

α -Amylase Assay of *Bacillus Subtilis* KC3: Isolated from *Euphorbia Hirta* Rhizosphere

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Abstract. Microbial α -amylases with extensive commercial applications in various sectors occupy a quarter of the world enzyme market. Studies were carried out with a bacterial strain producing extracellular α -amylase isolated from rhizospheric soil of *Euphorbia hirta*. The isolate was gram positive motile rod with terminal spore. It exhibited >98% similarity with the reference strains in the GenBank. The phylogenetic tree constructed on the basis of 16S rRNA gene sequences revealed that it clustered with the closest members of *Bacillus subtilis* and identified as *Bacillus subtilis* KC3. Furthermore, the effect of incubation period, temperature, pH and different carbon and nitrogen sources of the medium was optimized. The maximum enzyme production was found after 48h (22.92 U/ml) at 40°C and pH 7. The optimal temperature and pH for enzyme activity were 50°C and 6.5 respectively. Barley starch (27.27 U/ml) was observed to be the best inducer followed by corn starch (24.30 U/ml) and maltose (19.10 U/ml). The addition of glucose to the culture medium greatly reduced the synthesis of α -amylase (5.45 U/ml) which demonstrates that a classical glucose effect is operative in this organism. These properties of *Bacillus subtilis* KC3, suggest that this is a promising isolate which merits further investigations for potential application in various biotechnological processes.

Keywords: α -amylase, *Bacillus subtilis* KC3, *Euphorbia hirta*, Rhizospheric soil

1. Introduction

In recent years the potential of microorganisms as bioresources for production of industrially important α -amylases have stimulated interest in the exploration of amylolytic activity in several microbes [3, 8, 13]. Amylases constitute a group of industrial enzymes, which alone covers approximately 25% of the enzyme market for their use in many commercial biotechnological processes including renewable energy, starch degradation, detergent, foodstuff, pharmaceutical, textile and paper industries [7, 12, 13, 20,]. Amylases are produced by a variety of bacteria and fungi [2]. In fact such industrially important microorganisms found within the genus *Bacillus*, can be exploited commercially due to their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the extracellular medium and safe handling [3]. *Bacillus* is endowed to produce thermostable α -amylase and large quantities of other enzymes. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus* i.e. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* [1]. Though, the growth pattern and the enzyme profiles of different *Bacillus* species have similarities but the optimized conditions vary, depending upon the strain. Although amylases are obtained from plant and animal sources, but microbial origin fulfils industrial demand due to their high yield and thermostability [3]. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields. The objectives of the present study are to screen *Bacillus* species isolated from *Euphorbia hirta* rhizosphere in order to study their suitability with regard to α -amylase production.

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2. Material and Methods

2.1. Sampling

The samples of rhizospheric soil of rooted *Euphorbia hirta* were collected from the Research Garden, Department of Botany, Patna University, Patna (25°36'39.6"N 85°08'38.4"E) and stored for further study.

2.2. Isolation, Screening and Characterization

Bacteria were isolated by serial dilution and streak plate methods. The aliquots (0.1 ml) were plated in triplicates on nutrient agar medium [(w/v) 0.5% peptone; 0.3% beef extract; 0.5% NaCl; 1.5% agar, pH 7] and incubated at 30±2°C for 72h. The nutrient agar plates containing 1% starch (Starch Agar plates) were inoculated with test bacterial isolates and incubated at 30±2°C. The α -amylase producing bacteria were screened out by flooding the plates with Lugol's iodine solution [w/v 1% iodine in 2% potassium iodide] after 48h of incubation. The isolate showing maximum clear halo zone was designated as KC3 and further analysed. The culture was maintained on NA slants at -20°C.

