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FIRST REPORT ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5 ISOLATED FROM AGRICULTURAL FIELD SOILS

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ABSTRACT: For the present study, 10 bacterial strains were isolated from agriculture field soils collected from Guntur, Andhra Pradesh, India, and thereafter screened for amylase activity. Among them, the strain DS5 showed a maximum zone of clearance on the starch hydrolysis test was selected for this study. Identification of bacterial strain DS5 was confirmed by 16S rRNA sequencing analysis. The phylogenetic tree constructed on the basis of 16S rRNA sequences revealed that it clustered with the closest members of *Bacillus pumilus* DS5 and the sequences were deposited in NCBI Gen bank along with accession number MK430437. Further the effect of incubation period, temperature, pH, carbon and nitrogen sources was optimized. Highest amylase production was found after 48 h of incubation at pH 7.0 and 40 ºC temperature. Starch proved to be the best carbon source and peptone as nitrogen source for the highest production of amylase by *Bacillus pumilus* DS5. Supplementation with different nitrogen and carbon source favoured the increased enzyme yield by submerged fermentation.

INTRODUCTION: Amylases are one of the prominent enzymes that hydrolysis starch molecules to give diverse products, including dextrin and progressively smaller polymers composed of glucose units [1]. Hydrolytic enzymes like alpha-amylase, lipase, Chitinase protease and Cellulases were obtained from microorganisms. However, amylases from bacillus species are ubiquitous; non-fastidious nutritional requirements and high productivity of most of the alpha-amylases were organism choice [2, 3]. Amylases are among the most important enzyme and account for about 30% of the world's enzyme production [4].

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Although amylases can be derived from several sources, such as plants, animals, and microorganisms, those obtained from microbial sources generally meet industrial demands. The amylase family of enzymes has been characterized well by studying various microorganisms. Many microorganisms utilize starch and other reserve polysaccharides in their metabolic activities. The action of enzymes called amylases brings about the degradation of starch. The end product of starch breakdown is glucose, which is used in many microorganisms directly.

Amylase is majorly produced by bacterial species of *Bacillus* like *B. subtilis* and *B. licheniformis* are among the species that have been widely studied [5]. The other most widely used *Bacillus* species such as, *B. amyloliquefaciens*, *B. megaterium* and *B. licheniformis* are extensively used for the commercial production of the enzyme. Other

Bacillus species that have been explored to produce the enzyme include *B. cereus* and *B. subtilis* to name a few from the available literature. Amylases can be produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens and* show promising potential in a number of industrial applications in processes such as food, fermentation, textiles (used as desizing agent) and paper industries [6, 7]. However there were no reports on amylase production by *Bacillus pumilus* DS5. For the first time we are reporting the amylase production by *Bacillus pumilus* DS5 isolated from agriculture field soils. Hence the present study was aimed at the production and optimization conditions for amylase-producing *Bacillus pumilus* DS5 isolated from agriculture field soils.

MATERIALS AND METHODS:

Isolation of Bacteria: One gram representative soil sample was suspended in 10 ml of sterile distilled water and shaken thoroughly for 10 minutes. Starch utilizing microorganisms was isolated from collected samples by the serial dilution plate technique using Starch Agar Medium (SAM). Serial dilutions of 10-5 of each sample were prepared using sterilized water [8]. Sample dilutions were plated (in triplicates) on SAM and incubated at 35ºC for 24 to 48 h. Pure Colonies were picked and maintained on SAM slants at 4ºC and further assessed for enzyme production in a liquid medium.

Production of Amylase: The amylase production was carried out in 250 ml Erlenmeyer flasks in each containing 100 ml of Starch Agar Medium. The medium was adjusted to pH 7.0 and sterilized in an autoclave for 15 min at 121 ºC. The bacterial strains were inoculated into the amylase production medium and incubated at 35 ºC for 48 h. The culture filtrate was centrifuged at 10000 rpm for 15 min, until a clear suspension was used as crude enzyme solution, which was stored for further studies.

Enzyme Assay: One ml of crude enzyme supernatant was taken in the test tube, and 1.0 ml of substrate (starch solution) was added. The test tubes were covered and incubated at 35°C for 15 minutes in a water bath. 2.0 ml of DNS reagent was added to each tube, and the reaction was stopped by boiling the reaction mixture in a water bath for 10 minutes. After cooling at room temperature, the absorbance (O.D) was measured at 540 nm by spectrophotometer, and the released sugar was determined from the standard maltose curve [9]. One unit of amylase activity was defined as the amount of enzyme that released 1µmol reducing sugar equivalent to maltose per minute under the assay condition.

Amount of reducing sugar = absorbance at 540 nm / Slope of maltose standard / Amount of reducing sugar x 1000

Enzyme activity (iu/ml/min) / Molecular weight of maltose × time

Optimization Studies for Amylase Production by *B. pumilus* DS5:

Effect of pH and Incubation Temperature for Amylase Production by *B. pumilus* DS5: To determine the influence of pH on growth and bioactive metabolite production, the potent strain was cultured in the medium with different pH levels (4-10) and at different temperatures (20 - 60°C). The biomass and bioactive metabolite production were estimated to determine the optimal pH and temperature.

