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ISOLATION AND CHARACTERIZATION OF BACTERIAL STRAIN PRODUCING THERMOSTABLE α- AMYLASE

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ABSTRACT

The use of thermostable enzymes in industrial applications has been increasing rapidly due to the fact that they are more active and stable at higher temperatures, and have a longer shelf life. The present study was aimed at isolating and identifying thermostable α-amylase producing bacteria. A total of fourteen bacterial strains were isolated, of which three were found to be potent amylase producers and showed maximum enzyme activity. The three isolates were identified as *Bacillus species*. The cultures were optimized for maximum enzyme activity on different parameters such as pH and temperature. All three isolates showed maximum growth and produced maximum amount of enzyme after 48 hours of incubation at pH 8.0 in a medium containing nutrient agar and 1% starch. Further, it was found that B7 was stable up to temperature of 80ºC with optimum activity at 60°C. B11 and B13 showed maximum activity at 40°C but were found to be stable up to 70°C.

Keywords: Activity, α-Amylase, *Bacillus species*, optimization, thermostable.

INTRODUCTION

The role of enzymes in many processes has been known for a long time. With better knowledge and purification of enzymes, the number of applications has increased, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermophiles are the organisms which are adapted to live at high temperatures.[1] Thermophilic bacteria are widely distributed in nature.[2] The most important characteristic of thermophilic organisms is their ability to produce thermostable enzymes with a higher operational stability and a longer shelf-life.[3] Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms.[4] The enzymes from thermophiles find a number of commercial applications because of their thermostability and thermoactivity. Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes.[5] One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Other advantages of carrying out industrial processes at elevated temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and favourable equilibrium displacement in endothermic reactions.[6, 7, 8] The starch industry is one of the largest users of enzymes for the hydrolysis and modification of useful raw material.[9] The starch polymer, like other polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases. Thermostable α-

amylases have had extensive commercial applications in starch processing, brewing and sugar production.[10] Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery; therefore, there has been a need and continual search for more thermophiles and thermostable α-amylases.[11] The isolation of some thermophiles within our indigenous environment will be of benefit to different biotechnological processes. The aim of the present study was to isolate and identify a bacterial strain, which can produce thermostable α-amylase and to optimize culture conditions for maximum enzyme activity.

MATERIALS AND METHODS

Sampling: All lab ware and sampling apparatus were pre-soaked in distilled water for a day prior to experiment. Six soil samples were collected from kitchen waste, bakery waste and flour mill waste, Dehra Dun, using sterile plastic bags. All the soil samples were from the surface (0-15 cm depth). These plastic bags were maintained at 4^{o}C to ensure minimal biological activity. To provide homogenized soil samples the soil was thoroughly mixed.

Isolation of bacteria: Soil suspension was prepared by mixing 5gm of soil sample in 45ml distilled water. It was then stirred and allowed to settle. This allowed the microorganisms to come in water phase. The suspension was then serially diluted from 10^{-1} to 10^{-5}. Undiluted suspension and dilutions were used for isolation of microorganisms. Undiluted suspension and dilutions, each 100µl were spread on the surface of nutrient agar plates and incubated for 24hrs at 37˚C ± 1°C. The colonies so obtained on the plates were marked and numbered. They were then streaked on their respective medium from which they were isolated. Bacterial pure cultures were maintained on NAM slants and stored at 4˚C and sub cultured every month.

Screening: Distinct bacterial morphotypes from different sources of samples were found in nutrient agar plates. The different types of colonies were picked and screened on 1% starch agar medium by incubating at 37°C ± 1°C for 48h. Several amylase producing bacterial colonies were identified after flooding the plates with Gram's iodine solution. Those colonies which gave halo zone around their growth after addition of Gram's iodine were considered as amylase producers (Amy†) and others as amylase non- producers (Amy^{-}). The diameter of halo zone so formed was measured which represented the amylolytic activity of the strains. Bacterial isolates that formed large halo zone were selected for further experiments.[12]

Characterization of bacterial isolates: Isolated pure strains were identified on the basis of morphological and physiological characteristics in nutrient agar plates, slants and broth and by biochemical tests. Colony size, Margins, Forms, Texture, Elevation and Colour was studied. Simple and Gram staining was carried out. Catalase test, citrate utilization test, indole production test, methyl red-Voges Proskeur (MR-VP) test, Nitrate Reduction test, H_2S production test, oxidase test and urease tests were carried out for the identification.

