RESEARCH PAPER

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Isolation and phenotypic screening of alpha amylase producing bacteria from soils of Jammu region

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The enzymes from microbial sources generally meet industrial demand and are cheap. Amylase is the most abundant form of storage polysaccharides is of great significant in biotechnology, in various starch processing industries. About 60 isolates were isolated from four different soil samples of Jammu region of which 40 isolates were pure. In total out of forty isolates twelve isolates gave positive test for starch iodide test one from each source.

Key words : Alpha amylase, Phenotypic characterization, Media optimization

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INTRODUCTION

Soil bacteria and fungi play pivotal roles in various biogeochemical cycles (Molin and Molin, 1997; Wall and Virginia, 1999) and are responsible for the cycling of organic compounds. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (Timonen *et al.,* 1996) and soil fertility (O'Donnell *et al.,* 2001). Microorganisms constitute a huge and almost unexplained reservoir of resources likely to provide innovative applications useful to man. They represent by far the richest repertoire of molecular and chemical diversity in nature.

The biological diversity of the Indian subcontinent is one of the richest in the world owing to its vast geographic area, varied topography and climate. Because of its richness in overall species diversity, India is recognized as one of the 12 mega diversity regions of the world (Virdi *et al.,* 2007; Manikandan *et al.,* 2008). The enzymes from microbial sources generally meet industrial and are more stable than with plant and animal amylases and obtained cheaply (Haq *et al.,* 2002). Starch, which is the substrate of amylase, is the most abundant form of storage polysaccharides is of great significant in biotechnology, in various starch processing industries.

RESEARCH METHODOLOGY

Sample collection:

Samples were collected from different environment sources. Soil samples were taken from potato field, bating method (garden soil), wheat field and maize field from different Jammu areas. These samples were brought to laboratory in plastic bags (Table 1).

Table 1: Samples and their location

Sample type	Location	Sample number
Potato field	Akhnoor	G1
Baiting method	Jammu	K1
Wheat field	Akhnoor	B1
Maize field	Akhnoor	M1

Isolation method:

Amylolytic microorganisms were enriched by inoculating 5g of soil in 250 ml of Erylenmeyer flask containing 100 ml of MEB (Mineral Enrichment Broth) medium composed of yeast extract - 0.5%, peptone- 0.5%, starch- 0.5%, KH_2PO_4- 0.5%, pH- 7.0. The flasks were incubated for 24 hrs at 37 ºC in an incubator shaker. After 24 hrs the culture broth was serially diluted up to 10^{-3} to 10^{-7}. Then 0.5 ml of the diluted suspension from each tube was transferred to MEB plates. The plates were

gently rotated clockwise and anticlockwise for uniform spreading of diluted suspension and were incubated at 37 ^{0}C for 24 hrs in an incubator. After 24 hrs, independent colonies were picked and again transferred to fresh MEB plates for getting pure cultures. Then colonies with different morphologies were streaked on plates containing MEM medium (Yeast extract- 0.5%, starch- 0.5%, peptone - 0.5%, KH_2PO_4 - 0.5%, agar - 2.0%, pH -7.0) and incubated at 37 ºC to identify their starch utilizing potential. Pure isolates were maintained on nutrient agar plates.

Phenotypic characterization:

Gram staining:

Using Gram staining kit the cells were then examined under the light microscope. A loop full of overnight culture was then suspended on the slide. Smear was prepared by spreading the drop of autoclaved water with a toothpick. After drying, the heat fixed smear was first stained with crystal violet for 1 min. The slide was rinsed under the tap water for few seconds and then was stained with safranin. It was again rinsed under tap water and dried on paper towels. The cells were then examined under the light microscope.

Catalase test:

Isolates were grown in MEM tubes for 48-72 h at 37^{0}C. Then 3.0 % hydrogen peroxide was poured onto the colonies. The presence of catalase was indicated by bubbles of free oxygen gas. The absence of bubble formation was a negative catalase test.