Effect of Carbon and Nitrogen Sources on Amylase Production by *B. pumilus* DS5: To determine the effect of carbon sources on amylase production of the strain, different carbon sources like maltose, lactose, fructose, sucrose, dextrose, starch, mannitol, arabinose, xylose, glycerol and inositol 1% were added separately to the optimized medium [10]. Furthermore, the effect of varying concentrations of the best carbon source (0.5 - 5%) on amylase production was also determined. Similarly, the influence of various nitrogen sources on amylase production was evaluated by supplementing inorganic as well as organic nitrogen sources like sodium nitrate, ammonium sulfate, ammonium oxalate, peptone, yeast-extract, tryptone, casein, tyrosine, phenylalanine, glycine and glutamine 0.5% to the medium containing optimum level of the superior carbon source as determined above. Furthermore, the impact of varying concentrations of optimized nitrogen source (0.1- 2%) was studied to standardize the maximum amylase production.

Effect of Metal Ions on Amylase Production by *B. pumilus* DS5: The effect of metal salts on α-

amylase production was studied by adding different metal salts like $CaCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$, and $CuSO_4$ and in the medium at 0, 0.1, 0.2, 0.3, 0.4 and 0.5 % concentration. The estimation of the enzyme was determined in the presence of 1% soluble starch as substrate. The relative enzyme activity was measured under standard assay conditions [11].

Statistical Analysis: Three replicates were maintained for each treatment in this research work. Values were given as means ± standard deviation for triplicate samples.

RESULTS AND DISCUSSION: Amylase-producing *Bacillus* species were isolated from the banana rhizosphere soils collected from different cultivated lands in and around Guntur district, Andhra Pradesh. All ten *Bacillus* isolates turn blue on hydrolysis of the starch test. Among them, *Bacillus pumilus* DS5 showed clear zones on the starch hydrolysis test.

Similar reports [12] related that 5 isolates were selected by screening test on the basis of morphological and biochemical characterization; the isolates were identified as *Pseudomonas* sp, *Bacillus* sp, *E. coli* sp, *Proteus* sp, *Shigella* sp. Microbial isolates like *Brevibacillus laterosporous* and Enterobacter cloacae are also produced amylase enzymes [13, 14].

Maximum Parsimony Analysis of Taxa: The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [15]. The tree with the highest log likelihood (-2129.36) is shown. The percentage of trees in which the associated taxa clustered is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value.

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1474 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [16].

Effect of Incubation Period: Maximum enzyme production could be obtained only after a certain incubation time, allowing the culture to grow at a study state. Enzyme production of each strain is based on the specific growth rate of the strain. The culture's growth rate and enzyme synthesis are the two main characteristics mainly influenced by incubation time **Table 1**. The growth pattern revealed increased enzyme production from 12 h to 48 h. On further incubation, the amylase production declined, indicating 48 h as the optimal incubation period for the isolated strain. The amylase activity by the isolated *Bacillus pumilus* DS5 strain was observed at 48 h, and the maximum amylase activity obtained was 102.4 U/ml. *Synorhizobium kostiense* MRR 104 produced the maximum amount of EPS and growth after 48 hours of incubation [10].

TABLE 1: EFFECT OF INCUBATION ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5

Incubation period (h)	Amylase production (U/ml)
12	32.9
24	45.8
36	51.2
48	102.4
60	68.3
72	52.4

The F- Value for incubation period and interactions are all significant with $p<0.05$.

Effect of pH: Present study Amylase activity was observed at different pH levels, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 **Table 2**. Optimum pH of 7.0 with maximum enzyme activity (97.0 U/ml) was recorded in *B. pumilus* DS5. The enzyme activity was decreased in acidic and alkaline pH. Generally, the medium's pH enormously affected the bacteria's growth was also reported [17].

TABLE 2: EFFECT OF PH ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5

pH	Amylase production (U/ml)
6.0	31.8
6.5	44
7.0	97
7.5	92
8.0	65
8.5	20.1

The F- Value for incubation period and interactions are all significant with $p<0.05$.

Optimization parameters reveal that the acidic and alkaline pH influences the amylase production by microorganisms such as *Acinetobacter johnsonii, Pseudomonas ludensis, Arthrobacter polychromogens* and *Pseudomonas fragi* was reported by [18].

Effect of Temperature: The results indicated that the *B. pumilus* DS5 had the potential to grow in all the tested incubation temperatures and produce amylase enzyme **Table 3**. The maximum growth was observed at 40° C with amylase production of (105.4 U/ml) in 48 h. Relatively *Bacillus licheniformis* was capable of maximum amylase production at 50°C [19]. The growth and enzyme production in maximum temperatures was also reported in *Bacillus licheniformis* DS3 [20].

TABLE 3: EFFECT OF TEMPERATURE ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5

Temperature	Amylase production (U/ml)
25º C	35.2
30º C	52.9
35º C	99.4
40º C	105.4
45º C	68.5
50º C	35.2

The F- Value for incubation period and interactions are all significant with $p<0.05$.

