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Amylase producing efficiency of *Bacillus* species isolated from Jammu soil

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Now-a-days the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms. In this study, our purpose was to isolate *Bacillus* bacteria that have the ability to secrete amylase and its production optimization with variation of pH and high temperature. Fifteen bacteria, isolated from mill disposal soil samples of Jammu were identified as *Bacillus* spp. on the basis of their growth on selective media i.e Mannitol Egg yolk agar base and biochemical tests (*viz*. positive in starch hydrolysis, gelatin liquefaction, simmon's citrate, casein hydrolysis and H_2S production test but negative in urease test). Amylase production was qualitatively detected by the disappearance of blue colour in the starch agar medium around the microbial colonies. Quantitative estimation of amylase activities were determined by DNS method at different pH and temperatures and the results revealed that highest amylase activity in *Bacillus* spp. was observed at pH 7.0 and at temperature between 40-60°C. The *Bacillus* spp. labeled as NBS-9 showed maximum amylase activity (2.851 µmole maltose/min/mg protein) among the fifteen isolates and was considered as most efficient isolate for industrial application at high temperature.

Key words: *Bacillus* spp., amylase, temperature, pH

INTRODUCTION

Bacillus species are gram positive, rod-shaped, aerobic and endospore forming in nature (Rao *et al*. 1998). Many species of this genus exhibit a wide range of physiologic abilities that allow them to live in many natural environments (Ahn *et al.* 2001). *Bacillus* spp. are known to produce versatile extracellular enzymes such as amylases which have got tremendous application in paper industries, food, sugar production etc. (Bolton *et al*. 1997). *Bacillus* species such as *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus licheniformis* are used as bacterial workhorses in industrial microbial cultivations for the production of a variety of enzymes as well as fine biochemicals for decades (Saxena *et al*. 2007). Amylases are among the most important enzymes used in biotechnology, particularly in process involving starch hydrolysis, industries such as brewing, food, paper, textile and pharmaceuticals. Alpha amylase is an extra cellular enzyme, which is used in the starch processing industry where it breaks starch into simple sugar constituents (Reddy *et al.* 2003). α-amylase hydrolyses the internal α-1, 4 linkages in starch and related substrates in an endo-fashion producing oligosaccharides including maltodextrins, maltose, and glucose (Calik and Ozdamar,2001). Amylases are the most important enzymes and account for about 30% of the world's enzyme production (Kandra 2003). Amylases can be produced from several sources such as plants, animals and microorganisms like fungi and bacteria (Ivanova *et al.* 2001), the major advantage of using microorganisms for the production of amylase is its economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane *et al*. 1990). Almost all microorganisms of the *Bacillus* genus synthesized alpha amylase. This genus has the potential to dominate the enzyme industry (Pretorius *et al*. 1986). Microorganisms are important producers of industrial enzymes, due to their diversity. Production of industrially important enzymes from

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eukaryotes has been replaced with microorganisms. The genus *Bacillus* produces a large range of industrial important extracellular enzymes (amylases and proteases) (Morgan *et al*. 1981). The production of microbial alpha amylase by bacteria dependent on the type of strain, composition of medium, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermostability. Temperature is one of the major criteria used in the selection of an industrial enzyme. Most enzymes used in industrial applications are preferred as thermostable (Godffrey and West, 1996)**.** The present study deals with the isolation of *Bacillus* spp. on the basis of biochemical tests and their amylase production potential and the effect of culture conditions *viz.* temperature and pH on its activity.

MATERIALS AND METHODS

Collection of soil samples and isolation of bacteria

From disposal sites of different mills, soil samples were collected from different areas of Jammu region of Jammu and Kashmir. For the isolation of the starch degrading bacteria, serial dilutions (10^{-1} to 10^{-6}) of the soil sample were prepared using distilled water. It was then cultured on specific medium (Mannitol Egg Yolk Agar Base pH 7.2) using the spread plate technique and incubated overnight at 37°C. After incubation, colonies produced were observed and finally cultured on nutrient agar slants using the streak technique and pure cultures thus obtained were maintained and stored in refrigerator at 4°C for further use.

