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Characterization of α-amylase Producing *Bacillus cereus* Strains from Marine Waters of Bay of Bengal, Visakhapatnam

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Marine microbes have been attracted more attention as a source for novel enzymes because they were relatively stable and active. Bacteria particularly have been regarded as treasures of many useful enzymes: amylases, proteases, lipases, hydrolases and reductases. The bacterial genus *Bacillus* proved to be an important source of amylase in food, paper, 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°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. Six isolates of *Bacillus cereus*; *B.cereus r1, B.cereus r4, B.cereus a5, B.cereus F6, B.cereus f1 and B.cereus g1* 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°C for 24 hours. The Amylase activity of B. cereus f1 was found to be maximum, 1430 μg/ml and three isolated *B.cereus a5, B.cereus F6* and *B.cereus g1* showed within a range of 1000 to 1162 μg/ml and other two showed < 1000 μg/ml.

Keywords: *Bacillus cereus*, a-amylase, Cultural, Morphological, Biochemical characterization.

Marine bacteria have a diverse range of enzymatic activity and were capable of catalyzing various enzymatic reactions with novel enzymes, secondary metabolites and therapeutics[1]. Amylase has widened application in many sectors such as clinical, medical & analytical chemistry. Besides their use in starch saccharification, they also 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.* was ubiquitous in terrestrial, fresh water and marine habitat[3]. Bacillus α-amylases isolated and characterized earlier in soil and marine waters by[4-18]. Therefore, the present study was aimed to isolate and identify potent *Bacillus cereus* strains showing high amylase activity from marine coastal waters of Bay of Bengal, Visakhapatnam.

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MATERIALS AND METHODS

Collection of Samples

Marine water samples were collected from coastal areas of Visakhapatnam across the Bay of Bengal at four sites; Rushikonda (r), Appugur (a), Fishing harbor (F & f) and Gangavarum (g). Visakhapatnam was situated in the east cost 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 Method. 0. l ml 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°C, then 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 were 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 were characterized by their Cultural, morphological and biochemical characters by adopting standard techniques from laboratory manual[21]. The isolated 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°C, discrete colonies were observed showing zone of Starch hydrolysis as indicated by Iodine staining. In the present studies, 6 potent amylase producing isolates of *Bacillus* were selected based on the zone of starch hydrolysis showing more than 10mm and labeled according to the four different sites of coastal waters of Visakhapatnam as designated, Rushikonda (r1 & r4), Appugur (a5), Fishing harbor (F6 & f1) and Gangavarum (g1)**.** The isolated six isolates were identified as *Bacillus* (Table 1) 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 based on Bergey's Manual[27-29].

All isolates of *Bacillus* the amylase production was estimated by DNS method after incubation in nutrient broth, pH 7, at 37°C for 24 h (Table 1). Out of 6 isolates tested, *B. cereus* f1 showed maximum production that was 1,430 µg/ml where as three isolates *B. cereus a5, B. cereus F6* and *B. cereus g1* showed 1000 to 1162µg/ml. Niziolek[30] have studied 41 strains of the genus *Bacillus*, he 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] isolated 86 strains of the *Aspergillus* producing maximum acid stable alpha-amylase.

Identification of 6 isolates

All the 6 isolates of bacilli were further classified at genus and species level by referring Bergey's manual of determinative bacteriology and

Table 1. Primary screening and Nomenclature of high α-Amylase yielding Bacterial isolates

S. No.	Bacterial Isolates	*Bacilli* classified using Bergey's Manual of Determinative Bacteriology	Zone of starch Hydrolysis in mm	Amylase activity in U/ml (Nutrient Broth, pH: 7 & 37°C)
1	*Bacillus r1*	*Bacillus cereus r1*	12	942
2	*Bacillus r4*	*Bacillus cereus r4*	10	882
3	*Bacillus a5*	*Bacillus cereus a5*	13	1003.3
4	*Bacillus F6*	*Bacillus cereus F6*	18	1021.6
5	*Bacillus f1*	*Bacillus cereus f1*	21	1430
6	*Bacillus g1*	*Bacillus cereus g1*	20	1162.3

identified as *Bacillus cereus r1, B. cereus r4, B. cereus a5, B. cereus f1, B.cereus F6, and B.cereus g1* (Table 1). Pretorius *et al*., [25] reported 134 alpha amylase strains of *Bacillus* were divided into 12 groups by their biochemical and morphological characterizations.