Effect of Carbon Sources: Several carbon substrates like Glucose, Maltose, Arabinose, Lactose, Fructose, Starch, mannitol, Sucrose, and xylose were tested to evaluate the enzyme production by submerged fermentation. On supplementation of various carbon substrates, maximum enzyme production was exhibited by starch (1% w/v). Results showed different impacts on enzyme production with different substrates.

TABLE 4: EFFECT OF CARBON SOURCES ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5

Carbon sources	Amylase production U/ml
Control	23.9
Glucose	103.4
Maltose	113.8
Lactose	124.5
Starch	180.4
Arabinose	130.5
Fructose	100.2
Sucrose	131.4
Mannitol	145.1
Xylose	130.4

The F- Value for incubation period and interactions are all significant with $p<0.05$.

The maximum enzyme production obtained was 180.4 U/ml with 1% w/v Starch. When supplemented as additional carbon substrate to the medium, sucrose, and mannitol have enhanced enzyme production at 131.4 U/ml and 145.9 U/ml, respectively **Table 4**. This followed the reported maximum α-amylase production when starch was used as the carbon source [19]. The effect of carbon sources (Sucrose and galactose) greatly influenced enzyme production was also reported by [21].

TABLE 5: EFFECT OF NITROGEN SOURCES ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5

Nitrogen sources	Amylase production (U/ml)
Control	27
Ammonium sulphate	104.5
Beef extract	130.2
Peptone	170.4
Potassium chloride	104.5
Tryptone	90.5
L-Aspargine	95.4
Yeast extract	135.6
Ammonium nitrate	102.3

The F- Value for the incubation period and interactions are all significant with $p<0.05$

Effect of Nitrogen Sources: Various nitrogen sources like Ammonium sulphate, Ammonium nitrate, Beef extract, Peptone, Potassium chloride, Tryptone, L-Aspargine, and yeast extract were tested to evaluate the enzyme production by submerged fermentation **Table 5**.

On supplementation of different organic and inorganic nitrogen substrates *B. pumilus* DS5 showed that the maximum enzyme production (148.3) was exhibited by peptone (0.5% w/v) followed by Yeast extract and Beef extract enhanced the enzyme activity of 111.3 U/ml and 102.3 U/ml respectively. Various reports stated that the nitrogen source yeast extract and peptone influenced the enzyme production [22, 23].

Effect of Metal Ions: Metal ions were considered the best ions for optimum growth of the bacteria and the best inducer for amylase production **Table 6**. Different metal ions $CaCl_2$, $MgSo_4$, $MnSo_4$, $FeSo_4$, and $CuSo_4$ at 0.1 to 0.5% concentrations were studied for enzyme production. All the strains showed the maximal amount of enzyme in the presence of metal ions. The strain *B. pumilus* DS5 showed the supreme amount of enzyme in the presence of $CaCl_2$. Other metals also showed the maximal enzyme activity recorded by three strains. The presence of metal ions in the medium

influenced the enzyme production was also revealed [11].

TABLE 6: EFFECT OF METAL IONS ON AMYLASE PRODUCTION BY *BACILLUS PUMILUS* DS5

Metal ions	Amylase production (U/ml)
$CaCl_2$	160
$MgSO_4$	132
$MnSO_4$	105
Fe SO_4	97
Cu SO_4	102

The F- Value for the incubation period and interactions are all significant with $p<0.05$.

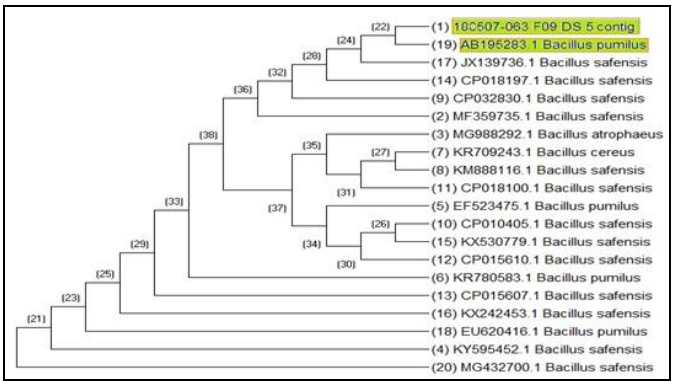

FIG. 1: PHYLOGENETIC TREE BASED ON 16S RRNA SEQUENCES OF THE GENUS *BACILLUS* OBTAINED FROM BLAST SEARCH SHOWING THE POSITION OF ISOLATE (ANU-MCB-DS5) AND RELATED STRAINS

CONCLUSIONS: The present study reveals the isolation and optimization conditions for amylase production by *B. pumilus* DS5 isolated from agricultural field soils. The optimization conditions of *B. pumilus* DS5 showed maximum enzyme activity at 42 hours of incubation, pH 7.0 and 40℃ temp. Highest production of enzyme production in starch medium was supplemented with starch and peptone as carbon and nitrogen sources.

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