Isolation of thermophilic *Bacillus* sp. strain EF_TYK1-5 and production of industrially important thermostable α-amylase using suspended solids for fermentation

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*Bacillus* sp. strain EF_TYK1-5 strain was isolated from hot water spring at Unkeshwar (19° 85’ N and 78° 25’ E), Nanded district, Maharashtra, India. The optimum activity of α-amylase extracted from the same was observed at 60 °C temperature. The enzyme showed maximum activity with Ca++ and Co++ at 60 °C temperature. The enzyme was stable in the pH range of 4.0-9.0 and 50-90 °C temperatures under SmF. The enzyme showed stability towards surfactant (SDS) at 5 mM concentrations. The thermostable enzyme isolated was compatible and works in the presence of detergents (*Surf excel*, *Ariel*, *Tide*, *Ghadi*, *Wheel* and *Nirma* at 1% concentration). Agricultural wastes as substrate such as rice hull, wheat bran, millet, soyabean husk, and tamarind seeds were studied for enzyme activation under suspension solid fermentation. Of these, rice hull was proved as the best substrate for α-amylase production (10 U/mg) by organism after 24 h incubation, 1,000 μm particle sizes, and 1% inoculum level (v/w), 55 °C temperatures. Optimum temperature and pH for enzyme production were 60 °C (85.56 U/mg), and 7.0 (46.71 U/mg) respectively. Additional carbon sources, 1% lactose (47.84 U/mg) enhanced α-amylase production. Among the various nitrogen sources tested, 1% beef extract (172.43 U/mg) was observed as the best nitrogen source for α-amylase production. The maximum α-amylase production was observed as 124 U/mg at 24 h, 1,000 μm particle size, and 20% inoculum level (v/w), pH 7.0, 1% lactose, 1% beef extract, temperature 65 °C. The values of $K_m$ and $V_{max}$ were 1.3699 mg/mL and 0.000074 mmol respectively.

**Keywords:** *Bacillus*, hot water spring, laundry detergents, MEGA 5, Rice hull, Unkeshwar

**Introduction**

Thermophilic microorganisms are the source of industrially important thermostable enzymes. The thermostability and compatibility of enzymes at high temperature is an interesting feature from an industrial point of view for biotechnological and agricultural industries. The successful implementation of enzymes as industrial biocatalysts requires the availability of suitable enzymes with high activity, specificity and stability under process conditions. Enzymes from thermophilic microorganisms display unique characteristics such as temperature, chemical and pH stability. Thermostable amylases, isolated mainly from thermophilic organisms, have been found a number of commercial applications because of their inherent stability. Thermostable amylases can be used in several industries such as detergent, agricultural, starch liquefaction and saccharification, textile, paper, food, baking, novel food applications and analysis in medical and clinical chemistry in which they replace chemicals or mesophilic amylases.

With reference to novel thermophilic microorganisms and their products, thermal springs offer tremendous potential; therefore we have selected hot water springs in Unkeshwar (19° 85’N and 78° 25’E), Nanded district, Maharashtra, India for investigation. The present paper describes isolation and identification of *Bacillus* sp. strain EF_TYK1-5 and production of thermostable amylase by organism. It also provides information on the effect of different parameters such as temperature; pH, substrates, particle size, inoculum size incubation time, and incubation temperature on α-amylase production.

**Materials and Methods**

Water samples were collected from two hot springs namely Mukhya kund hot spring and Surya kund hot spring located at Unkeshwar (19° 85’N and 78° 25’E), District Nanded, Maharashtra State (India). pH and temperature of samples were recorded at collection point. Water samples were analyzed for physical and chemical
properties. Starch agar (5 g peptone; 3 g beef extract; 20 g soluble starch; 25 g agar-agar; 1 L distilled water; pH 7.4) medium was used for cultivation of microorganisms. Number of colonies appeared and colony characteristics were recorded. Cultures were preserved on nutrient glycerol agar pH 7.0 in refrigerator at 4 °C. Bacterial pure cultures were obtained. Isolates were spot inoculated on same medium and incubated at 60 °C for 16 h. After incubation plates were flooded with 1 mL Gram's iodine reagent (0.01 M I2-KI). Zone of clearance around colony was observed. Of the various colonies, the one that exhibits highest degradation of starch was selected. Identification of selected isolates was carried out using biochemical methods. To confirm results, bacterial DNA was extracted using phenol chloroform extraction method. 16S rRNA gene was amplified by PCR with pair of primer (forward primer Bac 8F: 5'AGAGTTTGATCCTGGCTCAG 3' & reverse primer 1492 R: 5'GGTTACCTTGTTACGACTT 3'). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.2. Phylogenetic tree was constructed using the neighbor joining method. Bootstrap analysis (1000 replications) was applied. Nucleotide sequence of 16S rRNA genes from EF_TYK1-5 strain was submitted to NCBI database.