Plate assay

Morphological characterization

All the isolates were examined for their colony morphology i.e. shape, size, nature of colony on the basis of gram staining technique based on Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994).

Biochemical characterization

Biochemical characterization of all the isolates was done as per the procedures of Cappucinu and Sherman , (1992).

Starch hydrolysis

The test cultures were spotted on the Starch Agar plates and incubated at 28±2°C for 24 hours. After incubation, the plates were flooded with 1% iodine solution. A colourless halo around the growth and blue color in the rest of the plate showed utilization of starch by the microorganism.

Urease test

The overnight cultures were inoculated to the test tubes containing sterilized Urease Broth and incubated for 24-48 hours at 28±2°C. After incubation, the development of pink colour was taken as positive for the test.

Gelatin hydrolysis test

The test cultures were inoculated to the pre-sterilized Nutrient gelatin deep tubes and incubated at 28±2°C for 24 hours. After incubation tubes were kept in a refrigerator at 4°C for 20 mins. The tubes that remained liquefied were taken as positive for the test and the tubes that solidify on refrigerator were taken as negative for the test.

Simmons citrate test

The overnight cultures were streaked on Simmons citrate agar slants and incubated for 24-48 hours at 28±2°C. Some organisms required up to 7 days of incubation due to limited rate of growth on citrate medium. The development of intense Prussian blue were taken as positive for the test.

Casein hydrolysis test

The overnight cultures of the test isolates were spotted on skimmed milk agar plates and incubated at 28±2°C for 24- 48 hours. The production of holo zone around the colony was taken as positive for the test.

Hydrogen sulfide (H_2S) gas production test

The overnight cultures of the test isolates were streaked on triple sugar iron agar slants and incubated at 28±2°C for 24-48 hours. The change of red colour to yellow colour of the slant was taken as positive for the test.

Estimation of crude extracellular amylase activity

The isolates was propagated in Tendler's Non-Synthetic Broth Medium supplemented with 1% (w/v) starch medium and incubated in a shaker (120

rpm,) at 37°C for 48 hrs. After incubation, resultant broth was centrifuged at 10000 rpm for 10 min. and the supernatant was collected as the source of crude enzyme. Amylase activity was determined by measuring the reducing sugar formed by the enzymatic hydrolysis of soluble starch. 1 ml of 1% (w/v) soluble starch was taken in a test tube and 10 ml of 0.1M phosphate buffer (pH 7.0) was added to it. 50 µl of the crude enzyme was added to the test tube containing reaction mixture and incubated at 55°C for 10 min. The reaction was stopped by adding 3 ml DNS reagent. The reaction mixture was heated for 10-15 min. in boiling water bath and absorbance was read at 540 nm to estimate reducing sugars released (Miller,1959). Unit of enzyme activity was defined as the number of µmole of reducing sugar (maltose) produced in 1 minute under the assay condition.

Protein content assay

The protein content of samples was measured by the Bradford method (Bradford, 1976).

Optimization of cultural conditions

Effect of high temperature on amylase activity of isolates of Bacillus spp.

Effect of temperature on amylase production activity was assessed by determining enzyme activity of the isolates of *Bacillus* spp. grown at different temperatures following above mentioned Miller's method. 1 ml of 1% (w/v) soluble starch was taken in a test tube and 10 ml of 0.1M phosphate buffer (pH 7.0) was added to it. 50 µl of the crude enzyme was added to the test tube containing reaction mixture and incubated at different temperatures *viz*. room temperature 30°C, 40°C, 50°C and 60°C for 10 min. The reaction was stopped by adding 3 ml DNS reagent. The reaction mixture was heated for 10-15 min. in boiling water bath and absorbance was read at 540 nm to estimate reducing sugars released. One unit of enzyme activity was defined as the amount of enzyme that liberated 1µM of reducing sugar as maltose equivalents in 1 minute under the assay condition.

Effect of pH on amylase activity of isolates of Bacillus spp.

