Production of Extracellular α-Amylase by Thermophilic Bacillus sp. Isolated from Arid and Semi-arid Region of Rajasthan, India

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ABSTRACT

Thermophiles are the organisms which are adapted to live at high temperatures. The enzymes from thermophiles find a number of commercial applications because of their thermostability and thermoactivity. One of the most attractive attributes of thermophiles is that they produce enzymes capable of catalyzing biochemical reactions at temperatures higher than those of mesophilic organisms. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial thermophilic enzymes. Hot Arid and Semi Arid region of Rajasthan could serve as a good source for new thermophilic microorganisms with novel industrially important properties. The aim of this research is the isolation and identification of industrially important extracellular α-amylase enzyme producing thermophilic bacteria from Arid and Semi arid Region of Rajasthan. In the present investigation, soil samples were collected from Jhunjhunu, Sikar and Churu districts of Rajasthan, India. Total 20 bacteria were isolated from the collected soil samples. Among all isolated bacterial population only two thermophiles were able to produce amylase enzyme by utilizing starch as carbon source and yeast extract as nitrogen source. The enzyme was estimated by qualitative and quantitative experiments. The bacterial isolates were identified as Bacillus sp. by microscopic, biochemical and molecular characteristics. The best enzyme activity was observed at pH 7, 5% salt concentration and 55°C temperature. So these bacterial strains can be potentially used in a number of industrial processes such as food (baking, brewing, dairy industries), fermentation, textile, detergent, paper industries, starch processing industries and biodegradation processes.

Key words: Thermophiles, Thermophilic enzyme, Amylase, Arid region, Semi-arid region

INTRODUCTION

Microbial species exist in many environments like extremes of temperature, pH, chemical content and pressure. This existence of microbes is due to certain genetic and/or physiological adaptations [1, 2, 3]. Thermophiles are the organisms which are adapted to grow optimally at high temperatures [4]. The enzymes from thermophiles are known as thermophilic enzymes and these enzymes find a number of commercial applications because of their thermostability and thermoactivity. One of the most attractive attributes of thermophiles is that they produce enzymes capable of catalyzing biochemical reactions at temperatures higher than those of mesophilic organisms [5, 6]. These thermostability of enzymes occur because of some changes like thermostability appears to be achieved by proteins using the same forces involved in folding acquired by small structural modifications [7], alterations in the amino acid composition of proteins bring about additional electrostatic interactions, formation of hydrogen and
Disulfide bonds, enhancement of hydrophobic interactions or compaction of the structure and there are only a few cysteine residues in thermophilic enzymes or they are completely absent etc.

Amylases are enzymes which utilize and hydrolyse starch and glycogen as substrate. On the basis how amylase break down starch molecules and produce glucose, these are classified as α- amylase (which breaks down the bonds at random manner), β- amylase (which act on the glucose-glucose bonds and remove two glucose unit at a time and produce maltose and Amyloglucosidase (AMG)). β- Amylase breaks the nonreducing end of the straight chain and produce glucose. Amylase producing industries need a temperature tolerating amylase producing bacterial strains which are able to produce thermostable amylase enzyme, because temperature and pH controls are critical during some stages of production. α-Amylase (endo 1, 4-α D glucan glucohydrolase) is an extracellular enzyme which act on substrate starch and degrade it in to disaccharide and trisaccharide [8]. Amylase can be derived from various sources such as microorganisms (bacteria and fungi), plants and animals. Microbial production of amylase is more fruitful than that of others sources like plant or animals because microbial amylase is fast, cost efficient, easy and moderate for obtaining enzymes of desired characteristics [9]. There is much significance of enzymes in atmosphere or human life, because of the interest and demand of enzymes with novel properties is quite high in several industries [10]. It leads our interest towards the discovery of various types of amylases with new or unique properties. Amylases find potential applications in a number of industrial processes such as food (baking, brewing, dairy industries), fermentation, textile, detergent and paper industries [11]. Microbial amylases have replaced the chemical hydrolysis of starch in starch processing industries. These would also be useful in the pharmaceutical and fine chemical industries [12].

