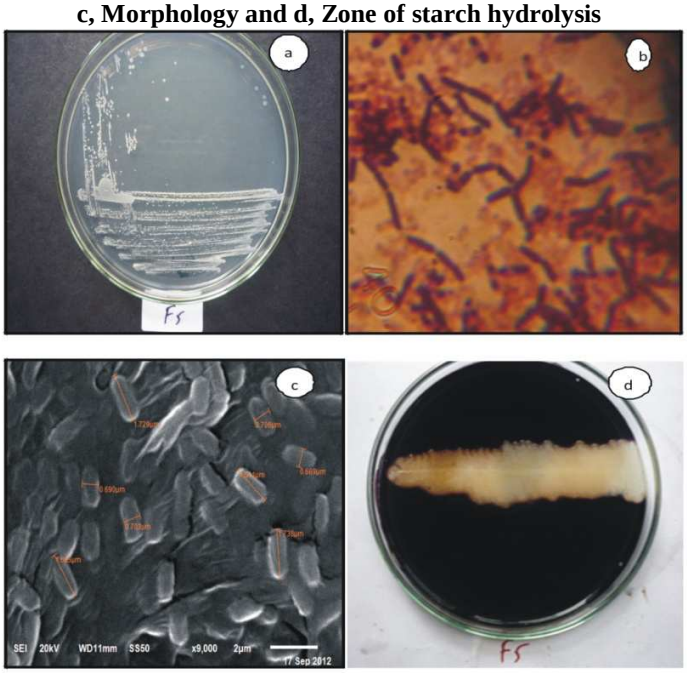


Fig. 3: *Bacillus mycoides* F5*:* a, Colony characterization cultured on nutrient agar; b, Gram staining (2000x); c, Morphology and d, Zone of starch hydrolysis



CONCLUSION

In the primary screening, two strains of *Bacillus mycoides* isolated from coastal waters of Bay of Bengal, Visakhapatnam, have showed greater potential to produce large amounts of α-amylase. Isolation of α-amylase producing *Bacillus* from marine environment in this coast provides ample scope for exploration in biotechnological, medical, sewage treatment, textile and industrial applications.

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