

Thermophilic Actinomycetes from Hot Water Spring Capable of Producing Enzymes of Industrial Importance

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Abstract: *With the increasing demand for industrial enzymes with novel properties, the production of enzymes tolerant to extreme conditions has also increased. Due to their unique macromolecular properties, thermophiles possess high metabolic rates, physically and chemically stable enzymes and higher end product yields than similar mesophilic species. Actinomycetes are bacteria which require minimal nutritional requirement for their growth and hence are preferred over thermophilic bacteria as source of industrial enzymes. In this study, thermophilic actinomycetes were isolated from hot water springs situated at Vajreshwari near Mumbai, Maharashtra and screened for the production of cellulase and amylase. Five isolates of thermophilic actinomycetes obtained, tested positive for both amylolytic and cellulolytic activity. Two out of the five isolates were chosen for large scale production of the enzyme. The enzyme activity of the crude extract was determined using DNSA assay for reducing sugar. The optimum conditions for activity of amylase from isolate no. 4 were 55°C at pH 4.8 and for cellulase were 55°C at pH 9. Amylase and cellulase with high stability and good activity at elevated temperature and acidic /alkaline conditions may find commercial applications in various fields such as starch processing, food industries and detergents industries.*

Keywords: *Thermophile, Actinomycetes, Cellulase, Amylase, Hot springs*

1. INTRODUCTION

Extremophiles have provided an interesting and challenging platform for researchers since the time of their discovery. Besides growth under the extreme conditions, their ability to produce industrially valuable compounds (such as enzymes, antibiotics etc.) has fascinated researchers. Among the various classes of microorganisms, thermophilic bacteria and actinomycetes are the organisms which produce variety of such compounds. In addition, cultivation of thermophiles at high temperature is technically and economically beneficial as it reduces risk of contamination and viscosity which leads to high degree of substrate solubility [1].

Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Due to ever increasing industrial growth, the demand for thermostable enzymes such as proteases, amylases, cellulases, lipases, xylanases and DNA polymerases has also increased [2]. Microbes are important sources of enzymes and are preferred over sources of plant and animal origin. More than 50% of the enzymes used in industrial processes are obtained from bacteria. [1]. Amylase is an important enzyme used in industries and accounts for 25% of the enzyme market. Thermostable α -amylase have extensive commercial application in starch processing (stability at low pH), brewing and sugar production, desizing in textile industries and in detergent manufacturing processes (stability at high pH) [3,4]. Amylase with application in these industrial processes is characterized by being stable at 60-70°C and pH variation. Therefore, amylase extracted from thermophilic organisms could be useful for these applications.

Cellulose, the most abundant organic source of feed, fuel and chemicals, consists of glucose units linked by β -1, 4-glycosidic bonds in a linear mode. In the current industrial processes, cellulolytic enzymes are employed in detergents, for color brightening and softening, stoning of jeans, pre-treatment of cellulosic biomass and industrial wastes [5,6]. In order to attack the native water insoluble cellulose, thermostable cellulases active at high temperature and alkaline pH would be desired.

Actinomycetes consist of gram positive bacteria that show filamentous and branching growth pattern. They constitute a significant component of the microbial population in most soils and produce

extracellular enzymes which can decompose various materials. Enzymes of Actinomyces are more attractive than enzymes from other sources because of their high stability and unusual substrate specificity. A wide array of enzymes and their products applied in biotechnological industries and biomedical fields has been reported from various genera of actinomycetes. In recent years, thermophilic actinomycetes have been subjected to intensive investigations in search for producers of new thermostable enzymes with activity at high temperature (50-65°C) [7]. The constant search for novel enzymes with robust characteristics or applications in industrial processes, are key to profit growth [1]. To get enzymes with unique properties, screening programs for choosing rare actinomycetes are being adopted.

Actinomycetes found in extreme habitats have attracted considerable research interest due to their ability to produce novel products with huge commercial potential. However, many of the rare genera of actinomycetes have not been explored as prospective enzymes producer. Studies on unique ecological environments could yield enzymes that could be of great commercial importance in near future. In the present scenario, we focused attention on isolating thermophilic actinomycetes from natural habitat (hot spring), and then selected the best of isolates for the production of cellulase and amylase. We have also defined the optimum conditions for the activity of extracted enzymes through variations of pH and temperature.