Phenotypic characterization of the isolate was done by different tests referring to Bergey's Manual of Determinative Bacteriology and Agriculture handbook [14, 22]. For genotypic characterization, genomic DNA was extracted from the isolate using Chromous Genomic DNA isolation kit (RKT09). The amplification of 16S rRNA gene was carried out by using Thermal cycler (ABI 2720) in 100 μ l reaction mixture containing 2.5mM each of four dNTP, 10X PCR buffer, 3U of Taq DNA polymerase, 10 ng template DNA and 400ng each of primer (F) 5'-AGA GTR TGA TCM TYG CTW AC-3' primer (R) 5'-CGY TAM CTT WTT ACG RCT-3'. The amplification programme was set as initial denaturation at 94°C for 5min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min and a final extension at 72°C for 5 min. The sequencing was performed according to the manufacturer's protocol using Big Dye Terminator Cycle Sequencing Kit (v3.1, Applied Biosystems) and analyzed in an Applied Biosystems Analyzer. The sequence of 16S rDNA (1468 bp) was aligned by using the BLASTN program to identify the most similar sequence in the database [25] and has been submitted to the GenBank under accession number HM195191. 16S rDNA sequences of different strains of *Bacillus subtilis* and its phylogenetically related species and genera were downloaded from GenBank database (<http://www.ncbi.nlm.nih.gov/entrez>) and aligned to construct a neighbour-joining phylogenetic tree using Clustal W algorithm with the help of MEGA software version 4.1 [15].

2.3. Inoculation and Optimization of Production Medium

Erlenmeyer flasks (250 ml) containing 60 ml of amylase producing broth medium [(w/v) 0.6% peptone; 0.05% MgSO₄; 0.05% KCl; 1% starch; pH 7] were inoculated with 1% overnight (24h) grown culture (approximately 2x10⁶ CFU/ml) and incubated at 30±2°C in triplicates. At constant intervals (24h), the samples were harvested and the growth was determined by measuring the absorbance at 600 nm. The enzyme was extracted from the stationary phase culture. Cells were removed by centrifugation (8,000 rpm for 20 min) and supernatants were used for enzyme assay [11]. α -amylase was assayed by adding 1 ml of enzyme to 1 ml soluble starch (1%) in 0.1M phosphate buffer (pH 6.5) and incubated at 50°C for 10 min. The reaction was stopped by adding 3 ml of 3, 5-dinitrosalicylic acid reagent [11]. Following the color development, the absorbance was measured at 575 nm using double beam UV/VIS spectrophotometer (Systronics, 119). One enzyme unit (unit/ml) is defined as the amount of enzyme which releases 1 μ mole glucose. The effects of incubation period, pH, temperature, carbon (barley starch, corn starch, wheat starch, maltose and glucose) and nitrogen sources (peptone, tryptone, yeast extract and sodium nitrate) each at 1% concentration (w/v) were investigated on amylase production.

2.4. Effect of pH and Temperature on Enzyme Activity

The pH optimum of the enzyme was assayed by varying the pH (5.5 to 9.0) at intervals of 0.5 units of the reaction mixture by using 0.1 M phosphate buffer. The temperature optimum of the enzyme was evaluated by measuring the α -amylase activity at different temperatures (30-70°C) in 0.1 M sodium phosphate buffer at optimum pH.

2.5. Statistical Analyses

Data were statistically analysed using Statistics Package for the Social Sciences, SPSS-X Chicago, USA. All analyses were performed at $p \leq 0.05$.

3. Results and Discussion

The rhizospheric isolate KC3 was selected for α -amylase activity on the basis of maximum halo zone developed on the Starch Agar plates flooded with Lugol's iodine solution. The isolate was gram positive rod with terminal endospore, aerobic, catalase positive having distinct fermentation profiles of different carbon sources. It exhibited high level of similarity (>98%) with the closest known species in the database. A phylogenetic tree based on bacterial 16S rDNA sequence and closest reference strains downloaded from the database, showed a significant relationship with *Bacillus subtilis* (Fig. 1). Henceforth, this isolate was identified as *Bacillus subtilis* KC3.