Citrate-agar utilization test:

This is the test for an organism's ability to use citrate as a sole carbon source and ammonium ions as the sole nitrogen source. Pure culture was allowed to grow on citrate agar plates. The plates were placed in incubator at 37 ^{0}C for 24 hours. Use of citrate increases the pH of the medium. The increase in pH then causes color change in the bromothymol blue used as an indicator, turning it blue. Change in coloration was observed.

Triple-sugar iron test:

Triple sugar iron test is the test performed to check the ability of microorganism to ferment sugars and produce hydrogen sulphide. Isolates grown on nutrient agar slants were taken. They were allowed to grow on triple-sugar iron slants at 37 ^{0}C for 24 hours placed in incubator. Following incubation the fermentive activities of the organism were noticed as:

– Alkaline slant (red) and acid butt (yellow) with or without gas production(breaks in the agar butt)indicates only glucose fermentation has occurred.

– Acid slant (yellow)and acid butt (yellow) with or without gas production indicates that lactose or/and sucrose fermentation has occurred.

– Alkaline slant (red) and alkaline butt (red) or no change (orange-red) butt indicates that no carbohydrate fermentation has occurred.

Following incubation some cultures of organisms, capable of producing H_2S showed an extensive blackening in the butt because of the precipitation of the insoluble ferrous sulphate.

Amylase activity (Starch iodine test):

This test is based on the principle that as amylase is hydrolyzed the iodine binding sites will be released, leaving products unable to form the blue complex. This complexing of iodine with amylase is presumed to be a stearic absorption. As amylase is further hydrolyzed, it will not absorb iodine. The starch is hydrolyzed from a native molecule to small fragments and the color intensity produced by the iodine reagent changes from blue, to purple, and finally to colorless (Takeshita and Hehre, 1975).

All the pure isolates were streaked on to individual MEB agar plates and were incubated at 37 ^{0}C. Then from each plate single colonies were picked up and streaked on the starch agar plates with starch as the only carbon source. The plates were incubated at 37 ^{0}C for 24-48 hrs. Then the individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5 g potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. If a strain is amylolytic then it starts hydrolyzing the starch present in the plate nearby its growth and in the zone of degradation no blue color forms, which is the basis of the detection and screening of an amylolytic strain. The zone of decolorization becomes visible within few seconds of addition of I2-KI solution and removing excess of the solution. The colonies, which were showing zone of clearance in starch agar plates, were maintained on to MEM agar plate as well as on to nutrient agar slants.

RESULTS AND ANALYSIS

Isolation was carried out using soil samples taken from different sources on MEB and MEM agar plates. Enrichment method was used and single colonies were

Table 2: List of pure isolates

Sample	Number of isolates	Isolate name	Pure isolates
Potato field	19	G1 toG19	G4, G7, G9, G12, G13, G15, G17
Baiting	19	K1 to K19	K1 to K19
Wheat field	16	B1 to B16	B3, B9, B11,B13, B14, B15, B16
Maize field	14	M1 to M14	M2, M3, M5, M8 M10, M12, M13

purified by repeated streaking and incubating at 37^0C for 24 hrs whereas, Ramesh and Lonsane (1997) suggested that the production of bacterial amylase using the SSF technique requires less fermentation time of 24-48h. In total 68 isolates were obtained. Out of which 40 isolates were pure (Table 2).

Gram staining:

After staining all the pure isolates, the cells were examined under the light microscope. Gram (+) cells were

Table 3: Reaction of forty pure isolates towards different phenotypic screening tests

