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Research article

CHARACTERIZATION OF AMYLASE PRODUCING BACTERIA

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ABSTRACT: The screening led to the isolation of 36 amylase producing bacterial isolates. Among these, 12 isolates formed halo zones of 1 cm or more and they were selected for further biochemical investigations. All the isolates were unable to ferment xylose. The isolates showed good growth in presence of NaCl. BCH9, BCH13 & BCH25 showed growth in 2.6M NaCl, 2.5M NaCl and 2.6M NaCl respectively. On the basis of various biochemical tests, BCH9 & BCH25 were identified as *Bacillus licheniformis* while BCH34 was identified as *Bacillus cereus*. BCM9 & BCM25 are of halophilic nature and hold promise in biotechnological industries.

Key Words: Halo zone, Halophilic, amylase, NaCl *Bacillus licheniformis Bacillus cereus*

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INTRODUCTION

Amylases constitute one of the most important groups of industrial enzymes and account for nearly 25% of the total sale of enzymes (Burhan et al. 2003). The amylase superfamily can be generally subdivided into two groups, emdoamylases (α-amylase) and exoamylases (β-amylases, glucoamylase and α-glucosidase). Endoamylases hydrolyse the interior of a glucose chain in starch to generate oligosaccharides of various lengths. Exoamylases act at the non-reducing ends of polysaccharides and produce low molecular weight products, e.g., glucose and maltose (Pandey et al. 2003). Enzymes obtained from microbial sources are widely used in various industrial processes because of their low cost, productivity, chemical stability, plasticity and vast availability (Burhan et al. 2003; Mishra & Behera et al. 2008).Today a large number of microbial enzymes are commercially available and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey et al. 2000). Thermostable α-amylases are available from different sources and they have extensively commercial applications in starch processing, brewing and sugar production (Leveque et al. 2000), designing in textile industries (Hendriksen et al. 1999) and in detergent manufacturing processes (Hewitt & Solomons, 1996, Lin et al. 1998). Each application of α-amylase requires unique properties with respect to specificity, stability and temperature and pH value dependence (Tigue et al. 1995). Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to industrial applications (Gupta et al. 2003, Wanderley et al. 2004). The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases are of considerable industrial importance (Swain et al. 2006). Prominent *Bacillus* species used for industrial applications are *Bacillus subtilis, Bacillus amyloliquefaciens and Bacillus licheniformis* (Das et al. 2013).

Soil is a rich source of amylase secreting microorganisms and present investigation was designed to screen for bacterial isolates for amylase production and their characterization, which could pave way for advanced research on amylase activity and stability under various conditions such as temperature, pH, metal ions and organic solvents leading to its industrial applications.

MATERIALS AND METHODS

Soil samples were collected from different localities of Patna namely Kankarbagh, Rajendra Nagar and Patna City in sterilized plastic bags and stored at 4° C for the preliminary screening for amylase producing bacteria. For isolation of bacterial isolates, serial dilution method was employed. In this method, the soil sample was weighed (1 gram) and was mixed in 10 ml of normal saline by proper vortexing.

Six test tubes were labeled namely A, B, C, D, E and F, each containing 9 ml of normal saline.1 ml of the sample was transferred to tube A making the concentration 10^{-1}ml.1 ml from tube A was transferred to tube B making the concentration 10^{-2} ml and similar transfers were made to obtain concentrations in order of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Each tube was vortexed for even distribution of sample. Six nutrient agar (NA) plates consisting of peptone (0.5%), NaCl (0.5%),beef extract (0.3%) and agar (1.5%) were made and labelled as A, B, C, D, E and F. 0.1ml from each tube containing the sample in decreasing concentrations was transferred aseptically to each NA plate and the sample was evenly spread on the plates. The plates were incubated for 24 hours at 37°C. After 24 hours colonies were selected based on the basis of cultural characteristics and subculturing was done by streaking. Successive subculturing was done to obtain pure cultures.

Screening for amylase activity

Pure cultures were streaked on starch agar plates consisting of soluble starch (2%), NaCl (0.5%), beef extract (0.3%) and agar (1.5%) and plates were incubated for 37°C for 48 hours. After 48 hrs, the plates were flooded with Lugol's iodine, which is an indicator of starch. When iodine comes in contact with a medium containing starch, it turns blue. If starch is hydrolyzed, the medium will have a clear zone next to growth. Excess Lugol's iodine was drained of and plates were observed for clear halo zone around the colony against blue-black background. A clear halo zone around the colony indicates amylase production. Size of the zone corresponds to amylase production.