Amylase activity was also determined from isolates of *Bacillus* spp. grown at different pH following DNS method. The reaction mixture containing 1 ml of 1% (w/v) soluble starch in 10 ml of 0.1M phosphate buffer of different pH *viz*. 5.8, 6.2, 6.6, 7.0, 7.4 separately and 50 µl of the crude enzyme was incubated at 55°C for 10 minutes. The reaction was stopped by adding 3 ml DNS reagent. The reaction mixture was heated for 10 min. in boiling water bath and absorbance was read at 540 nm to estimate reducing sugars released. One unit of enzyme activity was defined as the amount of enzyme that liberated 1µM of reducing sugar as maltose equivalents in 1 min. under the assay condition.

RESULTS AND DISCUSSION

In this present investigation, fifteen isolations were made from soil samples on the basis of their growth on specific solid medium (MYP Agar Base) and single colonies were picked up and streaked on the Nutrient Agar slants. These isolates were characterized by Gram staining, selective medium and biochemical tests. The bacterial isolates showing superior amylase production gave the following preliminary characterization: gram-positive spore forming bacilli, approximately one micron in length, Agar colonies were small, smooth and round, or intermediate in size, flat, rough, and irregular, or sometimes rather large, spreading, and mucoid. Provisionally, they all appear to fit into the *Bacillus* spp. group. Bacterial isolates were subjected to different biochemical tests to verify and characterize the isolates. All the isolates showed positive tests for starch hydrolysis, casein hydrolysis, Simmon's citrate , Gelatin hydrolysis and H_2S-gas production tests , but were negative for urease reaction with varying intensities (Table 1). The characterization process was done followed by the recently used methods by other researchers (Niazi *et al*. 2010; Kaur *et al.* 2012).

Amylase activity was preliminarily checked by plating the *Bacillus* spp. on the Starch agar medium. The medium was flooded using iodine indicator, clear zone around the colony indicated the presence of amylase activity. Members of the genus *Bacillus* are heterogeneous and they are very versatile in their adaptability to the environment. There are various factors that influence the nature of their metabolic processes and enzymes production (Kaur *et al.* 2012). A great deal of attention is being given to thermophilic and extremely thermophilic microorganisms and their enzymes (Kunal *et al*. 2011). *Bacillus* species produce a large variety of extra cellular enzymes,

Table 1: Biochemical characterization of isolated *Bacillus* spp.

Isolates	Starch hydrolysis	Casein hydrolysis	Simmons's Citrate test	Urease test	Gelatin hydrolysis test	H_2S production
NBS-1	+++	++	+ve	-ve	+ve	+ve
NBS-2	+++	+++	+ve	-ve	+ve	+ve
NBS-3	+++	+++	+ve	-ve	+ve	+ve
NBS-4	+++	++	+ve	-ve	+ve	+ve
NBS-5	++	++	+ve	-ve	+ve	+ve
NBS-6	+	+++	+ve	-ve	+ve	+ve
NBS-7	++	+	+ve	-ve	+ve	+ve
NBS-8	+	+++	+ve	-ve	+ve	+ve
NBS-9	+++	++	+ve	-ve	+ve	+ve
NBS-10	+	++	+ve	-ve	+ve	+ve
NBS-11	++	++	+ve	-ve	+ve	+ve
NBS-12	+++	++	+ve	-ve	+ve	+ve
NBS-13	+	++	+ve	-ve	+ve	+ve
NBS-14	+++	++	+ve	-ve	+ve	+ve
NBS-15	+++	++	+ve	-ve	+ve	+ve

+= low positive reaction , ++ = moderate positive reaction, +++ = highest positive reaction, -ve = negative reaction, +ve = positive reaction

Table 2: Amylase production efficiencies of isolates of Bacillus spp. at ambient temperature (30°C) and pH(7.0)