Amylase production
Rice hull (RH), Wheat bran (WB), Millet (M), Soyabean husk (SH) and Tamarind seeds (TS) were collected from farm yards located in Maharashtra State and Andhra Pradesh. The materials were dried and powdered. The powder of materials was used as suspended solid for amylase production. 3 gm of pre-sterilized solid material was mixed with 100 ml sterile mineral medium containing 11 g Na2HPO4·2H2O, 6.1 g NaH2PO4·2H2O, 0.3 g KCl, 0.1 g MgSO4·7H2O, pH 7.0 per 100 ml. The flasks were incubated in shaking incubator after inoculation of 1% Bacillus sp. strain EF_TYK1-5 for 24 h at 120 rpm.

Optimization of parameters
Effect of temperature, metals, pH, activators, inhibitors, and chelator at 1 mM concentration on activity thermostable amylase were determined. Effect of SDS (analytical detergent) at 1 mM and 5 mM concentration on activity thermostable amylase were studied. Effect of substrate concentration on amylase activity was studied. Particle size of substrate was optimized by observing enzyme production using different particles of 1000 μm and 1500 μm size. Effect of temperature and pH on amylase production was studied. Effect of inoculum size (10-40% by mass per volume) on enzyme production was determined and optimum inoculum size was measured. The rice hull (RH) mineral salt media supplemented with various carbon sources (such as starch, sucrose, glucose, lactose, maltose and galactose at w/v) and nitrogen sources (such as beef extract, peptone, tryptone, yeast extract, ammonium chloride, and ammonium nitrate 1% w/v) to determine their effect on production of thermostable amylase. Effect of temperature on enzyme production was determined by using varying incubation temperature. The production was carried out at temperatures 45, 55, and 65 °C. Effect of incubation time (24-120 h) on amylase production was determined and optimum incubation period was calculated.

Precipitation, partial purification and characterization of thermostable amylase
After production of the thermostable amylase, it was precipitated, partially purified and the protein content was determined. The amylase activity was determined according to the Bernfeld method. The compatibility of Bacillus sp. strain EF_TYK1-5 amylase with local laundry detergents was studied. Detergents used were Ariel™ (Procter and Gamble, India), Ghadi™ (Rohit Surfactants Pvt. Ltd., India), Surf Excel™ (Hindustan Lever Ltd., India), Wheel™ (Hindustan Lever Ltd., India), Tide™ (Procter and Gamble, India), and Nirma™ (Nirma Ltd., India). Detergents were dissolved in distilled water (1% w/v) and boiled for 1 minute to denature native enzymes present in the solution. To check the compatibility and stability of α-amylase with laundry detergents, the detergents solution was pre-incubated with amylase (1:1) for 10 minute at 60 °C, and the effect on enzyme activation was determined. The residual activity was compared with control sample incubated under same conditions (without any detergent). The enzyme activation of control sample was taken as 100%.

Enzyme kinetics
Substrate concentration is one of the most fundamental factors affecting enzyme activity. The $K_m$ and $V_{max}$ values were determined by varying the substrate concentration from 500 to 5000 μg/mL. The effect of
substrate concentration on activity is expressed in $K_m$ and $V_{max}$ values using Lineweaver-Burk plot. The data was analyzed and plot was drawn using the Graph Pad Prism 5.0 software package.

Results and discussion

Hot spring water

The temperatures of the Mukhya hot spring and Surya hot spring were 61.7 ± 0.5 °C & 48.0 ± 0.5 °C respectively. The pH of the two hot spring water was 7.3 ± 0.2. Total solids, total dissolved solids and total suspended solids were 360 mg/L, 266.7 mg/L and 49.6 mg/L respectively. Dissolved oxygen content was 8.23 mg/L. Chemical oxygen demand was 120 mg/L. Total acidity (70.0 mg/L), total alkalinity (200 mg/L), total hardness as CaCO3 (123 mg/L), calcium hardness (28.1 mg/L), magnesium hardness (12.99 mg/L), chloride (38.22 mg/L), sulphate (62.0 mg/L), sulphide (16.67 mg/L), nitrate (3.7 mg/L) and phosphate content (410 mg/L) were determined. The total bacterial count was 8.3 x $10^3$ CFU/mL.