Biochemical characterization of the isolates

Gram's staining

Bacteria stained by the Gram method falls into two groups: gram positive bacteria, which retain the crystal violet and hence appear deep violet in colour, and gram-negative bacteria, which lose the crystal-violet after treatment with decolourising agent ethanol or acetone. Gram negative bacteria are counter-stained by the saffranin and hence appear red in colour. This difference in staining is related to structure and composition of the cell wall of both groups.

Salt tolerance test

The selected isolates were grown in 6.5% NaCl nutrient broth for 48 hours and growth was monitored. The growth in 6.5% NaCl is an indicator of halophilic character of any bacterial isolate.

Carbohydrate Fermentation Test

One of the primary tests for the identification of bacterial species is carbohydrate fermentation test. This test is done to evaluate the ability of a particular bacterial isolate in using a particular sugar for energy. The broth consists of 0.5% sugar base, 1% peptone, 0.5% yeast extract and 0.2% phenol red as pH indicator in 1liter.The carbohydrate fermentation broths were prepared and inoculated with 100 µl of bacterial suspension prepared from 18-24 hrs old bacterial culture. The inoculated broths were incubated for 24 hrs at 37°C. The fermentation causes change in colour of the medium from orange-red to yellow as production of acid lowers the pH of the broth and phenol red acquires yellow colour at acidic pH.

Catalase test

The selected isolates were screened for presence of catalase enzyme. The enzyme catalase breaks down hydrogen peroxide (H_2O_2) (v/v) to form oxygen and water. Few drops of 3% H_2O_2 were added to 18-24hrs old growth of an organism. Production of bubbles indicates presence of catalase.

Citrate Utilization test

This test determines the ability of bacteria to utilize citrate as a single carbon source. The Simmon's citrate slants were inoculated lightly and incubated at 35 to 37°C for upto 4 days. The growth of organism on slant is an indicative of its ability to utilize citrate and conversion of ammonium phosphate to ammonia results in a pH change which turns pH indicator (bromophenol blue) from green to blue.

Methyl Red-Voges Proskauer Test

The methyl red (MR) test was used to determine an organism ability to produce both strong acid from glucose and to maintain a low pH after prolonged (48-72 hrs) incubation.MR-VP medium was inoculated with the isolates and incubated for at least 48hrs. After incubation 5 drops of 0.02% methyl red reagent was added to 1ml aliquot of test broth. Production of red colour in the medium indicates a positive test. Voges–Proskauer (VP) test was used to determine the ability of an organism to produce acetylmethylcarbinol (acetoin) from glucose metabolism.MR-VP broth was inoculated with test organism and incubated for 48hrs. 0.6ml of 5% α-napthol and 0.2ml of 40% Potassium hydroxide were added to a 1 ml aliquot of test broth with gentle mixing. Production of red colour in the medium indicates a positive test.

Thioglycollate broth test

This determines the growth pattern in a broth according to oxygen gradient.This is used to cultivate strict anaerobes and microaerophiles

RESULTS AND DISCUSSION

Soil is a rich source of amylase producing bacteria. Soils rich in starchy contents were collected in sterilized polypropylene bags from different areas of Patna and were stored in refrigerator at 4°C. Serial dilution and Spread plate methods were used for the preliminary isolation of bacteria and different colonies were selected on the basis of cultural characteristics. Pure cultures were obtained after many rounds of streaking. Pure cultures were stored at 4°C and were subcultured after every 15 days. The pure isolates were screened for amylase production. The screening led to the isolation of 36 amylase positive isolates of bacteria (Table 1). The most efficient isolates were selected on the basis of size of the halo zone around the streaked colony and the isolates showing the largest halo zones were selected for further studies.Out of different localities it was seen that soil sample of Kankarbagh was highly efficient in amylase producing bacteria. In present investigation 12 isolates formed halo zone of 1 cm or more and it has been observed that the size of the halo zone is generally proportional to amylase production (Jahir Alam Khan, 2011, Mohammed et al. 2011, Ghasemi et al. 2010, Sasmita Mishra, 2008, Kishore et al. 2004).