Isolates	Protein content (mg)	µ mole of maltose per min i.e 1 U	Specific activity i.e 1 U/mg of protein
NBS-1	0.0156	0.00534	0.342^{abc}
NBS-2	0.0226	0.00547	0.242^{bc}
NBS-3	0.0227	0.0108	0.475^{abc}
NBS-4	0.0246	0.0187	0.760^{bc}
NBS-5	0.0218	0.0142	0.651^{bc}
NBS-6	0.017	0.0078	0.458^{abc}
NBS-7	0.0218	0.0176	0.807^{c}
NBS-8	0.0248	0.0076	0.306^{ab}
NBS-9	0.027	0.077	2.851^{e}
NBS-10	0.0261	0.0047	0.180^{a}
NBS-11	0.0243	0.0185	0.761^{bc}
NBS-12	0.0246	0.0116	0.477^{abc}
NBS-13	0.0208	0.031	1.490^{d}
NBS-14	0.0223	0.0357	1.600^{d}
NBS-15	0.0252	0.0376	1.492^{d}

Values with same letter within the column were not significantly different at á=0.05 by Tukey's test.Results are the mean of three replication for each treatment

such as amylases, which have significant industrial importance (Oyeleke *et al*. 2010). In the same vein, bacterial enzymes are known to posses more thermostability than fungal amylases (Daniel *et al*. 2010). The amylase activities of all fifteen isolates of *Bacillus* spp. were assayed from starch degrading abilities and were found statistically varied among the isolates. The data presented in Table 2 revealed that the specific activities of the isolates ranged from 0.180 to 2.851 µmole maltose /min/mg protein at ambient temperature and pH (at 30°C and pH 7.0); in which isolate labeled with NBS-9 showed highest amylase activity (2.851 µmole/ min/mg protein) followed by NBS-13, NBS-14, NBS-15 with amylase activities 1.490, 1.600, 1.492 µmole/min/mg protein at pH 7 and temperature 30°C. Kunal *et al*. (2011) also reported amylase activity of *Bacillus* isolates in the range of 2.33 and 2.00 IU.

In this present research, the effect of temperature on amylase production by *Bacillus* spp. using starch as substrate was carried out at various temperature *viz*., 30°, 40°, 50° and 60°C. The effect of high temperature (30°C, 40°C, 50°C & 60°C) on amylase activity of *Bacillus* isolates at pH 7.0 revealed that the isolates NBS-1, NBS-4, NBS-6, NBS-7, NBS-8, NBS-10, NBS-11 showed maximum amylase activity at temp 30°C, while the isolates NBS-2 ,NBS-14 at 40°C and NBS-3, NBS-5, NBS-9, NBS-12, NBS-15 at 50°C respectively. However, only one isolate NS-13 (0.556 µmole maltose/min/

Table 3: Effect of high temperature on amylase production efficiency of isolates of *Bacillus* spp.

Isolates	Specific activity (µmole/min/mg of protein) at different temperature (^{o}C)			
	30	40	50	60
NBS-1	0.208^{d}	0.069^{b}	0.040^{a}	0.021^{a}
NBS-2	0.413^{i}	0.685^{o}	0.663^{l}	0.502^{j}
NBS-3	0.463^{l}	0.553^{k}	0.685^{m}	0.647^{l}
NBS-4	0.390^{h}	0.203^{f}	0.186^{d}	0.176^{f}
NBS-5	0.539^{m}	0.605^{m}	0.597^{j}	0.143^{e}
NBS-6	0.456^{k}	0.294^{h}	0.237^{e}	0.218^{h}
NBS-7	0.225^{e}	0.132^{d}	0.103^{c}	0.035^{b}
NBS-8	0.131^{b}	0.089^{c}	0.083^{b}	0.038^{b}
NBS-9	0.569^{n}	0.573^{l}	0.654^{k}	0.643^{l}
NBS-10	0.193^{c}	0.161^{e}	0.102^{c}	0.095^{d}
NBS-11	0.467^{l}	0.460^{j}	0.391^{g}	0.364^{i}
NBS-12	0.011^{a}	0.035^{a}	0.100^{c}	0.054^{c}
NBS-13	0.269^{g}	0.345^{i}	0.403^{h}	0.556^{k}
NBS-14	0.449^{j}	0.666^{n}	0.527^{i}	0.192^{g}
NBS-15	0.235^{f}	0.245^{g}	0.309^{f}	0.174^{a}