Microorganisms isolated from soil sources are used for enzymes production at industrial level, although the potential for synthesis of several novel enzymes by terrestrial microorganisms have been recognized. The physical and chemical parameters like temperature, pH, incubation time duration, salt concentration etc. affect the enzyme production and enzyme activity strongly, so optimization of these parameters are very useful for good production of enzyme by microorganisms. Screening of soil microorganisms with higher amylase activity could attracts the discovery of novel amylases suitable for new biotechnological and industrial applications [13]. Many microorganisms produce amylase enzyme, most commonly used amylase producing bacteria for industrial production are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquifaciens* [14, 15]. Starch bioprocessing usually involves two steps liquefaction and saccharification, both require high temperature. α-Amylases from *Bacillus licheniformis* have been typically used this step cause of their thermostability [16]. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial enzymes.

In keeping with this view, hot Arid and Semi Arid region of Rajasthan could serve as a good source for new thermophilic microorganisms with novel industrially important properties, so we selected these areas for the research study.

Aim of the present research is the isolation and identification of industrially important extracellular amylase enzyme producing thermophilic bacteria from Arid and Semi arid region of Rajasthan and further optimization of physical and chemical parameters which affect the amylase enzyme production and activity.

**MATERIALS AND METHODS**

**Collection of soil sample**

Arid and Semi-arid region of Rajasthan were selected for soil sampling. Samples were collected from 3 to 4 cm depth with the help of sterile spatula and transferred in to sterile plastic bags. They were brought to the Molecular and Microbiology Research Laboratory of MITS University in aseptic conditions and stored in a refrigerator at 4°C for further analysis.

**Analysis of physico-chemical characterization of soil**

Soil samples were analyzed for physico-chemical characteristics including water holding capacity (WHC), humidity/moisture content, pH, electric conductivity (EC), total dissolve solids (TDS), specific gravity, organic carbon (OC) and chloride contents [17, 18].
Isolation of thermophilic bacterial cultures
Isolation of thermophilic soil bacteria was performed by serial dilution and spread plate method. 1g of soil sample was serially diluted in sterilized distilled water to get a concentration range from $10^{3}$ to $10^{6}$. A volume of 0.1 ml of each dilution was spread on nutrient agar plates uniformly using a glass spreader. These plates were incubated at 55°C for 24 hr. The bacterial isolates were further subcultured by streaking method on fresh basal medium (nutrient agar) to obtain pure culture. Pure bacterial isolates were maintained at 4°C in refrigerator for further processing [19].

Screening of amylase producing bacteria
Bacterial isolates were screened for amylase production by starch hydrolysis test on starch agar plate. Pure bacterial isolates were streaked on the starch agar plate and incubated at 55°C for 24 hrs. After incubation 1% iodine solution (freshly prepared) was flooded on the starch agar plate. Presence of blue color around the bacterial growth on starch agar plate indicated negative result and a clear zone around the bacterial growth indicated hydrolysis of starch (positive result) and were considered as amylase producing bacterial strains [12, 20].

Identification of amylase producing bacteria
Colony morphology
The bacterial colony morphology was recorded with respect to color, size, shape and pigmentation.

Microscopic observation
Gram staining, endospore staining, capsule staining and motility test were performed and analyzed under the microscope.

Biochemical characterization
Bacteria were biochemically characterized on the basis of Catalase test, Oxidase test, Urease test, Indole test, Methyl red test, Simmons citrate test, Starch hydrolysis test, Voges Proskauer test, H$_2$S production test, Nitrate reduction test, Gelatin hydrolysis test etc. [21].