B.cereus r1 showed moderate, rough, lobate, raised, irregular, and opaque colonies. sediment growth in nutrient broth and filiform on NA slant (Fig. 1). They were Gram positive, Strepto bacilli, 2 µm x ≥1µm in size (Fig. 2), bipolar spore forming and motile. They ferment lactose, dextrose, and sucrose without gas production. Produced amylase (Fig 3), protease and lipase. Alpha-hydrolysis on blood agar, catalase positive, oxidase positive, VP positive, hydrolyzes urea, reduce nitrate to nitrite, and resistance to bile salts.

B.cereus r4 colonies were moderate, mucoid, butyrous, serrate, raised and irregular edge (Fig. 4). Gram positive, Strepto bacilli, 2 µm x e"1 µm in size, (Fig. 5), central spore forming and showed amylase hydrolysis (Fig. 6).

B.cereus a5 showed moderate, mucoid, butyrous, undulate margin, raised, irregular, opaque colonies, beaded on NA slant (Fig. 7). Gram positive, Strepto bacilli, 2.5 µm x ≥1 µm in size (Fig.

Table 2. Colony Characterization of *Bacillus cereus*

Character	*B.cereus* r1	*B.cereus* r4	*B.cereus* a5	*B. cereus* f1	*B. cereus* F6	*B. cereus* g1
Size	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Surface texture	Rough; dull	Mucoid	Mucoid	Shiny, glistening	Shiny, glistening	Mucoid
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Chromogen pigmentation	white	white	white	white	light yellow	white
Margin	Lobate	Serrate	Undulate	Undulate	Undulate	Lobate
Elevation	slightly elevated	slightly elevated	slightly elevated	slightly elevated	slightly elevated	Flat
Form	indented peripheral edge	indented peripheral edge	indented peripheral edge	indented peripheral edge	indented peripheral edge	indented peripheral edge
Optical character	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Nutrient broth culture	Sediment	Sediment	Sediment	Sediment	Uniform fine turbidity	Sediment
Growth form on slant	Filiform	Filiform	Beaded	Beaded	Filiform	Echinulate

Table 3. Morphological Characterization of *Bacillus cereus*

Character	*B. cereus* r1	*B.cereus* r4	*B.cereus* a5	*B.cereus* f1	*B.cereus* F6	*B.cereus* g1
Morphology	Strepto bacilli	Strepto	Strepto	Strepto	Stout	Strepto bacilli
Shape		bacilli	bacilli	bacilli	bacilli	
Size	≥1µm					≥1 µm
width	2 µm	≥1 µm	≥1 µm	≥1 µm	≥1 µm	4 µm
Length		2 µm	2.5 µm	2 µm	2 µm	
Gram staining	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
Spore staining	Positive	Positive	Positive	Positive	Positive	Negative
Type of spore	Bipolar spore	Central	Bipolar spore	Bipolar spore	Bipolar spore	
Acid fast	Staining	Negative	Negative	Negative	Negative	Negative
Negative						
CapsuleStaining	Negative	Negative	Negative	Negative	Positive	Positive
Motility	Positive	Positive	Positive	Positive	Positive	Positive