Maximum amount (22.92 U/ml) of α -amylase was produced after 48h of incubation (Fig. 2). Incubation beyond the optimum time course was generally accompanied by a decrease in the growth rate and enzyme productivity, which gradually declined to 5.48 U/ml after 96h. The decreased activity in the later phase of growth was probably due to catabolite repression by glucose [16]. The short incubation period for *Bacillus sp.* than other bacteria and fungi offers unique potential for inexpensive enzyme production [4, 21]. Similar results had been reported in *Bacillus flavothermus* after 24h [10] and in *Bacillus amyloliquefaciens* after 72h [9]. At pH 7, optimum amount (23.30 U/ml) of amylase was produced and below and above this pH, the production gets either decreased or denatured (Fig. 3). Different organisms have different pH optima, and decrease or increase in pH, on either side of the optimum value results in poor microbial growth [19]. The pH of 6 and 7 had been reported for normal growth and enzyme activity in *Bacillus* strain isolated from soil [17, 24]. The results illustrated in Fig. (4) showed a positive correlation between the growth/enzyme production and the incubation temperature up to 40°C, followed by a gradual decrease. At higher temperature the bacterial growth gets suppressed and consequently enzyme formation also gets inhibited [3]. The effect of different carbon sources suggest that α -amylase was an inducible enzyme and gets induced in the presence of carbon sources (Fig. 5). Potato starch (27.27U/ml) and corn starch (24.30U/ml) appeared to be the best inducers followed by maltose (19.10U/ml). From the present findings, it is evident that the induction of α -amylase requires substrates having α -1, 4 glycosidic bond, including starch and maltose, but glucose represses its production (Fig. 5). The biosynthesis of α -amylase in most species of the genus *Bacillus* is repressed by readily metabolizable substrates, especially glucose, by a mechanism of catabolite repression, mediated by the protein encoded by the CreA gene [5, 18]. Among the organic nitrogen sources, peptone (24.64 U/ml) proved to be the most suitable followed by tryptone (21.66 U/ml) as compared to inorganic N₂ sources i.e. 12.34 U/ml (Fig. 5). It has been previously found that organic nitrogen sources like peptone and yeast extract usually have stimulating effects [6] and our findings are similar to them.

In the present communication the enzyme was optimally active and stable at pH 6.5 (23.65 U/ml) which is within the range of values for most starch degrading bacterial strains [23]. A considerable amount of activity (19.26 to 22.02 U/ml) was obtained at alkaline pH (Fig.3) showing the wide application of enzyme. The optimum temperature for α -amylase activity was 50°C. At 60°C the maximal enzyme activity was recorded (80.07%) but the activity sharply declined (38.36%) at 70°C (Fig. 4). Amongst the physiological parameters, temperature and pH of the growth medium plays an important role in production and activity of microbial enzymes [26]. The present study shows that barley starch is a good substrate for amylase production by *Bacillus subtilis* KC3. The characteristic temperature, slightly acidic pH, short lag and initial stationary phase are novel features which could be commercially exploited for production of α -amylase in starch and other food industries.

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5. References

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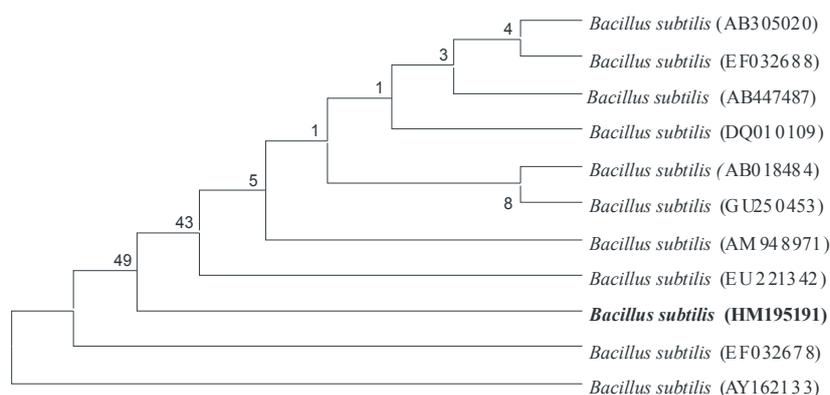


Fig.1: Phylogenetic tree showing genetic relationship of *Bacillus subtilis* with taxonomically similar strains, species and genus based on 16s rDNA sequence. GenBank accession number of each isolate is given in parentheses. Bootstrap values based on 1000 replicates are shown next to the branches.