Table 1: Colony Characterization of Bacteria screened for amylase production

Colony No	Colour	Configuration	Margin	Elevation	Mucous	Size of Halo Zone[a]
BCH01	White	Round	Smooth	Convex	Dry	**0.7cm**
BCH02	White	Wrinkled	Wavy	Flat	Dry	**1.1cm**
BCH03	White	Wrinkled	Wavy	Flat	Dry	**1.0cm**
BCH04	Whitish	Wrinkled	Branching	Flat	Mucoid	**0.5cm**
BCH05	White	Wrinkled	Wavy	Raised	Dry	**1.2cm**
BCH06	Whitish	Round	Wavy	Flat	Mucoid	**0.4cm**
BCH07	Transparent	Round with raised margins	Smooth	Flat	Mucoid	**0.9cm**
BCH08	Whitish	Irregular & Spreading	Lobate	Flat	Dry	**0.7cm**
BCH09	Whitish	Round with raised margins	Smooth	Flat	Dry	**1.5cm**
BCH10	Blackish	Round	Smooth	Flat	Dry	**0.8cm**
BCH11	White	Y-shaped	Smooth	Flat	Dry	**0.9cm**
BCH12	White	Irregular	Branching	Flat	Mucoid	**1.2cm**
BCH13	Whitish	Round with raised margins	Smooth	Partially raised	Dry	**1.3cm**
BCH14	Whitish	Irregular	Ciliated	Flat	Mucoid	**0.6cm**
BCH15	Yellow	Round with radiated margin	Branching	Convex	Mucoid	**0.5cm**
BCH16	Whitish	Irregular	Wavy	Raised	Mucoid	**0.4cm**
BCH17	White	Rhizoid	Branching	Flat	Dry	**0.8cm**
BCH18	Whitish	Round	Smooth	Convex	Mucoid	**0.7cm**
BCH19	White	Irregular & spreading	Irregular	Flat	Mucoid	**1.7cm**
BCH20	Whitish	Irregular & spreading	Irregular	Flat	Mucoid	**1.1cm**
BCH21	Whitish	Round	Smooth	Raised	Mucoid	**0.2cm**
BCH22	White	Irregular & spreading	Branching	Flat	Mucoid	**0.6cm**
BCH23	Transparent	Round	Smooth	Flat	Dry	**0.8cm**
BCH24	Whitish	Round	Smooth	Flat	Dry	**0.9cm**
BCH25	White	Round	Smooth	Flat	Dry	**1.6cm**
BCH26	Whitish	Wrinkled	Branching	Flat	Mucoid	**0.9cm**
BCH28	White	Wrinkled	Wavy	Raised	Dry	**1.2cm**
BCH29	Whitish	Round	Wavy	Flat	Mucoid	**0.8cm**
BCH30	White	Round	Smooth	Convex	Dry	**0.8cm**
BCH31	Whitish	Irregular & spreading	Irregular	Flat	Mucoid	**1.1cm**
BCH32	White	Y-shaped	Smooth	Flat	Dry	**0.8cm**
BCH33	White	Irregular	Branching	Flat	Mucoid	**0.4cm**
BCH34	Whitish	Round with raised margins	Smooth	Raised	Dry	**1.3cm**
BCH35	White	Y-shaped	Smooth	Flat	Dry	**0.8cm**
BCH36	White	Y-shaped	Smooth	Flat	Dry	**0.8cm**

a= Halo zones were calculated as a mean of triplicates. After many rounds of screening,36 isolates showed positive amylase test and out of which 12 namely BCH 02, 03, 05, 09, 12, 13, 19, 20,25, 28, 31&34 formed halo zones of 1 cm or more around the streaked colonies andwere selected for further biochemical investigation.

Out of 12 isolates, 10 were Gram positive while 2 were Gram negative (Table 2). All the test isolates were catalase negative (Table 3). All the isolates were not able to ferment xylose (Table 4).BCH 03 gave positive result for MR-VP test (Table 5).BCH 02,03, 09, 25& 28 were facultative anaerobic while BCM was strictly aerobic (Table 6). All the isolates were able to utilize citrate (Table 7). Two isolates namely BCH 09 & BCH 25 showed growth at 55°C (Table 8) and were identified as *Bacillus licheniformis*. All the isolates showed good growth in presence of NaCl with BCH09&BCH25 showing growth in 2.6MNaCl nutrient broth (Table 9). This is reflective of their halophilic character and halophilic amylases are tolerant to high salt concentrations and certain type of organic solvents that would easily denature common amylases (Shafiei & Amoozeger, 2011). A number of alkalitolerant α-amylases have been isolated from bacteria primarily from *Bacillus lichenformis* and *Bacillus halodurans* (Hmidet et al. 2008, Marakami et al. 2008). BCH34 was identified as *Bacillus cereus* and thermostable, alkaline tolerant α-amylase has been previously extracted and purified from it (Annamalai et al. 2011).