Table 4. Biochemical Characterization of *Bacillus cereus*

Experimental procedure	Observation	*B.cereus* r1	*B.cereus* r4	*B.cereus* a5	*B.cereus* F6	*B.cereus* f1	*B.cereus* g1
Amylase activity	Halo zone of hydrolysis	Positive	Positive	Positive	Positive	Positive	Positive
Protease activity	Halo zone of hydrolysis	Positive	Positive	Positive	Positive	Positive	Positive
Lipase activity	Halo zone of hydrolysis	Positive	Positive	Negative	Positive	Positive	Positive
Blood agar	Incomplete hydrolysis	Alpha-hydrolysis	Alpha-hydrolysis	Alpha-hydrolysis	Gamma-hydrolysis	Alpha-hydrolysis	Alpha-hydrolysis
Chocolate agar	Growth mucoid gray colonies	Positive	Positive	Positive	Positive	Positive	Positive
Cetrimide agar	No color change	Negative	Negative	Negative	Negative	Negative	Negative
Macconkey agar	Pink or red color colonies	Positive	Positive	Negative	Positive	Negative	Positive
Mannitol salt agar	No color zone	Negative	Negative	Negative	Negative	Negative	Negative
Eosin methylene blue agar	Yellow zone	Negative	Negative	Negative	Positive	Negative	Negative
Bile esculine agar	Color less colonies	Positive	Positive	Negative	Positive	Positive	Positive
Heltoen enteric agar	No green color	Negative	Negative	Negative	Negative	Negative	Negative
Thiosulphate citrate bile salt sucrose agar	Green color colonies	Positive	Positive	Negative	Positive	Positive	Negative
Deoxycholate agar	Pink color colonies	Negative	Positive	Positive	Positive	Positive	Negative
Indole test	No ring formation	Negative	Negative	Negative	Negative	Negative	Negative
Methyl red test	Yellow	Negative	Negative	Negative	Negative	Negative	Negative
Voges-proskauer test	Pink/red color	Positive	Positive	Positive	Positive	Positive	Positive
Cirtate utilization test	Green slant	Negative	Positive	Negative	Negative	Negative	Negative
Hydrogen sulfide test	Black ppt.	Negative	Negative	Negative	Negative	Negative	Negative
	Motility	Nil	Nil	Nil	Nil	Nil	Nil
Urease test	Yellow	Positive	Positive	Negative	Positive	Negative	Negative
Catalase test	Bubbles	Positive	Positive	Positive	Positive	Positive	Positive
Oxidase test	Purple color	Positive	Positive	Positive	Positive	Positive.	Positive
Carbohydrate fermentation	Gas	Nil	Nil	Nil	Nil	Nil	Nil
Lactose	P^H	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
	Turbidity	Positive	Positive	Positive	Positive	Positive	Positive
Dextrose	Gas	Nil	Nil.	Nil.	Nil.	Nil.	Nil
	P^H	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
	Turbidity	Negative	Negative	Negative	Negative	Negative	Negative
Sucrose	Gas	Nil	Nil	Nil	Nil	Nil	Nil
	P^H	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
	Turbidity	Negative	Negative	Positive	Negative	Negative	Negative
Nitrate reduction	Cherry red	Positive	Positive	Positive	Positive	Positive	Positive
Gelatin liquefaction	No liquefaction	Negative	Negative	Negative	Negative	Negative	Negative
Triple suger iron (acid)agar test (acid)	Slant	Red - alkaline	Yellow (acid)	Yellow (acid).	Yellow (acid)	Yellow (acid)	Yellow
	Butt	Yellow (acid)	Yellow (acid)	Yellow (acid)	Yellow (acid)	Yellow (acid)	Negative
	H_2s	Negative	Negative	Negative	Negative	Negative	
Coagulas test	Serum liquidifies	Negative	Negative	Negative	Negative	Negative	Negative
Pheyl alanine deaminase test	Yellow color	Negative	Negative	Negative	Negative	Negative	Negative

Fig. 1. Colony Characterization of B. *cereus r1* cultured on Nutrient agar

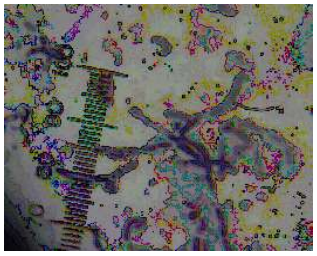

Fig. 2. Gram Staining, Length and width of *B. cereus r1* (2000x)

Fig. 3. Zone of Starch Hydrolysis by *B. cereus r1*

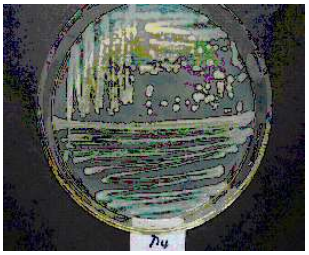

Fig. 4. Colony Characterization of B. *cereus r4* cultured on Nutrient agar

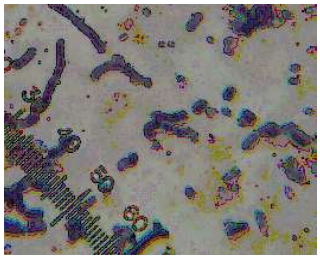

Fig. 5. Gram Staining, Length and width of *B. cereus* r4 (2000x)

Fig. 6. Zone of Starch Hydrolysis by *B. cereus r4*

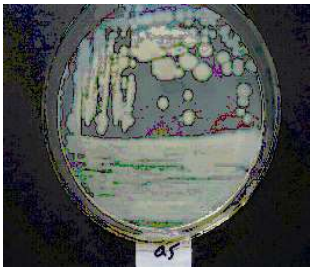

Fig. 7. Colony Characterization of B. *cereus a5* cultured on Nutrient agar

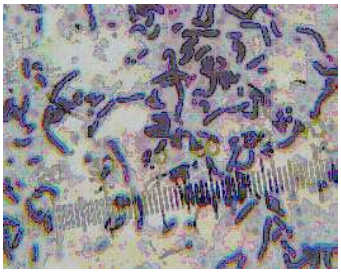

Fig. 8. Gram Staining, Length and width of *B. cereus f1* (2000x)

