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# EXTRACELLUAR AMYLASE PRODUCTION UNDER SUBMERGED FERMENTATION BY *BACILLUS SUBTILIS* RK6

***Shilpi Kiran[1], Sushma Kumari [2], Abha Singh [3], Chander Prabha [4], Sonali Kumari[5]**

**[1, 2, 3, 5] Microbial Biodiversity Lab., Department of Botany, Patna University, Patna-800005, Bihar, India.**
**[4]Tissue Culture Lab., Department of Botany, Patna Science College, Patna University, Patna-800005, Bihar, India.**

***Corresponding Author Email: shilpikpwc@gmail.com**

## ABSTRACT

*Microbial amylases are most important hydrolytic enzymes, sharing one-quarter of world enzyme market with prolific applications in various industries. Hot springs have been an important biotope of enzymes of industrial interest, encouraging exploration of new sources. The present study elaborates optimization of extracellular amylase production by a bacterial isolate RK6 isolated from Rishi Kund, a hot spring of Bihar. On the basis of morphological, biochemical and 16S rRNA sequence analysis, the potential bacterial isolate RK6 was identified as Bacillus subtilis RK6. Submerged fermentation technique was followed to investigate effects of different parameters like, incubation period, pH, temperature, nitrogen sources and carbon sources on amylase production. Enzyme production was optimized at 48 h of incubation, 50 °C temperature and 6.0 pH. Best enzyme production was observed with organic nitrogen source than that of inorganic ones, where peptone was found to be most suitable. Amylase production was induced by different starchy substrates, of which soluble starch appeared as best carbon source, with its concentration of 2.5 % (starch), giving maximum output.*

## KEY WORDS

*Bacillus subtilis, hydrolytic enzymes, microbial amylase, rishi kund, submerged fermentation.*

## INTRODUCTION

In recent years, there has been multifold increase in demand of microbial enzymes for industries. They are preferred over plant and animal sources because of their plasticity and vast availability. Hot springs are natural habitat of archea and eubacteria. In the last decade, hot springs have been an important biotope of thermophilic enzymes of industrial interest, encouraging further search for newer microorganisms. Amylases are hydrolases, which break down the O-glucosidic linkages in starch. There are two important groups of amylases; endoamylases (α–amylases) and exoamylases (β-amylases and glucoamylases) [1, 2]. They constitute a class of industrial enzymes contributing approximately 25-30% of the world enzyme market [3, 4]. They have a wide range of applications in food, syrup, baking, brewing, distilling, textile, detergent, pharmaceutical and paper industries [5, 6].

Submerged fermentation (SmF) is inevitable to enable bulk production of commercially important enzymes [7, 8], because of better control over multiple factors namely, pH, temperature, aeration and moisture. Improvement of amylase yield and consequent cost reduction depends on selection of strain, optimization of factors affecting biosynthesis, kinetic studies and characterization of enzymes. The optimization of fermentation conditions particularly, physical and chemical parameters, are important for maximum microbial growth and enzyme production [9, 10], thus

becomes necessary for practicability of the process and its impact on industrial economy.

Considering the wide potentials of amylases in various industries and importance of optimization for industrial aspects, the present study elaborates the optimization of extracellular amylase production, of bacterial origin, isolated from hot spring of Rishi Kund, Munger, Bihar, India.

## MATERIALS AND METHODS

### Sampling and isolation of bacteria

Water samples were collected from hot spring of Rishi Kund, Munger (Latitude- 25.38, Longitude- 86.47), Bihar, India and brought to Microbial Biodiversity Laboratory, Patna University, under aseptic condition. The samples were serially diluted up to $10^{-6}$ dilutions by the serial dilution method. The aliquots (0.1 ml) were plated on Nutrient Agar (NA) medium [(w/v) 0.5% peptone; 0.3% beef extract; 0.5% NaCl; 1.5% agar, pH 7] and incubated at 50±2 °C for 72 h.

### Screening for amylase activity (Stach Iodine test)

Isolated pure cultures were screened for amylase activity by employing zone clearing technique, using starch agar medium [11]. Pure culture of different isolates were streaked on starch agar plates. After incbution at 50 °C for 24 h, plates were flooded with Gram's iodine. Isolates producing clear halo zone around the colony were considered as amylase producers.