Values with same letter within the column were not significantly different at á=0.05 by Tukey's test.Results are the mean of three replication for each treatment

mg protein) recorded highest value at 60^{o}C (Table 3). Besides, isolates NBS-3, NBS- 9 and NBS-2 also maintained high value of amylase production up to 60^{o}C. Oyeleke *et al*. (2010) also reported the effect of temperature on activity of amylase produced by *B. megaterium*. The amylase activity in *B. megaterium* was found to rise due to rise of temperature from 30^{o}C to 40^{o}C but followed by a sharp decrease in at 50^{o}C. Senthilkumar *et al.* (2012) studied that amylase production by *Bacillus* spp. was maximum at 60^{o}C and minimum at 30°C.

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. In this study, out of 15 isolates, most of the isolates showed optimal amylase production at pH 7.0 with highest value 1.437 µmole/min/mg protein (Table 4). However, pH 5.8-7.4 also supported amylase production. Similar results were reported earlier by number of researchers. Maximum amylase production was observed at pH range between 6 and 8 (maximum amylase yield 1.67 mg/ml/min) in different isolates of *Bacillus* species; but in most of cases amylase

Table 4 : Effect of different pH on amylase production abilities of isolates of *Bacillus* spp.

Isolates	Specific activity (µ mole/min/mg of protein) at different pH				
	5.8	6.2	6.6	7.0	7.4
NBS-1	0.056^{a}	0.342^{ab}	0.422^{a}	0.123^{ab}	0.066^{a}
NBS-2	0.069^{a}	0.123^{ab}	0.188^{a}	0.232^{abc}	0.018^{a}
NBS-3	0.421^{a}	0.413^{ab}	0.218^{a}	0.211^{abc}	0.130^{a}
NBS-4	0.110^{a}	0.332^{ab}	0.449^{a}	1.004^{ef}	0.154^{a}
NBS-5	0.235^{a}	0.488^{ab}	0.568^{a}	0.637^{bcde}	0.384^{a}
NBS-6	0.102^{a}	0.194^{a}	0.257^{a}	0.314^{abc}	0.031^{a}
NBS-7	0.238^{a}	0.564^{ab}	0.596^{a}	0.931^{def}	0.191^{a}
NBS-8	0.380^{a}	0.065^{a}	0.062^{a}	0.056^{a}	0.046^{a}
NBS-9	0.044^{a}	0.138^{a}	0.261^{a}	0.575^{abcde}	0.225^{a}
NBS-10	0.031^{a}	0.068^{a}	0.143^{a}	0.111^{ab}	0.087^{a}
NBS-11	0.679^{a}	0.748^{ab}	0.790^{a}	0.962^{def}	0.242^{a}
NBS-12	0.149^{a}	0.305^{ab}	0.707^{a}	0.723^{cde}	0.418^{a}
NBS-13	0.220^{a}	0.725^{ab}	0.846^{a}	1.437^{f}	0.716^{a}
NBS-14	0.054^{a}	0.964^{b}	0.986^{a}	1.071^{ef}	0.116^{a}
NBS-15	0.054^{a}	0.223^{a}	0.357^{a}	0.453^{abcd}	0.440^{a}

Values with same letter within the column were not significantly different at á=0.05 by Tukey's test.Results are the mean of three replication for each treatment

activity was found at pH 7 (Oyeleke and Oduwole, 2009; Daniel *et al*. 2010). In another study, optimal pH for amylase production *in Bacillus* species recorded at pH 8.0 (Olajuyigbe and Ajele, 2005).

Thus, *Bacillus* spp. are rod-shaped, gram positive aerobic bacteria and are famous for production of extracellular amylases having great industrial importance. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable. Amylases are examples of hydrolases and function in the hydrolysis of molecules and are most important enzymes used in biotechnology (Burhan *et al*. 2003). Their use includes hydrolysis of starch to yield glucose syrup, amylase-rich flour and in the formation of dextrin during baking in food industries. Furthermore, in

the textile industry, amylases are used for removal of starch sizing and as additives in detergents (Grass *et al.* 2004).

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