Enzyme assay

Plate assay: The plate assay was performed using agar plates amended with starch. The agar plates were prepared with 1% starch and 1.5% agar. After agar solidification, around 10mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with the culture filtrate and incubated at 37°C for 48hrs. 1% of iodine solution was over layered on the agar and the observation was made to see the hydrolytic zone around the well. The negative control was maintained by adding sterile water in the separate well.

Chemical assay: Amylase activity was determined by DNS (3,5-dinitro salicylic acid) method using starch as a substrate. Crude culture filtrate was used as enzyme sample. About 0.5 ml of culture filtrate was boiled in a water bath (100ºC) for 20 minutes and then cooled suddenly in an ice bath for 5 minutes in order to kill the enzyme. Both killed and active samples were taken for the assay. 1% starch substrate was prepared freshly in 0.1M phosphate buffer (pH 6.0). The reaction mixture containing 500µl of substrate (starch) and 500µl of enzyme solution was incubated at 37°C for 15 minutes for enzymatic reaction. After incubation, 1ml of DNS (1%) was added and heated for 15 minutes in a boiling water to obtain a coloured reaction mixture. Absorbency of the solution was measured at 540nm using UV–VIS

spectrophotometer (Beckman DU 40). Killed enzyme mixture served as a blank. 1 unit (U) is the amount of enzyme that catalyses the reaction of 1µmol of substrate per minute.

Parameter optimization

Optimization of process parameters was conducted to evaluate the effect of an individual parameter on enzyme activity at a time and incorporate it as a standard before optimizing the next parameter.

Effect of incubation period on amylase activity: Effect of incubation period on enzyme activity was studied by incubating the cultures for 96 hours at 42^{o}C. Samples were taken at regular time intervals and amylase activity was measured by the chemical assay method.

Effect of pH on amylase activity: pH in the range of 4.0–10.0 were examined for their effect on amylase production by the selected isolate grown in production media. The flasks were incubated at 42ºC for 48 hours. Samples were taken at regular time intervals for amylase activity.

Thermostability of α amylase: The effect of temperature was evaluated by incubating the reaction mixtures at different temperatures (40ºC-100ºC) for 48 hours. Samples were taken at regular time intervals and analysed for amylase activity.

RESULT & DISCUSSION

Isolation of bacterial strains: A total of 14 bacterial strains were isolated from six soil samples collected from kitchen waste, bakery waste and flour mill waste at Dehra Dun. The samples were serially diluted and spread on Nutrient agar plates. Isolates which showed different morphology on plates were purified and preserved as shown in figure 1.

Screening: Plate assay method was performed to screen for bacterial strains capable of degrading starch. Out of 14 isolates three i.e B7, B11 and B13 were observed to show maximum degradation of starch and clear zone formation was studied. Therefore, these three isolates were selected for further experiments. The results obtained are shown in figure 2, 3 and 4.

Identification of the bacterial isolates: Isolated pure strains were identified by Bergey's Manual of Determinative Bacteriology, on the basis of morphological and physiological characteristics. The results obtained are shown in table 1.

Optimization of culture conditions

Maximum α-amylase production was achieved by optimizing various parameters as pH, temperature and incubation period. Thermostability of the enzyme was checked.

Effect of incubation period on α amylase activity: The enzyme activity of B7, B11 and B13 was studied by measuring the optical density at 540nm after 4, 24, 32, 40, 48, 56, 64, 72, 80, 88 and 96 hours of incubation and the results were as shown in table 2 and figure 5.

Effect of pH on α amylase activity after 48 hours: Isolates B7, B11 and B13 achieved maximum α-amylase production at pH 8.0. pH 4.0 to 10.0 supported α-amylase production. However, considerable amount of activity was obtained at alkaline pH showing the wide application nature of enzyme. The result obtained are shown in table 3 and figure 6. Similar results were shown by, [13, 14] who reported that α-amylases were stable at pH ranges from 4 to 11.

