Partial characterization and optimization of extracellular thermostable Ca\(^{2+}\) inhibited \(\alpha\)-amylase production by *Streptomyces erumpens* MTCC 7317

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Culture parameters for optimum production of extracellular \(\alpha\)-amylase by moderately thermostable *Streptomyces erumpens* MTCC 7317 isolated from a brick-kiln soil were found to be pH (6.0), temperature (50°C) and incubation period (36 h). Enzyme production was more in soluble starch (1%), beef extract (1%) and glycerol (0.02%) as compared to other carbon (1%: wheat flour, cassava starch, potato starch, cassava flour, etc.), nitrogen (1%: yeast extract, peptone, casein, ammonium chloride, etc.) and surfactant [0.02%: Cween (Tween) 20, Cween 40, Cween 60, Cween 80, sodium lauryl sulphate (SLS) and triton x 100] sources tested, respectively. Glycerol concentration (0.02 %) was most stimulatory in enzyme production. Addition of Ca\(^{2+}\) (even at 10 mM) inhibited \(\alpha\)-amylase production. *S. erumpens* cells immobilized on sodium alginate beads produced more (9.0-23.4%) amylase as compared with free cells. Partially purified enzyme (by ammonium sulphate precipitation) had a molecular mass of 54,500 Da in native SDS-PAGE.

**Keywords:** Actinomycete, \(\alpha\)-Amylase, *Streptomyces erumpens* MTCC 7317, Thermostable

**Introduction**

Thermostable enzymes\(^1\text{-}^3\) are most widely exploited and are of considerable commercial interest in starch processing\(^4\text{-}^5\), leather, food, paper, textile, detergents, drugs and toxic waste removal industries\(^4\text{-}^6\). Among starch hydrolyzing enzymes, thermostable \(\alpha\)-amylase (EC 3.2.1.1) is of utmost significance\(^4\text{-}^6\). Most thermostable amylases are of bacterial origin\(^7\), especially from *Bacillus* spp. (*B. licheniformis*, *B. amyloliquefaciens*, *B. amyllovorans*, etc.). Among actinomycetes, about three-fourths of *Streptomyces* spp. produce antibiotics\(^8\), and also produce several extracellular enzymes such as chitinase, lipase and \(\beta\)-xylosidase\(^9\text{-}^{11}\).

This study presents production and characterization of thermostable Ca\(^{2+}\) independent \(\alpha\)–amylase by *S. erumpens* MTCC 7317 isolated from a brick kiln soil.

**Material and Methods**

**Isolation and Identification of Thermostable Microorganism Producing \(\alpha\)-Amylase**

Soil samples from brick-kilns (1000°C) were collected from outskirts of Bhubaneswar, Orissa, India. Soil samples (1 g each) were serially diluted with sterile distilled water to 10\(^{-6}\) dilution and one ml of soil suspension (10\(^{-4}\text{-}10^6\)) was pour-plated on Medium A\(^12\) (peptone 2.0, yeast extract 1.0, NaCl 1.0 and agar 2.0%; pH 7.0) and incubated at 50°C for 48 h. Three replicates were maintained for each dilution. Colonies (140) that appeared on agar surface were considered as thermostable microorganisms, which were inoculated on starch containing Medium B\(^12\) (soluble starch 1.0, yeast extract 0.2, peptone 0.5, MgSO\(_4\) 0.05, NaCl 0.05, CaCl\(_2\) 0.015 and agar 2.0%; pH 7.0) at 50°C for 48 h. After incubation, iodine solution (KI/1 0.1 N) was spread on agar surface (culture) plate (containing Medium B) to isolate amylase producers by measuring halo-zones\(^12\). Strain producing higher amount of amylase halo-zones (>3.6 mm) than others was selected and maintained on agar slants made up of Medium B at 4°C. Selected isolate was identified as *S. erumpens* Calot & Cercos (Institute of Microbial Technology, Chandigarh, India) based on microscopic and biochemical tests and was coded as MTCC 7317. It was further confirmed by 16S rRNA sequence analysis.

**Effect of Various Parameters on \(\alpha\)–Amylase Production**

In all experiments, three replicates were maintained and expressed as mean of three replicates.