### Identification of bacterial isolate

Phenotypic characterization of the isolate was done by different tests, referring Bergey's Manual of Determinative Bacteriology and Agriculture handbook [12, 13]. Identification of selected isolate was carried out according to their morphological and biochemical tests. For genotypic characterization, genomic DNA was extracted from the isolate using Chomous Genomic DNA isolation kit (RKT09). The amplification of 16S rRNA gene was carried out by using Thermal cycler (ABI 2720) in 100 µl reaction mixture containing 2.5 mM each of four dNTP, 10X PCR buffer, 3U of Taq DNA polymerase, 10 ng template DNA and 400 ng 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 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 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 (9.9 kb) was aligned by using the BLASTN program to identify the most similar sequence in the NCBI database and phylogenetic analysis was done by constructing phylogenetic tree (Neighbor-joining tree) by using ClustalW algorithm with the help of MEGA 6.0 [14] .

### Preparation of inoculum

A volume of 60 ml of starch broth [(w/v) 0.6% peptone; 0.05% $MgSO_4.7H_2O$; 0.05% $CaCl_2$, 0.2% $(NH_4)_2SO_4$, 0.05% KCl; 1% soluble starch; pH 7] taken in 250 ml Erlenmeyer flask was inoculated with a loopfull of cells from a 24 h old culture and kept at 40 °C in a rotary shaker.

### Amylase Production by submerged fermentation

Enzyme production was carried out in 250 ml Erlenmeyer flask containing 60 ml of starch broth medium, inoculated with $10^6$ cells of overnight (24 h) grown culture, centrifuged at 12,000× g for 10 min at 4 °C and supernatant was used for estimation of enzyme activity.

### Amylase Assay

Amylase assay was done by DNSA method using starch as substrate. The reaction mixture contained 1 ml substrate (1% in 0.1M phosphate buffer, pH 6.5) and 1 ml of suitably diluted crude enzyme and was incubated at 50 °C for 10 min. The reaction was stopped by adding 2 ml of 3, 5-dinitrosalicylic acid (DNS) reagent, followed by boiling for 5 min to develop color. The absorbance was measured at 540 nm using double beam UV/VIS spectrophotometer (Systronics, 119). The reducing sugar released was measured by the method of [15]. One enzyme unit (U/ml) is equivalent to the amount of enzyme needed to release 1µmol of reducing sugar per minute under standard assay condition.

### Optimization of culture conditions for amylase production

Various parameters viz., incubation period, temperature, pH, nitrogen sources, carbon sources and its concentrations were investigated for optimum amylase production. The effect of incubation period was studied for different time intervals (24, 48, 72, 96 h) at pH 7 and temperature 50 °C in the basal medium. Thereafter, effect of temperature at 40, 50, 60, 70 °C and different pH was studied by adjusting pH of production medium to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0. Effect of different nitrogen sources (0.6%) was studied by replacing peptone with tryptone, beef extract, yeast

extract, sodium nitrate and urea. Similarly, effect of different carbon source (1%) was studied by replacing starch with corn flour, wheat flour, rice flour, wheat bran and rice bran in production medium. Finally effect of concentration of starchy substrate (0.5, 1.0,1.5, 2.0, 2.5, 3.0 %) was optimized.

**Statistical analysis**

Each experiment was performed in triplicate and graphically represented as the mean ±SE (n=3).

## RESULTS AND DISCUSSION

**Isolation and screening for amylase activity**

In the present work, a total of seven bacterial colonies (RK1…RK7) appeared on NA plates were incubated at 50 °C from water sample of hot spring and screened for amylolytic activity. Amongst them, four isolates were found to be amylase positive which showed starch hydrolysis activity on starch agar plates. One of the isolate, designated as RK6 exhibited considerable halo zone 6.5 mm on starch agar paltes (Fig. 1) was selected for further study.

**Identification of isolate RK6**

Bacterial isolate RK6 was characterized on the basis of phenotypic and genetypic parameters. Colony appeared as small, irregular margin, lobate to undulate and dull white in color. The microscopic observation revealed it to be gram positive, motile rod, bearing terminal endospores. Considering the colony characteristics, microscopic characters and biochemical tests (Table I), the isolate RK6 was grouped into genus *Bacillus* sp. Genotypic characterization, done by 16S rRNA gene sequence analysis showed 99% homology with the type strains in the database and phylogenetic tree showed significant relation with *Bacillus subtilis* (Fig. 2) thus, RK6 identified as *Bacillus subtilis* RK6. The sequence was deposited in the GenBank of the NCBI (Accession no: KX247637).