Molecular characterization
Genomic DNA were extracted from the bacterial isolates using CTAB (Cetyltrimethylammonium Bromide) method [22] and amplified by PCR with the 16S rDNA universal primers (Forward Primer: 5’ - TGC GGC TGG ATC C C C TC C T T- 3’ , Reverse Primer: 5’- CCGGGTTTCCCCATTCGG-3’). The thermo-cycler was programmed as Table 1. The presence of PCR products was determined by electrophoresis of 10µl of the reaction product in 1% agarose gel [23].

| Table 1: Optimized PCR Thermal profile for 16S rDNA amplification |
|-----------------------------|----------------|--------|---------|
| Program No. I               | PCR Phase   | Temperature | Time | No. of cycle |
| Program No. II              | Denaturation| 94°C    | 2 minutes |
| Program No. II              | Primer annealing | 56°C | 2 minutes |
| Program No. II              | Extension   | 72°C    | 2 minutes |
| Program No. III             | Final Extension | 72°C | 9 minutes |

16S rDNA sequencing and data analysis:
Sequencing of DNA was performed by 1500 bp PCR product. For the sequence analysis ABI automated sequencer was used. The two 16S rDNA sequences were aligned and compared with other 16S rDNA genes in the GenBank by using the NCBI Basic Local Alignment Search Tools (BLAST).

Amylase production medium
For the production of amylase enzyme, production media was prepared. All chemicals were purchased by CDH and HIMEDIA. The Production media contained (g/L) 10g starch, 10g peptone, 20g yeast extract, 0.05g KH$_2$PO$_4$, 0.015g MnCl$_2$,4H$_2$O, 0.25g MgSO$_4$.7H$_2$O, 0.05g CaCl$_2$.2H$_2$O and 0.01g FeSO$_4$.7H$_2$O [16]. 20 ml of bacterial inoculums was transferred in 1000 ml of production medium containing Erlenmeyer flasks and were kept on a rotary shaker incubator at a speed of 220 rpm at 55°C for 24 hours. After incubation, fermented broth was centrifuged at 7000 rpm for 15 min in a cooling centrifuge and supernatant was collected. This supernatant was used for the estimation of amylase enzyme [24].
Optimization of Temperature, pH, Incubation period and NaCl concentration for amylase enzyme production

Effect of temperature on enzyme production and enzyme activity was studied by adjusting the incubation at different temperature (35, 40, 45, 50, 55, 60 and 65°C), different pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8.0, 8.5 and 9) and different NaCl concentrations (0.5, 1.5, 2.5, 3.5 and 4.5 %) in the production medium. Enzyme production was also studied at different incubation period (24, 48, 72, 96, 120hrs).

Assay of Amylase

1.0 ml of culture broth and 1.0 ml of substrate (starch) were mixed in each test tube. These were covered with foil paper and incubated at 35°C for 15 minutes on water bath. Then 2.0 ml dinitrosalicylic acid (DNS) reagent was added in tube to stop the reaction. After this, test tubes were kept on boiling water bath for 5 minutes. Then cooled at room temperature and the absorbance were observed at 540 nm by spectrophotometer (SYSTRONICS SPECTROPHOTOMETER 106). One international unit (IU) of amylase activity is the amount of enzyme, which liberates 1 mol of glucose per min [25].

RESULTS AND DISCUSSION

Physico-Chemical analysis of soil samples:

In the present investigation physical properties of soil from arid and semi-arid regions were analyzed. Soil sample of Churu region exhibited higher pH, total dissolved solids (TDS) and electrical conductivity (EC) among all samples. The water holding capacity (WHC) and moisture content of soil sample of Sikar region was found higher. The values of specific gravity for soils were found almost similar with a considerable difference (Table 2).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameter</th>
<th>Unit</th>
<th>Semi-arid region</th>
<th>Arid region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Churu District</td>
<td>Jhunjhunu District</td>
</tr>
<tr>
<td>1.</td>
<td>pH</td>
<td></td>
<td>8.02 ± 0.02</td>
<td>8.57 ± 0.02</td>
</tr>
<tr>
<td>2.</td>
<td>Moisture content</td>
<td>%</td>
<td>14.73 ± 0.23</td>
<td>6.5 ± 0.50</td>
</tr>
<tr>
<td>3.</td>
<td>TDS</td>
<td>ppm</td>
<td>1.12 ± 0.01</td>
<td>1.20 ± 0.01</td>
</tr>
<tr>
<td>4.</td>
<td>EC</td>
<td>dsm</td>
<td>1.04 ± 0.01</td>
<td>1.16 ± 0.01</td>
</tr>
<tr>
<td>5.</td>
<td>Specific Gravity</td>
<td>g/ml</td>
<td>1.05 ± 0.01</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>6.</td>
<td>Water Holding Capacity</td>
<td>%</td>
<td>24.25 ± 0.025</td>
<td>10.15 ± 0.2</td>
</tr>
<tr>
<td>7.</td>
<td>Chloride content</td>
<td>mg/100g</td>
<td>0.25 ± 0.20</td>
<td>0.15 ± 0.2</td>
</tr>
<tr>
<td>8.</td>
<td>Organic carbon</td>
<td>%</td>
<td>0.29 ± 0.42</td>
<td>0.19 ± 0.55</td>
</tr>
</tbody>
</table>

Isolation and Identification of amylase producing thermophilic bacteria:

Total, 20 bacterial isolates were isolated, among them only two strains (J1 and JIII) were screened as a potent degrader of starch and showing clear hydrolytic zone on starch agar plate at 55°C (Fig. 1). These two bacterial isolates were characterized on the basis of colony characteristics, microscopic appearance and biochemical tests (Table 3). Molecular characterization of these strains was done by DNA isolation (CTAB method) and 16S rDNA analysis (Fig. 2). Further these amplified 16S rDNA sequences of the bacterial strains was blasted using online tool (MEGA 4). The taxonomical identification was done by the phylogenetic tree construction and the comparison of these bacterial strain sequences with other homologous bacterial sequences. After morphological, physiological, biochemical and taxonomical identification, these two bacterial isolates were identified as *Bacillus licheniformis* (J1) and *Bacillus subtilis* (J3) bacterial strains (Fig. 3).
Figure 1: Isolation of amylase producing thermophilic bacteria at 50°C, clear zone indicates the hydrolysis of starch as a result of amylase production.

Table 3: Characterization of J1 and J3 bacterial strains

<table>
<thead>
<tr>
<th>Mode of characterization</th>
<th>Characteristics of bacterial isolates</th>
<th>J1 bacterial strain</th>
<th>J3 bacterial strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultural characteristics</td>
<td>Colony morphology on nutrient agar</td>
<td>Irregular, mucoid, creamy yellow and fast growing colonies</td>
<td>Small, round, regular, mucoid, creamy yellow and fast growing colonies</td>
</tr>
<tr>
<td>Microscopic characteristics</td>
<td>Spore staining, Gram staining and Motility</td>
<td>Spore forming and Gram positive</td>
<td>Spore forming, Gram positive and motile</td>
</tr>
<tr>
<td>Biochemical characteristics</td>
<td>Indole test</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Methyl Red test</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Voges Proskauer test</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Citrate utilization test</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Catalase test</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Oxidase test</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Urease test</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Nitrate reduction test</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td></td>
<td>Gelatin liquefaction test</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td></td>
<td>Starch hydrolysis test</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td></td>
<td>Hydrogen sulphate test</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td></td>
<td>Hydrogen peroxidase test</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Casein hydrolysis test</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Glucose fermentation</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Figure 2: 16S rDNA PCR amplification of the bacteria isolates
Effect of temperature on amylase production:
Production of amylase by the *Bacillus licheniformis* (J1) and *Bacillus subtilis* (J3) was optimum at 55°C temperature. When the temperature was increased or decreased, there was gradual decrement in enzyme activity was recorded. At 65°C temperature, the production of amylase was extremely low. J1 and J3 bacterial strain showed maximum enzymatic activity 1.44 U/ml and 1.42 U/ml respectively at 55°C (Fig. 4).
Effect of pH on amylase production:
Optimum pH for the amylase production by Bacillus licheniformis (J1) and Bacillus subtilis (J3) was found 7. As pH was increased or decreased from 7, there was gradual decrease in growth of the organisms. These organisms did not grow in production medium below pH 5 and above pH 10. J1 bacterial strain showed 2.16 U/ml and J3 bacterial strain showed 1.99 U/ml maximum enzymatic activity at 7 pH (Fig 5).