Effect of temperature on α amylase activity after 48 hours: The organisms were grown at different temperatures ranging from 40ºC to 100ºC. The results showed that the optimum temperature for maximum yield of α-amylase for B7 was 60^{o}C. The enzyme was active up to a temperature of 80^{o}C. For B11 and B13 the optimum temperature for maximum yield of α-amylase was observed to be 40^{o}C. The result obtained are shown in table 4 and figure 7. The effect of temperature on α-amylase action has previously been reported to be 65°C at low substrate concentration and 75°C at high substrate concentration, respectively and the optimum temperature was recorded at 50°C-60°C. [15, 16]

CONCLUSION

A total of 14 bacterial strains (B1 to B14) which produced clear halos in the starch-nutrient agar medium were isolated. Among these, 3 i.e B7, B11 and B13 were found to be potent amylase producers and were identified as *Bacillus sp*. The cultures was optimized on different parameters such as pH and

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temperature. All 3 isolates showed maximum growth and produced maximum amount of enzyme after 48 hours of incubation at pH 8.0. Further, it was found that B7 was stable up to a temperature of 80ºC with optimum activity at 60ºC. Experiments can further be done to purify the secreted amylase and studies can be performed to enhance the application of enzyme to commercial level.

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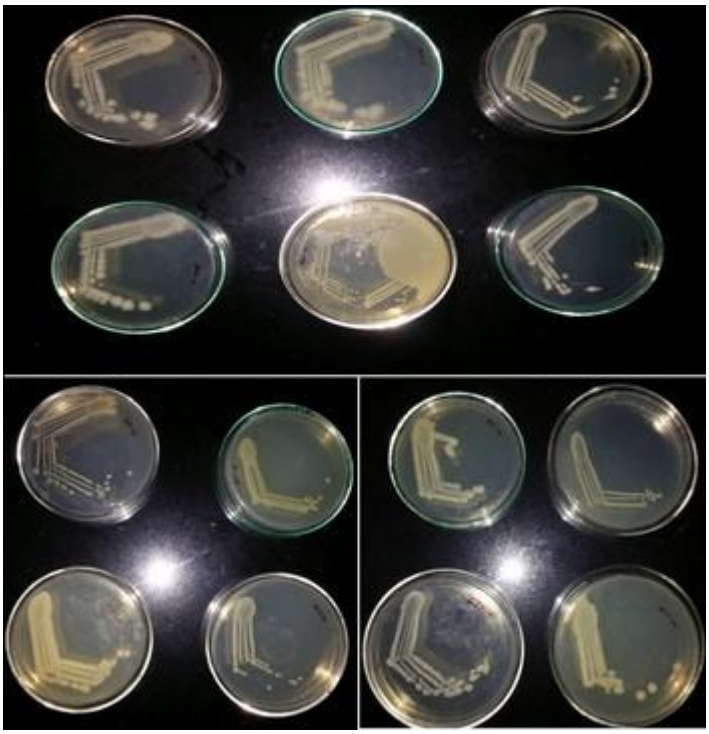