**Incubation Period**

Inoculum (1x10\(^{6}\) CFU/ ml) was prepared in soluble starch-peptone broth (soluble starch 1.0, peptone 0.5, peptone 0.5,
yeast extract 0.25, K$_2$HPO$_4$ 0.2 and MgSO$_4$ 0.1%; pH 7.0) by transferring a loop full of organism from a fresh culture and incubating at 50°C and 120 rpm for 24 h in an orbital incubator shaker (Remi Pvt Ltd, Bombay, India).

Amylase production was carried in a starch–beef extract (SB) liquid medium (soluble starch 1.0, beef extract 1.0, yeast extract 0.2 and MgSO$_4$ 0.01%; pH 7.0). Sterile SB medium (50 ml) taken in an Erlenmeyer flask (250 ml) was incubated with inoculum (2%) and agitated at 120 rpm for 60 h in an incubator shaker at 50°C. At an interval of 12 h, growth of microorganism was monitored by recording absorbancy [optical density (OD)] at 600 nm. Then culture broth was centrifuged at 8000 rpm in a refrigerated centrifuge (Model C-24, Remi Pvt Ltd, Bombay, India) for 20 min at 4°C. The clear cell free supernatant was used for amylase assay.

**pH**

SB medium of different pH (4.0-9.0) was incubated at 50°C for 36 h at 120 rpm in an incubator shaker. pH stability of amylase was also studied after incubating enzyme at various pHs of assay buffer (4.0-9.0) for 10 min. pH were measured with a pH meter (Systronics, Ahamadabad, India) using glass electrode. pHs (4.0-6.0) were maintained with acetate buffer (0.2 M) while higher pH (6.0-9.0) were achieved with phosphate buffer (0.1 M).

**Temperature**

Culture of *S. erumpens* was incubated at 30-90°C in SB medium for 36 h in an incubator shaker at 120 rpm. After 36 h of incubation, cell free supernatants were prepared and analyzed for amylase activity. Temperature stability of enzyme was determined by incubating cell free supernatants between 40-90°C for 30 min, using 0.1 M phosphate buffer (pH 6.0).

**Carbon and Nitrogen Sources, and Surfactants**

*S. erumpens* was grown in several variants of SB medium prepared by: (i) substituting starch with various carbon sources (1%); (ii) replacing beef extract with different organic and inorganic nitrogen sources (1%); and (iii) supplementing with different surfactants (0.02%). Medium was incubated at 50°C for 36 h at 120 rpm. After 36 h of incubation, cell free supernatants were used for amylase assay. In continuation of this experiment, concentrations of soluble starch (1-5%), beef or yeast extract (0.25-2.00%) and glycerol (0.01-0.05%) were studied for amylase production.

**Ca$^{2+}$ Ions**

Ca$^{2+}$ (10-40 mM) was introduced into production medium (SB) at 50°C and incubated for 36 h in an incubator shaker at 120 rpm. Amylase was assayed in cell free supernatant.

**Cell Immobilization**

*S. erumpens* cell suspension (1 x 10$^8$ CFU/ ml) was added to 4% (w/w) sodium alginate solutions (1:1 vol) and mixed thoroughly. Cell alginate mixture was then casted into beads by dropping from a hypodermic syringe into 0.1 M CaCl$_2$ solution. These beads (diam, 3.0 mm) were hardened by keeping in dilute (0.1 M) CaCl$_2$ solution for 24 h and washed with sterile distilled water. For high cell density, cells were grown on beads for 24 h before using for enzyme production. Gel beads containing immobilized *S. erumpens* cells were immersed in soluble starch-beef extract broth for 24 h at 50°C and cell population in immobilization increased from 10$^4$ to 10$^8$ cells/ g gel.

**TLC (Thin Layer Chromatography)**

The end products liberated by the action of amylase on starch were identified by spotting starch digest and standard sugars (glucose and maltose) on a silica gel plate activated at 80°C for 30 min. The plates were developed in butanol: ethanol: water (50:30:20) and dried overnight at room temperature (32 ± 2°C). Individual sugars were visualized by spraying with acetone-silver nitrate solution (0.1 ml saturated solution of AgNO$_3$ in 20 ml of acetone).

**Amylase Assay**

Reaction mixture consisted of 0.2 ml enzyme (cell free supernatant), 0.25 ml of 0.1% starch solution and 0.5 ml of phosphate buffer (0.1 M, pH 6.0) incubated at 50°C for 10 min. The reaction was stopped by adding 0.25 ml of 0.1 N HCl and colour was developed by adding 0.25 ml of I/KI solution (2% KI in 0.2% I). Optical density of blue colour solution was determined using a UV-Vis Spectrophotometer (Cecil Instrument, UK) at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that causes 0.01% reduction of blue colour intensity of starch iodine solution at 50°C in one minute per ml$^{13}$. 