**Table I. Phenotypic characteristic of amylolytic bacterial isolate (RK6)**

| | Characteristics of Bacteria | Result |
|---|---|---|
| Cultural Characters | Colony Morphology on NA plates | Irregular, lobate to undulate, dull white colonies |
| | Gram staining | Gram positive rods |
| Microscopic Characters | Spore staining | Spore forming |
| | Motility | Motile |
| | Catalase | + |
| | Oxidase | + |
| | Indole | - |
| | Methyl red (MR) | - |
| Biochemical Characters | Voges proskauer (VP) | + |
| | Citrate utilisation | + |
| | Starch hydrolysis | + |
| | Gelatin hydrolysis | + |
| | Nitrate reduction | + |

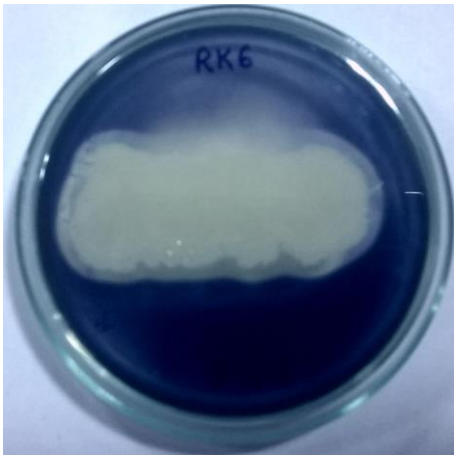


**Fig. 1 Starch Agar Plates showing Halo zone around bacterial isolate RK6**

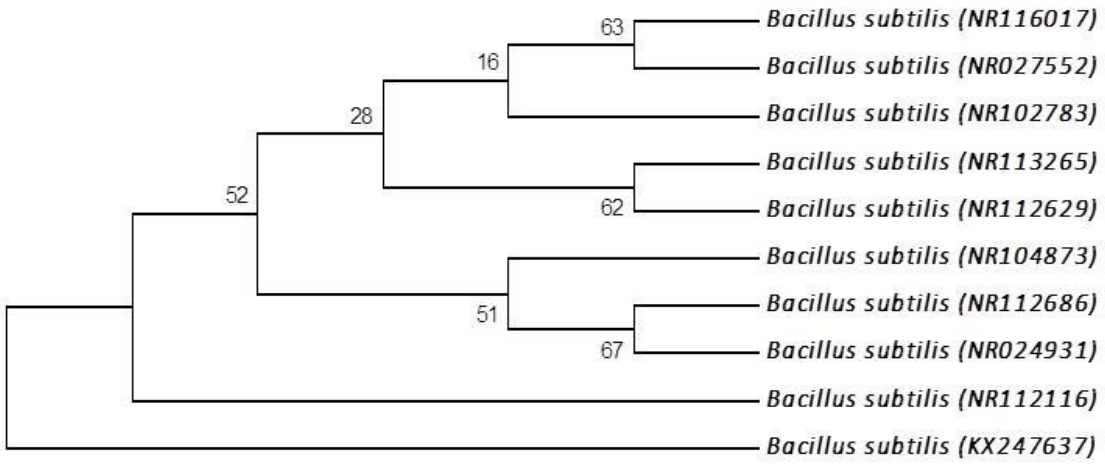


**Fig. 2- Phylogenetic tree showing genetic relationship of *Bacillus subtilis* with taxonomically similar strains, species and genus based on 16S rRNA gene sequences. Genbank accession number of each isolate is given in parentheses. Bootstrap values based on 1000 replicates are shown next to the branches Phylogenetic tree.**

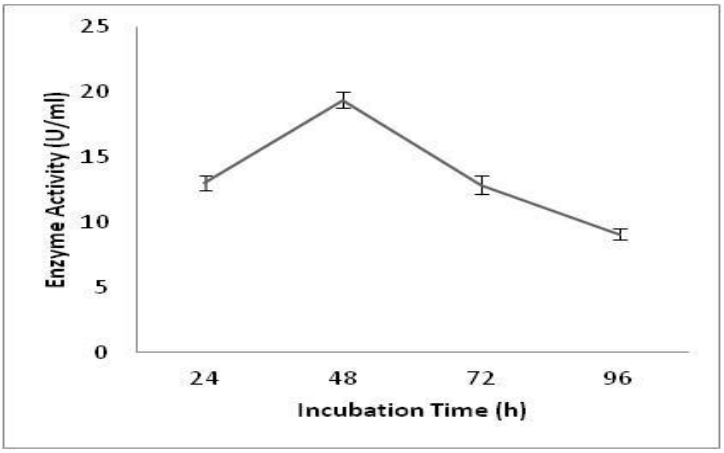


**Fig. 3 Effect of incubation time on amylase production from *B. subtilis* (KX247637). Bars represent mean ± SE (n=3).**