Isolate	Shape	Type	Catalase test	Citrate utilization test	Triple sugar iron test			
					Glucose	Lactose	Sucrose	H_2S
K1	Rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve
K2	Coccus	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K3	Coccus	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K4	Coccus	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K5	Rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve
K6	Rod	-ve	+ve	-ve	-ve	-ve	-ve	-ve
K7	Coccus	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K8	Rod	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K9	Coccus	+ve	+ve	+ve	+ve	-ve	-ve	-ve
K10	Rod	-ve	+ve	-ve	-ve	-ve	-ve	-ve
K11	Rod	-ve	+ve	-ve	-ve	-ve	-ve	-ve
K12	Coccus	-ve	+ve	+ve	-ve	-ve	-ve	-ve
K13	Coccus	+ve	+ve	-ve	-ve	-ve	-ve	+ve
K14	Coccus	+ve	+ve	+ve	+ve	-ve	-ve	-ve
K15	Rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve
K16	Rod	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K17	Coccus	-ve	+ve	-ve	-ve	-ve	-ve	-ve
K18	Coccus	+ve	+ve	-ve	-ve	-ve	-ve	-ve
K19	Rod	+ve	-ve	+ve	-ve	-ve	-ve	-ve
M2	Coccus	+ve	+ve	+ve	+ve	-ve	-ve	-ve
M3	Coccus	-ve	+ve	+ve	-ve	-ve	-ve	-ve
M5	Rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve
M8	Rod	-ve	-ve	+ve	-ve	-ve	-ve	-ve
M10	Rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve
M 12	Rod	-ve	+ve	-ve	-ve	-ve	-ve	-ve
M13	Rod	+ve	+ve	+ve	-ve	-ve	-ve	-ve
G4	Rod	-ve	+ve	+ve	+ve	-ve	-ve	+ve
G7	Rod	-ve	-ve	+ve	-ve	-ve	-ve	-ve
G9	Coccus	-ve	+ve	-ve	+ve	-ve	-ve	+ve
G12	Rod	+ve	-ve	-ve	+ve	+ve	+ve	+ve
G13	Coccus	-ve	+ve	-ve	-ve	-ve	-ve	-ve
G15	Rod	+ve	-ve	+ve	-ve	-ve	-ve	-ve
G17	Rod	+ve	+ve	+ve	+ve	-ve	-ve	-ve
B3	Rod	+ve	+ve	+ve	-ve	-ve	-ve	-ve
B9	Rod	+ve	+ve	-ve	-ve	-ve	-ve	-ve
B11	Rod	-ve	-ve	+ve	-ve	-ve	-ve	-ve
B13	Coccus	-ve	+ve	+ve	+ve	-ve	-ve	-ve
B14	Coccus	-ve	+ve	-ve	-ve	-ve	-ve	-ve
B15	Coccus	+ve	+ve	+ve	+ve	-ve	-ve	-ve
B16	Coccus	-ve	+ve	+ve	+ve	-ve	-ve	+ve

stained purple while Gram (-) cells stained pink or red (Table 3).

Catalase test:

Out of 40 pure isolates few isolates were found to be catalase positive (G4, G9, G13, G17, B3, B9, B13, B14, B15, B16, M2, M3, M5, M10, M12, M13) whereas samples collected from baiting method (K1 to K19) all were positive for catalase activity (Table 3).

Citrate-agar utilization test:

The isolates which showed positive test for citrate-agar utilization test were G4, G7 G15,G17, M2, M3, M5, M10, M12, M13, B3, B11,B13, B15, B16, K1, K5, K9, K12, K14, K15 (Table 3).

Triple-sugar iron test:

The isolates found to be positive for triple-sugar iron test were G4, G9, G17, B13, B15, B16, K1, K5, K9, K13, K14, M2, M6, M8, M12 (Table 3).

Starch iodine test:

Microbial alpha-amylase has most commonly been measured with an iodine reagent (Vera and Power, 1980). There were only twelve isolates out of forty pure isolates that gave a positive test (high zone of clearance) for starch iodide test. There was atleast one isolate from each source B15 (Wheat field), K9 (Baiting method), M12 (Maize field) and G17 (Potato field) that was positive for this test.

Conclusion:

Amylase producing bacteria has been isolated from different sources. The measurement of microbial alpha-amylase has most frequently been performed with starch as the substrate and iodine as the indicator. Of all the pure 40 isolates, four isolates *viz.,* B15, K9, M12 and G17 showing high zone of clearance (starch iodine test) were found to be catalase positive and were also positive for their ability to use citrate as a sole carbon source. Isolates B15 and K9 were *coccus* and Gram positive, isolate G17 was rod shaped and Gram positive whereas M12 was rod shaped and Gram negative. Amylase producing enzymes account for about 30 % of the world's enzyme production. Due to the increasing demand for these enzymes in various industries, there is enormous interest in isolating these enzymes from microbial sources.

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