Table 2: Gram Staining Test

Colony No	Result	Shape
BCH02	Positive	Bacilli
BCH03	Positive	Cocci
BCH05	Positive	Bacilli
BCH09	Positive	Cocci
BCH12	Negative	Cocci
BCH13	Positive	Bacilli
BCH19	Positive	Bacilli
BCH20	Negative	Cocci
BCH25	Positive	Bacilli
BCH28	Positive	Bacilli
BCH31	Positive	Bacilli
BCH34	Positive	Bacilli

Table 3: Catalase Test Test

Catalase	Result
BCH02	Negative
BCH03	Negative
BCH05	Negative
BCH09	Negative
BCH12	Negative
BCH13	Negative
BCH19	Negative
BCH20	Negative
BCH25	Negative
BCH28	Negative
BCH31	Negative
BCH34	Negative

Table 4: Carbohydrate Fermentation Test

Colony No	Glucose	Lactose	Maltose	Sucrose	Galactose	Xylose
BCH02	Acid	No Acid	No Acid	No Acid	No Acid	No Acid
BCH03	No Acid	No Acid	Acid	Acid	Acid	No Acid
BCH05	Acid	No Acid	No Acid	No Acid	No Acid	No Acid
BCH09	No Acid	No Acid	No Acid	No Acid	No Acid	No Acid
BCH12	No Acid	Acid	Acid	No Acid	No Acid	No Acid
BCH13	Acid	No Acid	No Acid	No Acid	No Acid	No Acid
BCH19	No Acid	Acid	Acid	No Acid	No Acid	No Acid
BCH20	Acid	Acid	Acid	No Acid	No Acid	No Acid
BCH25	Acid	No Acid	Acid	Acid	Acid	No Acid
BCH 28	Acid	No Acid	No Acid	No Acid	No Acid	No Acid
BCH31	Acid	No Acid	No Acid	No Acid	No Acid	No Acid
BCH34	Acid	Acid	No Acid	Acid	Acid	No Acid

Table 6: Thioglycollate broth Test

Colony No	Result
BCH02	Facultative Anaerobic
BCH03	Facultative Anaerobic
BCH05	Facultative Anaerobic
BCH09	Uniform growth
BCH12	Microaerophilic
BCH13	Uniform growth
BCH19	Aerobic
BCH20	Aerobic
BCH25	Facultative Anaerobic
BCH 28	Facultative Anaerobic
BCH31	Aerobic
BCH34	Aerobic

Table 5: MR-VP Test

Colony No	Result	
	MR	VP
BCH02	Negative	Negative
BCH03	Positive	Positive
BCH05	Negative	Positive
BCH09	Negative	Positive
BCH12	Negative	Negative
BCH13	Negative	Negative
BCH19	Negative	Negative
BCH20	Negative	Positive
BCH25	Negative	Negative
BCH 28	Negative	Negative
BCH31	Negative	Negative
BCH34	Negative	Positive

Table 7: Citrate Utilization Test

Colony No	Result
BCH02	Positive
BCH03	Positive
BCH05	Positive
BCH09	Positive
BCH12	Positive
BCH13	Positive
BCH19	Positive
BCH20	Positive
BCH25	Positive
BCH28	Positive
BCH31	Positive
BCH34	Positive

Table 8: Growth at 55°C

Colony No	Result
BCH02	Negative
BCH03	Positive
BCH05	Negative
BCH09	Positive
BCH12	Negative
BCH13	Negative
BCH19	Negative
BCH20	Negative
BCH25	Negative
BCH28	Negative
BCH31	Negative
BCH34	Negative

Table 9: Salt (NaCl) Tolerance Test

Colony No	6.5%	7.5%	8.5%	9.5%	10.5%	11.5%	12.5%	13.5%	14.5%	15.5%	16.5%	17.5%
BCH02	+++[a]	+++	+++	+++	+++	++	++[b]	+[c]	-[d]			
BCH03	+	+	+	+	++	+	-					
BCH05	+++	+++	+++	+++	+++	+++	++	++	+	-		
BCH09	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-
BCH12	+++	+++	+++	+++	+++	+++	++	++	+	-		
BCH13	+++	+++	+++	+++	+++	+++	+++	++	+	-		
BCH19	+++	+++	+++	+++	+++	++	++	+	-			
BCH20	+++	+++	+++	+++	+++	++	+	-				
BCH25	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-
BCH28	+++	+++	+++	+++	+++	++	+	-				
BCH31	+++	+++	+++	+++	+++	++	++	+	-			
BCH34	+++	+++	+++	+++	+++	+	+	-				

a= Good growth (O.D. between 0.9- 0.5 after 48 hrs of growth) b= Average growth (O.D. of below 0.5-0.3 after 48 hrs of growth) c= Poor growth (O.D. of below 0.3-0.1 after 48 hrs of growth) d= No Growth (O.D. of below 0.1 after 48 hrs of growth)

CONCLUSION

The present study was undertaken with aim of screening for biochemical characterization of efficient amylase producing bacteria and it was observed that soils of Patna are rich in amylase positive bacterial isolates which can with stand high salinity and hold immense promise in biotechnological industries.

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