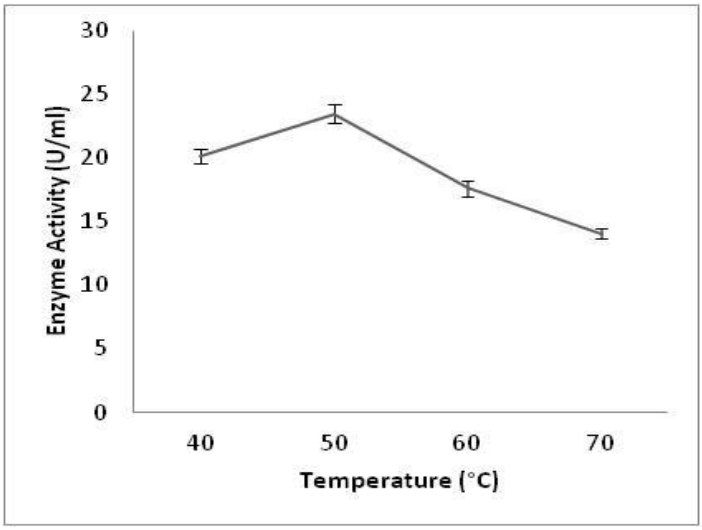


**Fig. 4 Effect of temperature on amylase production from *B. subtilis* (KX247637). Bars represent mean ± SE (n=3).**

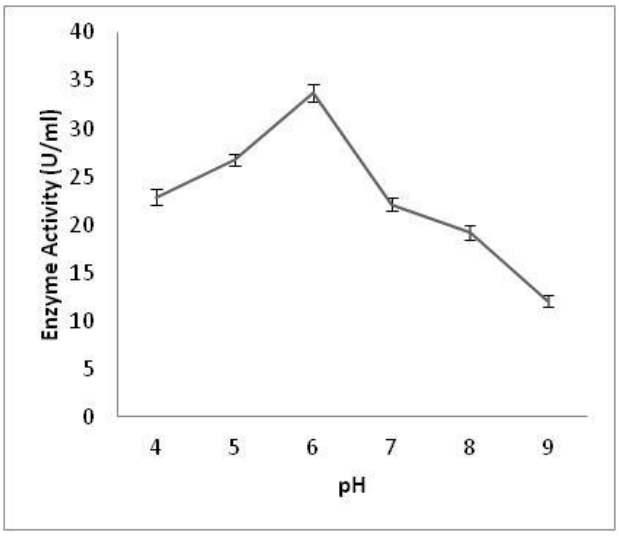


**Fig. 5 Effect of pH on amylase production from *B. subtilis* (KX247637). Bars represent mean ± SE (n=3).**

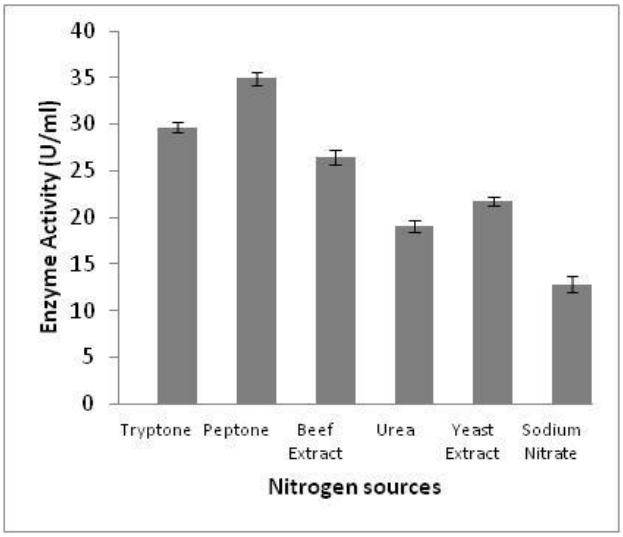


**Fig. 6 Effect of Nitrogen sources on amylase production from *B. subtilis* (KX247637). Bars represent mean ± SE (n=3).**