Fig. 9. Zone of Starch Hydrolysis by *B. cereus a5*

Fig. 10. Colony Characterization of B. *cereus f1* cultured on Nutrient agar

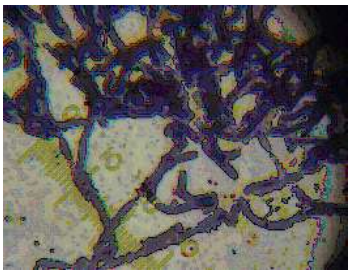

Fig. 11. Gram Staining, Length and width of *B. cereus a5* (2000x)

Fig. 12. Zone of Starch Hydrolysis by *B. cereus f1*

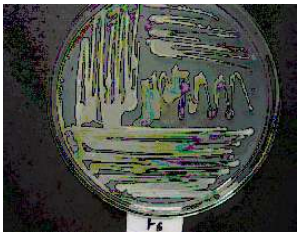

Fig. 13. Colony Characterization of B. *cereus F6* cultured on Nutrient agar

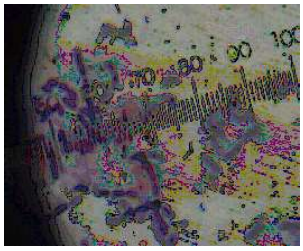

Fig. 14. Gram Staining, Length and width of *B. cereus g1* (2000x)

Fig. 15. Zone of Starch Hydrolysis by *B. cereus g1*

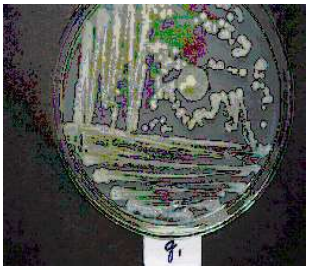

Fig. 16. Colony Characterization of B. *cereus g1* cultured on Nutrient agar

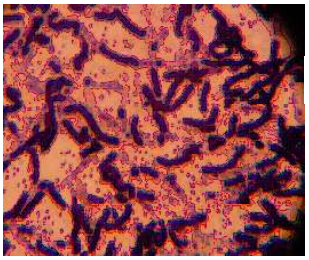

Fig. 17. Gram Staining, Length and width of *B. cereus F6* (2000x)

Fig. 18. Zone of Starch Hydrolysis by *B. cereus F6*

8). Produced amylase hydrolysis (Fig: 9) and no lipase and urea hydrolyzes.

B. cereus f1, smooth, shiny, glistening, butyrous, undulate, raised, irregular, opaque colonies and beaded colonies on NA slant (Fig.10) were observed. The cells were Gram- positive, Strepto bacilli, 2 µm x ≥1 µm in size (Fig. 11). Produced large zone of amylase (Fig. 12) but no urea hydrolysis.

B. cereus F6 colonies were moderate, smooth, shiny, glistening, light yellow, wavy indentations, slightly elevated, opaque colonies, uniform fine turbidity in nutrient broth (Fig. 13).They were Gram-positive, Stout bacilli, 2 µm x ≥1 µm in size (Fig.14), and capsulated. Gamma-hydrolysis on blood agar and amylase positive (Fig. 15).

B. cereus g1 showed moderate, gummy, butter like, white, marked indentation, flat, indented peripheral edge, opaque colonies, and echinulate on NA slant (Fig.16). Gram positive, Strepto bacilli, 4 µm x ≥1 µm in size, (Fig: 17), capsulated and produced zone of amylase activity (Fig.18).

Morphological and biochemical characterization was done by many earlier research workers, Gram-Staining[32], Coagulation and peptonization of milk[32], Starch hydrolysis[32-34], Casein hydrolysis[32, 35], Lipolytic Assay[34, 36], Nitrate reduction test[32], Gelatin hydrolysis[37]. Blood agar for haemolytic bacteria[38], Mannitol salt agar for halophiles[39], MacConkey agar for lactose fermentative bacteria[40].

CONCLUSION

In the primary screening, six strains of *Bacillus cereus* 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 and industrial applications.

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