2. MATERIALS AND METHODS

2.1. Collection and Processing of Sample from Hot Spring

The Hot-springs were situated at Akoli, Vajreshwari (near Mumbai) in Maharashtra state from India. The temperature of the water in the springs ranged from 45°C to 62°C with pH of 7 to 8. Following samples from four locations, (mainly from the area not tampered by human activities) were collected-

- i. Water from Hot springs: 100 ml in a sterile thermos.
- ii. Scrapings of the inner wall of vents: 10g in sterile petriplates.
- iii. Bottom sediment: 10 g in sterile flasks.

Soil and sediment samples were kept at 55°C for 10 minutes [8]. The pretreated sample was used for the isolation of actinomycetes.

2.2. Enrichment and Isolation of Actinomycetes

Suspension of soil sample was made by mixing 1g with 9 ml saline and the soil was allowed to settle. The supernatant was collected and 1ml was inoculated in 10ml Norrinson and Ribbon broth and incubated at 45°C for 24 hr. A loopful of the enriched sample from the broth was isolated Kenknight and Munaier's Agar plate (pH 8). Water sample collected was directly inoculated on the Kenknight agar plate by spreading 0.1ml of each sample. These were then incubated at 45°C for a one week [9]. Colony characteristic of all Isolates were studied. Isolated colonies showing good growth were purified by repeated isolation on Kenknight and Munaier's Agar plates. Microscopic examination of the isolates was carried out using slide culture technique.

2.3. Screening for Enzyme Activity

Pure Isolates showing very good growth rate were screened for enzyme activity.

To detect amylase producers: Sterile 1% Starch agar plate was divided into four parts and four isolates were spot inoculated on each plate. The plates were then incubated for 72 hours at 45°C. After incubation, the plates flooded with Gram's iodine solution. Amylase production was detected as zone of clearance on bluish purple background [10, 11].

To detect cellulase producer: 0.1 ml saline suspension of colony was added to 5ml Mac-beth's broth for enrichment of the Isolates. After incubation for five days at 45°C, loopful of the broth was spot inoculated on cellulose agar plate and incubated for 72 hours at 45°C. Cellulase Production was detected as zone of clearance after flooding the plates with solution 0.1% Congo red + iodine (1:1) solution [12, 13].

2.4. Enzyme Production

The isolates showing larger zone of clearance on both the plates were preserved on slants (4 °C) and used for further experiments. Suspension of each Isolates was separately inoculated in 1% starch broth (amylase production) and 0.5 % Carboxyl Methyl Cellulose broth (cellulase production) to get O.D 0.05 at 550nm. The organisms were grown on orbital shaker (250 rpm/min) at 45°C for 3 days in an incubator [14].

2.5. Extraction of Amylase/ Cellulase

Cells were removed by centrifugation at 3000 rpm for 20 minutes. This crude (supernatant) enzyme was further used for assay.

2.6. Enzyme Assay Protocol for Estimating Enzyme Activity

2.6.1. Assay for Amylase

The assay system had 2 ml of 1% starch with 1ml of phosphate buffer, 1 ml of 0.5% NaCl and 0.1ml of enzyme, which was incubated at 45°C for 20mins and 0.6ml of it was transferred to tubes containing 0.9ml of DNSA reagent [15]. The tube was then heated for 10mins in water bath at 100°C. They were cooled and 6ml of distilled water was added to it. Absorbance was measured at 530nm on photoelectric colorimeter. This assay was done in duplicate for crude enzyme from each isolate.

Preparing standards:

The standards graph of quantitative estimation of maltose by DNSA method (0.2 to 2 mg/ml of Maltose) was prepared for determining the amount of product formed due to activity of amylase [16].

2.6.2. Assay for Cellulase

The assay system was prepared in the same manner as mentioned above for amylase except that 0.5 ml of 2% CM cellulose in 0.05M sodium citrate buffer (pH 4.8) was used as substrate with 0.5mL of enzyme and incubation was done for 1hour at 45°C [17].

Standard graph of quantitative estimation of glucose by DNSA method (0.1 to 1 mg/ml) was prepared for determining the amount of product formed due to activity of cellulase [16].

Enzyme activity was calculated in terms of international units (IU) as μmol of product reducing sugars formed by 1ml of enzyme per minute [17].