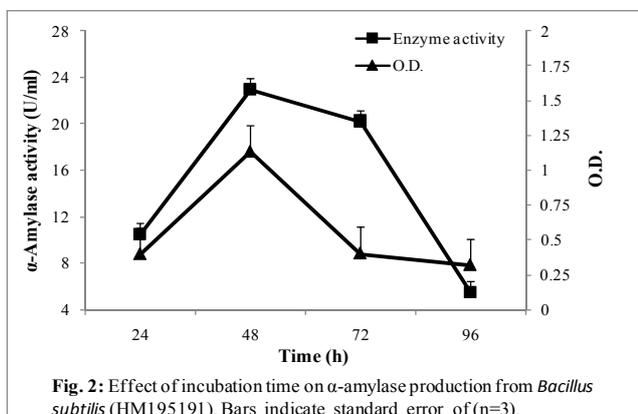


Fig. 2: Effect of incubation time on α -amylase production from *Bacillus subtilis* (HM195191). Bars indicate standard error of (n=3).

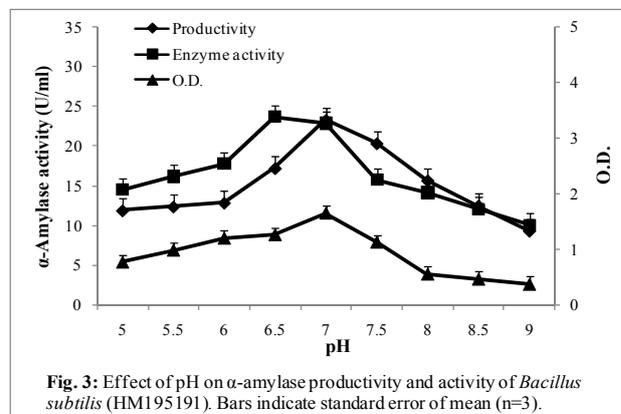


Fig. 3: Effect of pH on α -amylase productivity and activity of *Bacillus subtilis* (HM195191). Bars indicate standard error of mean (n=3).

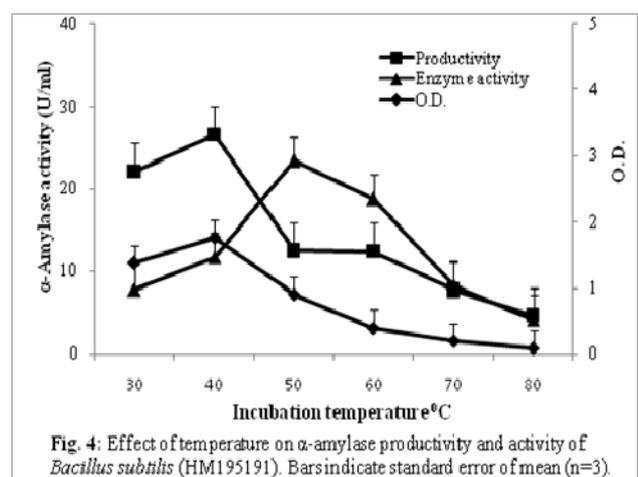


Fig. 4: Effect of temperature on α -amylase productivity and activity of *Bacillus subtilis* (HM195191). Bars indicate standard error of mean (n=3).

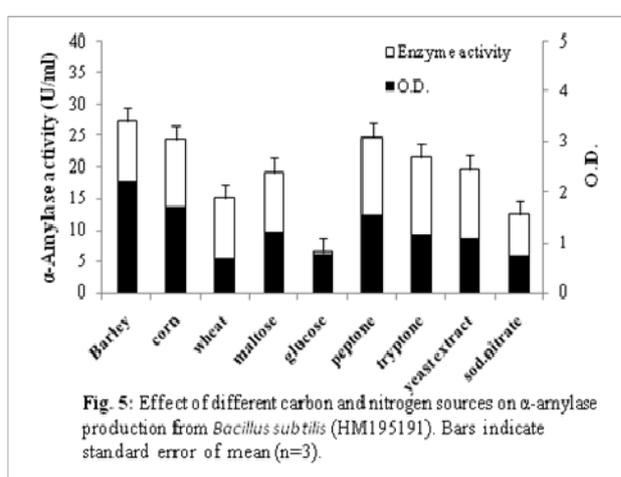


Fig. 5: Effect of different carbon and nitrogen sources on α -amylase production from *Bacillus subtilis* (HM195191). Bars indicate standard error of mean (n=3).