Screening of amylase producers

Of the 51 isolates, 12 have produced thermostable amylases. One of the 12 isolates showing effective production of thermostable amylase was selected further investigation. Strain EF_TYK1-5 was identified as *Bacillus* sp. (Figure 1). Cells of strain EF_TYK1-5 are Gram-positive, catalase-positive, oxidase-negative, aerobic rods, approximately 0.5-0.75 µm wide and 1.25-1.5 µm long, occurring singly and non motile with ellipsoidal endospores. Pellicle type of growth was observed in broth medium. Colonies were rhizoidal, white, flat and transparent, having filamentous margin and 1-3 mm in diameter after 24 h of incubation on nutrient agar (HiMedia, India). Growth occurred optimally with 0.5-2.0 % (w/v) total salt and at pH 7.0 and 55 °C. Methyl red and indole production test were positive. Acid was produced from glucose, xylose, sucrose, cellobiose, sorbitol, maltose, inositol, ribose and galactose. Acid and gas was produced from fructose. The isolated species were resistant to amphotericin B (20 mcg per disc) and bacitracin (10 units per disc). Starch, gelatin is hydrolyzed. Specificity of thermostable amylase towards different substrate was determined. The most suitable substrate for isolated bacterial amylase was soluble starch.

Thermostable amylase production

The 48 h of incubation have yielded 35.76 U of thermostable amylase/mg protein. Specific activity of amylase was 132.44 U/mg.
Effect of temperature and pH on production of enzyme

Optimum temperature for α-amylase production was 60 °C (85.56 U/mg), which is comparable to that described for other Bacillus α-amylases. Optimum pH for α-amylase production was 7.0 (46.71 U/mg) and peak activity was displayed in the pH range of 6.0-8.0.

Effect of different substrates and particle size on α-amylase production

Tamarind seeds (particle size 1500 µm) have yielded 26.86 U/mg of enzyme and showed production of amylase followed by Rice hull (particle size 1500; 9.15 U/mg), millet, wheat bran and soya bean husk. 1000 µm sized particles have yielded remarkable amylase and hence used in further investigation. Availability of large surface area could be the possible reason behind enhanced productivity.

Effect of inoculum size on amylase production

There was a significant increase in α-amylase production with an increase in inoculum size up to an optimum level (20%) after which enzyme yield decreased. Maximum enzyme production was obtained of 42.83 U/mg with 20% of inoculum size.

Effect of supplementation of carbon sources and nitrogen sources on amylase production

Effective production of amylase was recorded with lactose (47.84 U/mg) and beef extract (172.43 U/mg) as carbon and nitrogen source respectively. Considerable production of thermostable amylase was recorded by supplementation of other carbon and nitrogen sources.
Enzyme Kinetics

The enzyme activation increased with an increase in starch concentration from 500 to 2000 µg/mL, but further increase of starch concentration produced no significant increase of enzyme activation. The apparent values of $K_m$ and $V_{max}$ were calculated from a Lineweaver-Burk plot, and were approximately 1.3699 mg/mL and 0.000074 mmol respectively.

Conclusion

In this study, a new *Bacillus* sp. strain EF_TYK1-5 (accession no. JN392971) was isolated from hot water spring in Unkeshwar. Based on biochemical and 16S rRNA partial gene sequence analysis, the microorganism was closely related to *Bacillus* sp. with 97-98 % similarity. The bacterium was tested for amylase production under using suspended solids. These studies also showed that rice hull (RH) could be a good substrate for α-amylase production by thermophilic *Bacillus* sp. strain EF_TYK1-5. The *Bacillus* sp. strain EF_TYK1-5 isolated in present investigation have used rice hull like material and showed 126.24 U/mL of thermostable amylase production. Considerable amount of thermostable amylase production was also recorded with other agricultural waste products (such as tamarind seeds, wheat bran, soya bean husk, and millet). It produced α-amylase that was highly stable and active at temperature 30-90 °C and at pH 4.0-9.0. The thermostable α-amylase showed excellent stability and compatibility with various commercial detergents. Considering the high activity and stability at high temperatures, stability in the presence of surfactant and stability in the presence of commercial detergents, the *Bacillus* sp. strain EF_TYK1-5 α-amylase may find application in laundry detergents and to remove starch stains from clothing.

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