**Partial Purification of Enzyme**

α-Amylase was partially purified by ammonium sulphate fractionation followed by dialysis and gel filtration
A total of 100 ml of culture filtrate was centrifuged at 8000 rpm for 20 min at 4°C to remove cells. Supernatant was brought to 50% ammonium sulphate saturation at 4°C in an ice bath. Precipitated protein was collected by centrifugation at 8000 rpm at 4°C and dissolved in a minimum volume of phosphate buffer (0.1M; pH 6.0). Enzyme solution was dialyzed at 4°C against the same buffer for 24 h at 4°C with continuous stirring and three changes of same buffer.

DEAE cellulose-ion exchange column was pre-equilibrated with same buffer. Dialysate was concentrated through a rotary evaporator at 50°C and applied to DEAE cellulose column (flow rate 0.6 ml/min) with 50 ml linear NaCl gradient (0-0.5 M). Each fraction (10 ml) was analyzed for protein concentration and α-amylase activity. Active fractions were pooled and concentrated through a rotary evaporator at 50°C. Final concentrated enzyme solution was taken for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Optimal production and stability of partially purified enzyme at various pH (4.0-9.0) and temperature (30-90°C) were studied.

**Electrophoresis and Molecular Mass Determination**

SDS-PAGE was performed with 12% polyacrylamide gel using a Mini GEL electrophoresis system (Model No 0502, Bangalore Genei Pvt Ltd, Bangalore, India). Bacterial proteins were stained with 0.2% Coomassie Brilliant Blue. Molecular mass of partially purified amylase was estimated using a standard ‘protein marker’ (PMW-M) of known molecular mass (14300-97400 Da) (Bangalore Genei Pvt Ltd, Bangalore, India).

**Results and Discussion**

Although amylase is mainly a eubacterial product but the possibility of using Streptomyces spp. for enzyme production has been recently investigated.

**Isolation of Thermostable Amylase Producing Streptomyces sp.**

Isolated strain possessed the ability to hydrolyze starch and could grow on a variety of sugars (glucose, arabinose, mannitol, meso-inositol, raffinose, rhamnose, galactose and fructose). Strain could also grow on nutrient broth at 25-60°C (optimum 50°C) and pH 5.0-9.0 (optimum 6.0).

**Effect of Various Parameters on Amylase Production**

**Incubation Time**

Highest amylase production (3700 units) was obtained at 36 h of incubation (Fig. 1). Amylase production gradually progressed with growth of *S. erumpens*; enzyme production was highest when cell entered stationary phase (36 h). Similar results were obtained for many Bacillus spp. (*B. subtilis*, *B. licheniformis* and *B. coagulans*)

**pH**

pH (6.0-6.5) for optimal (Fig. 2A) amylase production and stability were similar to that reported (6.0-7.0) for most bacteria (*B. thermooleovorans* and *B. subtilis*), yeast (*Filobasidium capsuligenum* and *Candida antarctica*) and actinomycetes (*Streptomyces megasporus*, *S. rimosus* and *Thermoactinomyces vulgaris*).

**Temperature**

Optimum α-amylase production (maximum activity, 3480 units) at 50°C (Fig. 2B) is characteristic of a moderately thermostable microorganism. Beyond 50°C, there was sudden decrease in enzyme production. Optimum α--amylase production for actinomycetes, *Thermoactinomyces vulgaris* and *S. rimosus*, were found at 62.5°C and 45°C, respectively.
Maximum production of α-amylase was achieved when soluble starch (1%) as carbon (3500± 103.4 units) and beef extract (1%) as nitrogen source (3680± 106.2 units) were used in SB (production) medium. Other carbon sources (1%) resulted in enzyme production (in units) were as follows: wheat flour, 3090± 98.0; cassava starch, 2788± 110.4; potato starch, 2690± 103.4; cassava flour, 2338± 121.3; sweet potato flour, 2155± 87.0; and sweet potato starch, 1990± 121.1. Similarly, other nitrogen sources (1%) resulted in enzyme production (in units) were as follows: yeast extract, 3547± 93.23; peptone, 3480± 65.2; casein, 3377± 121.0; ammonium chloride, 1936.5± 95.0; asparagines, 1173.5± 123.0; urea, 1396.5± 115.0; ammonium sulphate, 1396.5± 105.5; potassium nitrate, 1948.5± 121.0; ammonium molybdate, 1408.5± 103.0; and ammonium nitrate, 1173± 110.0.