2.7. Effects of pH and Temperature on Activity

2.7.1. Effect of Temperature

Optimum temperature is defined as the temperature at which the enzyme shows maximum activity. As the temperature increases the rate of reaction increases till the optimum is reached. At temperature higher than optimum, enzyme being protein gets denatured and hence there may be decrease or literally no activity. Effect of temperature was studied by performing the same assay system as mentioned above at incubation temperatures of 4°C, 28°C, 37°C, 55°C, 80 °C, and 100°C.

2.7.2. Effect of pH

Enzymes have an optimum pH at which they show maximum activity. Change in pH results in decrease in activity due to change in configuration of the active site. In our study we tested activity of amylase and cellulase extracted from the isolates at different pH using different buffers such as acetate buffer (pH 4), Sodium citrate buffer (pH 4.8), Phosphate buffer (pH 7), Tris-amine methane buffer (pH 9) and borate buffer (pH 10.2). The assay system used was same as previously mentioned but at optimum temperature of 55°C.



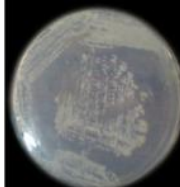


Enzyme activity at different pH and temperature was calculated in terms of amount of product formed by 1ml of enzyme per minute.

3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of Actinomyces from Hot Spring

The plates inoculated with samples from hot spring showed colonies with diverse morphologies. The colonies showing typical characteristics of actinomyces (i.e. small powdery dry, opaque) were selected and reisolated thrice on the Kenknight and Munaier's agar plates to obtain pure isolates (Table1). Twelve different types of isolates obtained, were maintained on Sterile Kenknight agar slants for further experiments. Sediment sample from hot spring, showed a good diversity of actinomycetes colonies while water sample had only two types. Several strains of actinomycetes have been previously reported from Hot-springs [18]. Thermophilic actinomycetes include the genera *Streptomyces*, *Micromonospora*, *Actinomadura*, *Saccharomonospora* and *Thermoactinomyces* [19].

Table1. Pure culture of *Actinomyces* species isolated from Hot spring

Colony Characteristics	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
On K & M Agar (Grown for 72hr At 45°C)					
Size	2mms	Pinpoint	2mm	3mms	3mm
Shape	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Pigmentation	White	Bluish Green	Light Brown	Grey	White
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Surface	Rough	Smooth	Rough	Smooth	Smooth
Consistency	Powdery	Dry	Powdery	Soft	Soft
Elevation	Flat	Flat	Flat	Centre Raised	Convex

Slide culture technique was done to observe the morphology and arrangement of the cells and spores (Figure 1c). The characteristics seemed to match the *Streptomyces* morphology, showing long mycelium with spores in different arrangements. Many plates had typical smell of wet soil, which are characteristic feature of *Streptomyces* [20]. Isolates were given numbers from 1 to 12 for easy identification. All of these isolates were screened for production of enzyme amylase and Cellulase.

3.2. Screening for Amylase Producer

On adding iodine to Starch agar plates with growth of isolates, a zone of clearance (on purple colour plate) was seen around Isolates 1,3,4,5,6,7,8 indicating that they produced amylase. Isolates 4, 7 and 8 showed bigger zone of clearance (Figure1a).

3.3. Screening for Cellulase Producer

On adding to Congo red solution to CMC agar plates with growth of isolates, a zone of clearance (on orange colour plate) was seen around isolates 2, 4,5,6,7,8,11. Since the medium contained cellulose as sole carbon source, the result of the test is a strong evident that cellulase was produced by isolates 2, 4,5,6,7,8,11. However the other isolates did not grow well. Here, again the Isolates 4, 7 and 8 showed distinct bigger zone of clearance than the rest (Figure1b).

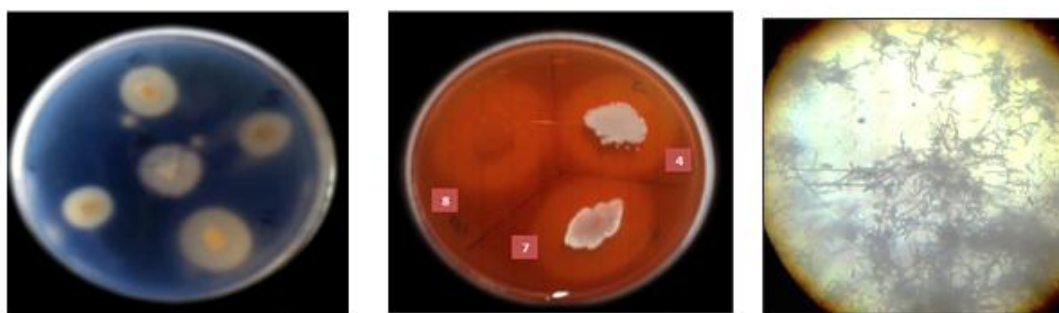


Figure1 a. Zone of starch hydrolysis produced by different isolates,
 b. Zone of cellulose hydrolysis produced by some Actinomycetes Isolates
 c. slide Culture observed under X45 showed filamentous *Streptomyces*.