Figure 1: Master plate of bacterial isolates

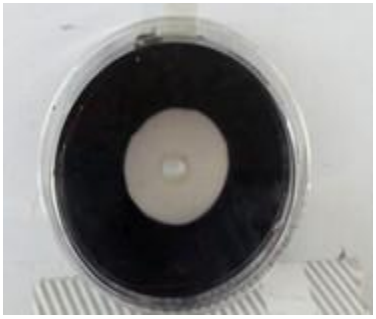

Figure 2: Zone formed by B7 isolate

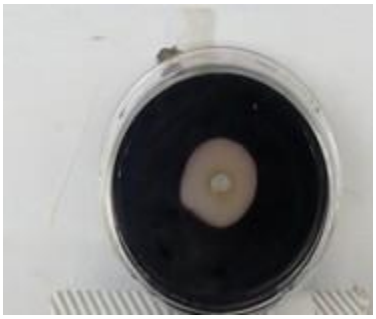

Figure 3: Zone formed by B11 isolate

Figure 4: Zone formed by B13 isolate

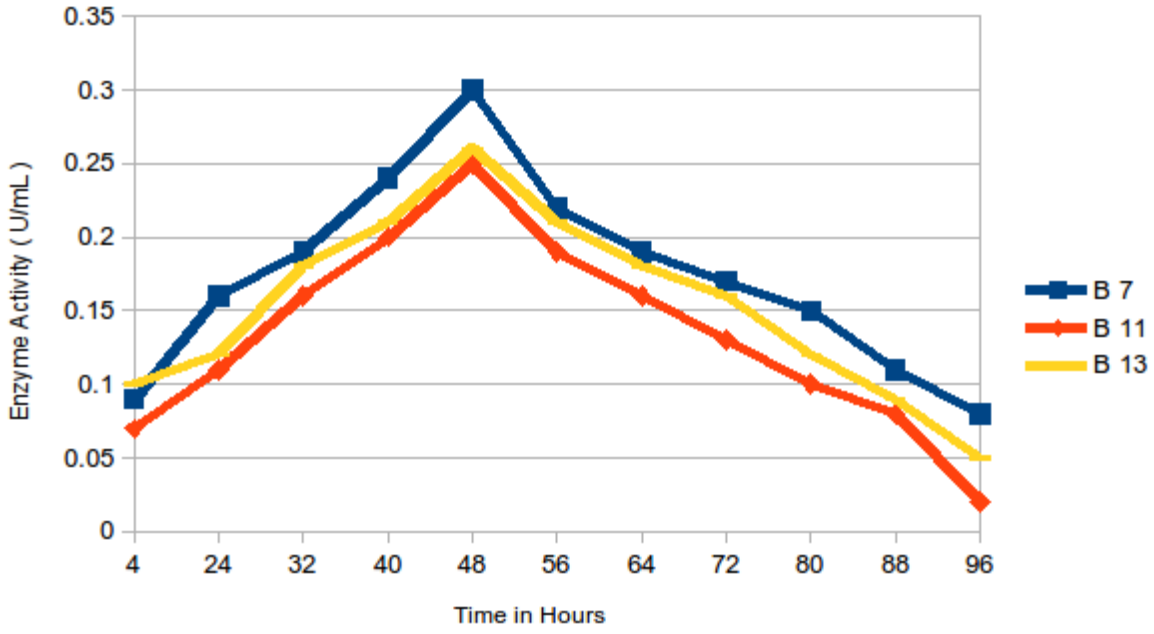

Figure 5: Effect of incubation period on α amylase activity

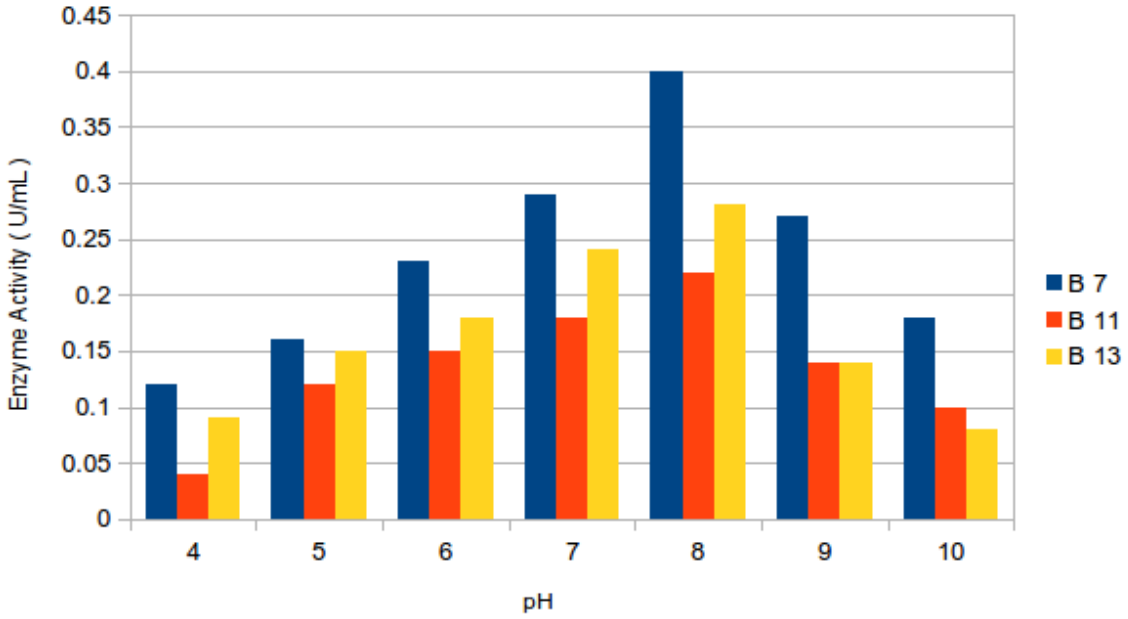

Figure 6: Effect of pH on α amylase activity after 48 hours

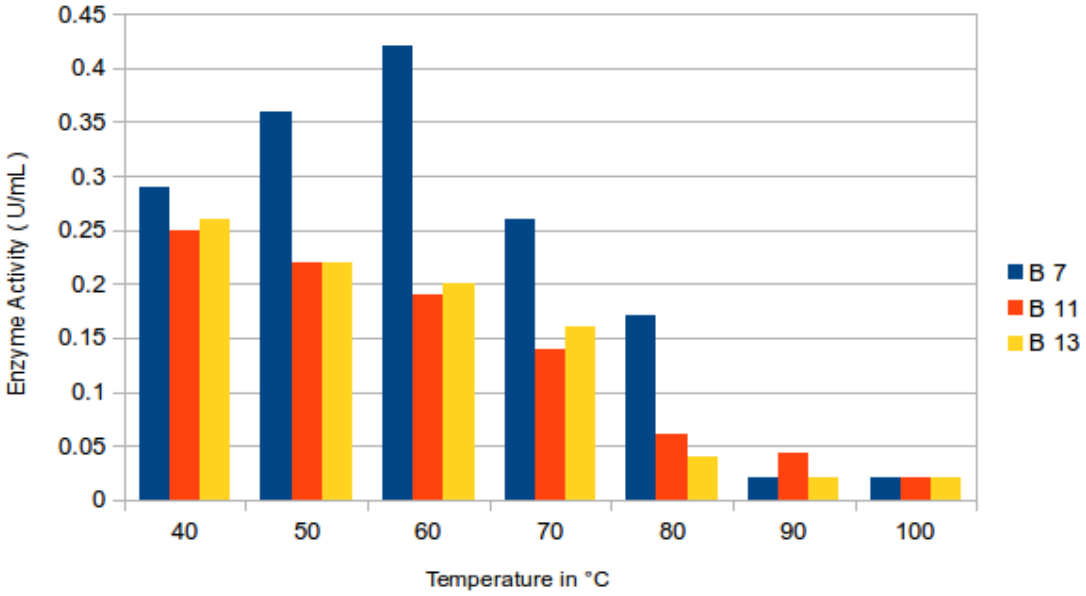

Figure 7: Effect of temperature on α amylase activity after 48 hours

Table 1[1]: Morphological and physiological characteristics of the bacterial isolates

Characteristics	***Bacillus sp.* (B7)**	***Bacillus sp.* (B11)**	***Bacillus Sp.* (B13)**
Gram Reaction	+	+	+
Morphology	Rod	Rod	Rod
Arrangement	Chains	Pairs	Chains
Motility	+	+	+
Oxidase	[+]	-	-
Indole	-	-	-
MR	-	-	-
VP	+	+	+
Citrate	+	[+]	[+]
Urease	[-]	-	[-]
H_2S	-	-	-
Nitrate Reduction	+	+	+
Catalase	+	+	+
Starch	[+]	+	+
Glucose	+	+	+
Lactose	-	[-]	d
Sucrose	+	+	+