Many species of *Streptomyces*\(^{18,23}\), *Bacillus* and other microorganisms\(^4,5\) gave similar results.

**Surfactants**

Surfactants often increase enzyme secretion and production\(^24\). Addition of glycerol had a marginal stimulating effect [9% more than the control (no surfactant added)] on α-amylase production whereas Cween 40 had innocuous effect and all others [(Cween 20, Cween 60, Cween 80, sodium lauryl sulphate (SLS), etc)] had inhibitory effect (Fig. 3). Glycerol (0.02%) showed highest amylase production (3680 units), whereas at 0.01, 0.03, 0.04 and 0.05% the enzyme yield was 3425, 3550, 3475 and 3400 units, respectively. Increased production of α-amylase from *Thermomyces lanuginosus* has been reported by addition of Tween 80\(^25\). It appears that same surfactant may stimulate enzyme production in one species and inhibit in another species. It is reported that growth and biochemical production of actinomycetes are stimulated by glycerol\(^8,26\), which solubilize membrane proteins that lead to an increase in cell membrane permeability, thereby enhancing secretion of biomolecules\(^27\).

**Ca\(^{2+}\) Ion Concentration**

For production and stability of α-amylase by *Bacillus* spp., addition of Ca\(^{2+}\) ion is often necessary\(^28\). However, in this study, with the increase in Ca\(^{2+}\) concentration (10-40 mM) in medium, α-amylase production was proportionally inhibited (Fig. 4). Thus, α-amylase production by *S. erumpens* was Ca\(^{2+}\) independent. Similar results were obtained for *Bacillus thermooleovorans*, *B. coagulans*, *B. licheniformis*, *Bacillus* sp. WN11, etc.\(^4,5,17,19\). There are reports that Ca\(^{2+}\) inhibit glucose isomerase\(^29\). Calcium-independent
Amylases merit consideration for starch liquefaction, especially in manufacture of fructose syrup, where calcium is a known inhibitor of glucose isomerase.

Cell Immobilization

Amylase production using immobilized *S. erumpens* cells was higher (9-23%) than free cells during 24-60 h of enzyme production (Fig. 5). Cells survived and were physiologically active on the support used for immobilization even after three cycles of operations, which could save considerable time and energy. Similar results were obtained on amylase production using *B. licheniformis*.

Partial Purification and Molecular Mass Estimation

Crude extract of α-amylase, partially purified with ammonium sulphate precipitation, contained 2.55 mg/ml protein and showed a specific activity of 1492.06 units/mg protein. After partial purification, specific activity increased to 3912.92 units/mg proteins with a yield of 20.7% and 2.6 fold purification (Table 1). Electrophoresis studies showed that molecular mass of partially purified enzyme was approx 54,500 Da.

Table 1 — Purification of α-amylase from *S. erumpens* MTCC 7317

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total volume ml</th>
<th>Total enzyme activity</th>
<th>Total protein mg</th>
<th>Yield</th>
<th>Specific activity Units/mg protein</th>
<th>Fold of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate (crude)</td>
<td>20</td>
<td>380700</td>
<td>255.2</td>
<td>100</td>
<td>1492.1</td>
<td>0</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>15</td>
<td>72390</td>
<td>24.8</td>
<td>19.0</td>
<td>2921.3</td>
<td>2.0</td>
</tr>
<tr>
<td>After dialysis</td>
<td>16</td>
<td>78884.48</td>
<td>20.2</td>
<td>20.7</td>
<td>3912.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Fig. 5 — Amylase production using normal and immobilized cell of *S. erumpens* MTCC 7317

Fig. 4 — Effect of Ca²⁺ concentration on α-amylase production by *S. erumpens* MTCC 7317
purified enzyme of *S. erumpens* showed similar pH (6.0-6.5) and temperature (50°C) optima and stability as in crude enzyme.

**Conclusions**

This study shows the production of thermostable α-amylase by free and immobilized cells of *S. erumpens* MTCC 7317 in submerged culture. α-amylase production from this actinomycete strain is significant as it is Ca²⁺ independent, which is useful in confectionary industries particularly in making of fructose syrups.

**Acknowledgement**

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**References**


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