All the isolates in the study were able to grow in the mesophilic range of temperatures but growth was better at higher temperature as seen by colony size.

3.4. Production of Enzyme

Results of the qualitative assay indicated that isolates 4, 5,6,7,8 produced both cellulase and amylase in detectable quantity. 5ml of the suspension (O.D₅₃₀ 0.05) of each was separately inoculated in 100 ml modified Kenknight and Munaier’s broth with starch or cellulose for large scale production of amylase and cellulase respectively. The production medium was incubated on shaker at 45°C for 3 days. Later the broth was centrifuged and the supernatant was used as crude enzyme.

3.5. Determination of Activity of Amylase and Cellulase from Actinomyces Isolate

To check the amount of enzyme produced, quantitative estimation of enzyme by colorimetric method was done. The assay was performed in triplicate and mean values are shown here for comparison. The enzyme substrate tubes in assay for both amylase and cellulase showed much higher reading than blank in DNSA assay confirming that the enzymes were active. Amylase and cellulase activity of the crude extract was determined and compared (Table 2). Since isolate 4 and 7 gave better enzyme activity for both the enzymes; they were selected for further studies. Comparison of enzyme units indicated that Isolate no. 7 produced more amylase, whereas Isolate 4 produced more amount of cellulase as compared to others.

Table2. Comparison of enzyme production by the isolated species of Actinomyces from Hot spring

Isolate number	Zone of clearance{mm}		Enzyme Activity in units/ml	
	on starch agar	on cellulose agar	Amylase	Cellulase
4	24.2	19.3	1.5	0.13
5	10.6	15.6	0.4	0.07
6	18.1	15.3	0.9	0.07
7	25.3	18.6	1.6	0.11
8	23.3	17.6	1.1	0.08

3.6. Effect of Important Parameters on the Amylase and Cellulase Activity

The parameters studied were pH and temperature. Both the enzymes from both isolates showed considerable enzyme activity at wide range of pH (from 4 to 9) and temperature (28 to 80 °C).

Amylase enzyme, from Isolate 4 showed maximum activity at pH of 4.8, and from Isolate 7 at pH of 7. Cellulase enzyme from both isolate 4 and 7 showed maximum activity at pH of 9. In assays to study effect of temperature on amylase and cellulase, maximum activity was seen at temperature of 55°C for both the isolates. Thus the optimum conditions for amylase from isolate 7 were 55 °C at pH 7 and for cellulase were 55 °C at pH 9. The optimum conditions for amylase from isolate 4 were 55 °C at pH 4.8 and for cellulase were 55 °C at pH 9 (Figure 2). These results demonstrate ability of the extracted enzymes to act under extreme environmental conditions (higher temperature and alkaline/acidic pH).