Effect of incubation period on amylase production:
Maximum production of amylase enzyme by Bacillus licheniformis (J1) and Bacillus subtilis (J3) was found at 24 hrs at 55°C, further there was gradual decrement in growth of the organism and enzyme production. The maximum activity of amylase was recorded at 24 hrs incubation period. The growth rate was observed at 120 hrs (5th day) very low. These organisms did not show growth and enzyme production after 120 hrs. 1.5 U/ml and 1.3 U/ml maximum enzymatic activities were observed by J1 and J3 bacterial strains respectively at 24 hrs incubation period (Fig. 6).
Effect of NaCl concentration on amylase activity:
Maximum enzyme activity was recorded at 5% NaCl concentration. As the salt concentration was increased or decreased, gradual decrement was observed in enzyme production, enzyme activity and growth of organisms. 1.8 U/ml and 1.6 U/ml enzymatic activities were recorded for J1 and J3 bacterial strain respectively at 5 % salt concentration (Fig. 7).

These results were similar to the Ashwini et al., [26]. They have achieved maximum α-amylase production by Bacillus sp. marini at 40°C and also optimized the physical conditions (pH, temperature and NaCl concentration), carbon source, nitrogen source etc. Zhou Hong-bo et al., [26] studied about moderate thermophilic bacterial strains for amylase production and concluded by their experiments that moderate thermophilic bacteria sustain higher enzymatic production at high temperature than mesophilic bacteria. Mohapatra et al., [13] studied about industrial production of amylase enzyme, they isolated total 56 bacterial strain from marine sample, out of them 46% bacterial strains was able to produce amylase enzyme. Strains showed high activity (> 0.5 IU/ml) of the amylase enzyme were recorded in bacterial strains belonging to the genera Alcaligenes and Bacillus sp. Through this research study we analyzed the α-amylase enzyme production by thermophilic Bacillus sp. at high temperature and optimized the conditions (pH, temperature, NaCl concentration and incubation period) for amylase enzyme production. On the other hand we also compared production of α-amylase enzyme by two thermophilic Bacillus sp. These isolated
bacterial strains produced highest α-amylose amount at 55°C which is not possible for mesophilic bacterial species. B. licheniformis and B. subtilis produced higher amount of amylase per liter of culture broth after optimization of cultural conditions. This amount of amylase was higher than before optimization. We have also found this fact that at 55°C temperature B. licheniformis produced high amount of amylase then B. subtilis.

CONCLUSION

Amylase enzyme is one of the most useful enzyme known and having great significance. It is already known that amylase having approximately 25% of enzyme market. These enzymes find potential applications in food, pharmaceutical and fine chemical industries. Biological production of amylase from B. licheniformis and B. subtilis microorganisms is very useful because it is economical, less time consuming and convenient to manage. This study reports biological production of thermostable α-amylose by soil bacteria B. licheniformis and B. subtilis isolated from arid and semi arid region of Rajasthan. Enzyme production was find maximum in presence of 5% NaCl concentration, temperature 55°C and pH 7.0. At the optimum conditions B. licheniformis and B. subtilis produced higher amount of amylase per liter of culture broth after optimization of cultural conditions. This amount of amylase was higher than before optimization. After compare to all studies and research we are able to conclude that moderate thermophilic amylase producing bacterial strains or thermophilic amylase producing bacterial strains contain thermostable amylase which degrade starch substrate and shows catalytic activity at high temperature.

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