Table 2: Effect of incubation period on α amylase activity

pH	**B7 Enzyme activity (U/ml)**	**B11 Enzyme activity (U/ml)**	**B13 Enzyme activity (U/ml)**
4	0.09	0.07	0.10
24	0.16	0.11	0.12
32	0.19	0.16	0.18
40	0.24	0.20	0.21
48	0.30	0.25	0.26
56	0.22	0.19	0.21
64	0.19	0.16	0.18
72	0.17	0.13	0.16
80	0.15	0.10	0.12
88	0.11	0.08	0.09
96	0.08	0.02	0.05

1 + 90% or greater positive; - 90% or greater negative; [+] 76-89% positive; [-] 76-89% negative.

Table 3: Effect of pH on α amylase activity after 48 hours

pH	B7 Enzyme activity (U/ml)	B11 Enzyme activity (U/ml)	B13 Enzyme activity (U/ml)
4	0.12	0.04	0.09
5	0.16	0.12	0.15
6	0.23	0.15	0.18
7	0.29	0.18	0.24
8	0.40	0.22	0.28
9	0.27	0.14	0.14
10	0.18	0.10	0.08

Table 4: Effect of temperature on α amylase activity after 48 hours

Temperature (^{0}C)	B7 Enzyme activity (U/ml)	B11 Enzyme activity (U/ml)	B13 Enzyme activity (U/ml)
40	0.29	0.25	0.26
50	0.36	0.22	0.22
60	0.42	0.19	0.20
70	0.26	0.14	0.16
80	0.17	0.06	0.04
90	0.02	0.044	0.02
100	0.02	0.02	0.02

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