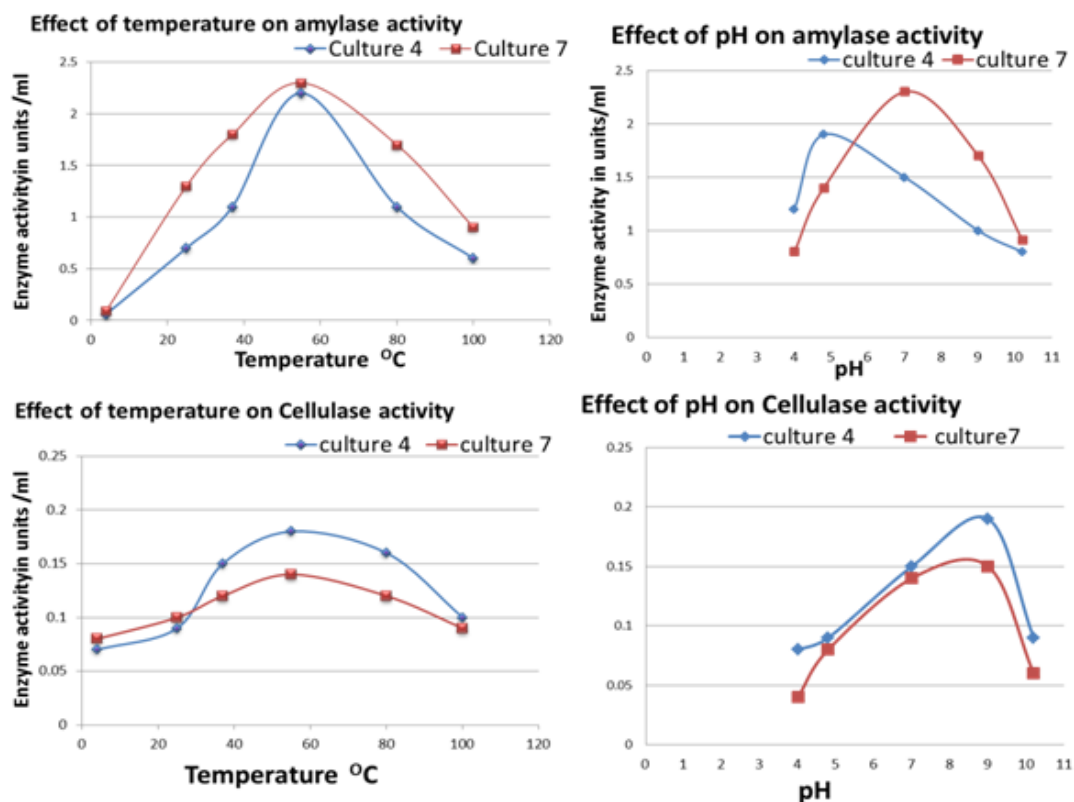


Figure2. Effect of pH and temperature on activity of amylase and cellulase from Isolate 4 and and isolate 7.

Thermophilic and acidophilic amylases from *Streptomyces* have applications in bakery, brewing, and paper industries. The amylase from *Thermobifida* sp. was used to produce maltotriose from raw sago starch [21]. In the production process of sweeteners from starch, the temperature used is 50°C or more and pH close to 7 to prevent the browning effects and to reduce the viscosity of starch pastes. Hydrolysis carried out at higher temperature also minimizes polymerization of D-glucose to isomaltose [22]. Thermostable amylases like the one extracted from Isolate7 are desired for this purpose as their use would also minimize contamination risk and reduce the reaction time. Cellulase-producing Actinomycetes have potential applications in converting cellulose into fermentable sugars fit for human consumption and in biofuel generation [1]. Cellulases from isolate 4 and 7, showing activity at high temperature and alkaline pH would be useful for this purpose.

It is evident from the results that the isolated species of *Streptomyces* were capable of producing considerable amount of both amylase and cellulase with temperature optimum at 55°C. The yield obtained can be increased by using nutrient rich medium for growth instead of a defined medium. Utilization of such enzymes is, however often limited by the cost of production. More studies need to be done to optimize the production cost of these important enzymes from actinomycetes isolates. Large scale cellulase production has been carried out on cheap substrates like rice and wheat straw [23] and fruit peels [24]. Growing the organisms on cheaper substrates or agricultural wastes will help in bringing down the cost. *Streptomyces* are now being recognized as the best known producers of array enzymes like isomerase, amylase, cellulase, lipase, protease, pectinase, keratinase, L-asparaginase, chitinases, and xylanases [25]. Several genes encoding extracellular enzymes have been cloned from *Streptomyces* species [26]. Recombinant enzymes with new characteristics and high productivity may help in lowering cost of production hence can also be the subject of future studies.

4. CONCLUSION

In the present study, we successfully isolated *Actinomyces* species from hot water spring which produce remarkable amount of thermostable amylase and cellulase. The interesting observation made in this study was the amylase exhibiting maximum activity at acidic pH and cellulases with optimum pH in the alkaline range. Amylase and Cellulase with such properties will be of great commercial importance and can be considered as probable candidates for industrial processes which use diverse range of pH and temperatures in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetic industries. Thermophilic actinomycetes play an important role in habitats where decomposition of organic matter takes place at elevated temperatures and under aerobic conditions. Using these isolates for direct hydrolysis agro-industrial waste and in biofuel generation also seems a promising strategy.

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