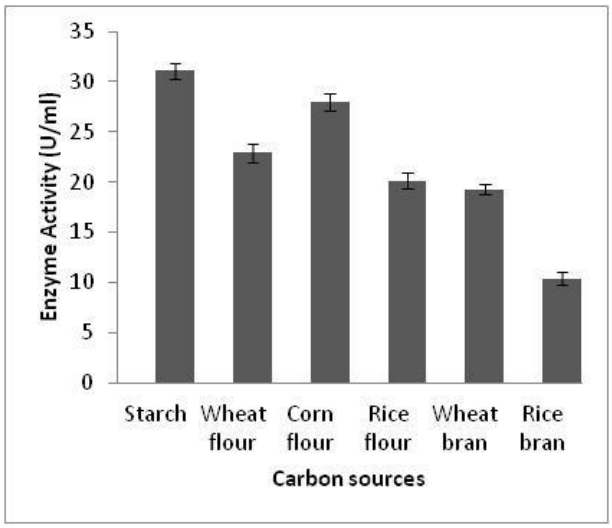

**Fig. 7 Effect of Carbon sources on amylase production from *B. subtilis* (KX247637). Bars represent mean ± SE (n=3).**

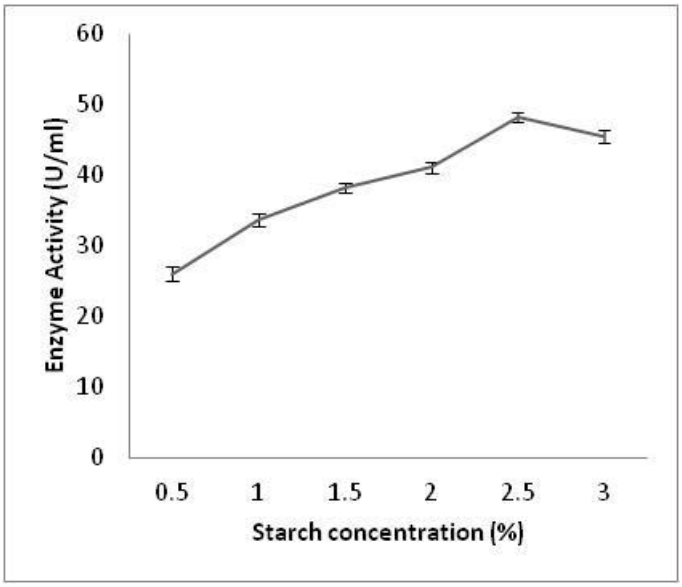


**Fig. 8 Effect of starch concentration on amylase production from *B. subtilis* (KX247637). Bars represent mean ± SE (n=3).**

**Optimization of culture conditions for amylase production**

The optimum incubation time for amylase production by *B. subtilis* RK6 was 48 h. It initiated at 24 h, maximal during 48 h (19.4 U/ml) and thereafter, declined to minimum 9.1 U/ml at 96 h of incubation (Fig. 3). [16] also made the similar observation in *Bacillus subtilis*, where a linear rise in amylase production upto 48 h was recorded, which merges with its sporulation pattern. Works carried by other researchers [17, 18] also support 48 h of incubation for maximum amylase production in *Bacillus subtilis* KC3 and *Bacillus subtilis* MTCC121 respectively. The probable reasons for the decrease in amylase production upon extended incubation may be due to reduction in nutrients, accumulation of waste products, cell death, and catabolite repression [19]. Generally, a higher concentration of primary metabolites is achieved in exponential growth phase, which is due to the availability of enough nutrients, important metabolites, non-accumulation of toxic products, etc. Amylase production increased with incubation temperature up to 50 °C (23.51 U/ml), thereafter it declined (Fig. 4), which could be attributed to suppression of bacterial growth beyond optimum temperature. A wide variability in temperatures i.e. 35 °C to 80 °C for optimum growth and amylase production has been previously reported [20, 21]. Maximum (33.71 U/ml) amylase was produced at pH 6.0, which decreased below and above the optimum pH (Fig. 5). Different organisms have different pH optima and even slight decrease or increase of optimum value results in poor microbial growth, ultimately reducing metabolite production. An optimum pH range between 6.0 to 7.0 has been reported for maximum growth of bacterial strains and enzyme production [10]. Our findings are in conformity with that of [22], who also achieved maximum enzyme production at pH 6.0 in *Bacillus amyloliquefaciens* strain 267CH.

Effect of different organic and inorganic nitrogen sources were tested in media for best enzyme production as shown in Fig. 6. The highest amylase production was noticed with peptone (34.89 U/ml) followed by tryptone (29.68 U/ml) and beef extract 26.43 U/ml, while lowest by sodium nitrate (12.86 U/ml). Besides, moderate amylase activity was recorded, when production media was supplied with yeast extract (21.74 U/ml) and urea (19.06 U/ml), respectively (Fig. 6). Present findings are supported by observations made by previous workers [23, 24, 25], who reported organic nitrogen sources for maximum α-amylase production in various bacteria. They attributed this phenomenon to the high nutritional amino acids and vitamins present in the production media.

Natural sources could serve as economical and readily available raw material for the production of valuable enzymes. The nature and amount of carbon sources in

culture media are important factor the production of extracellular amylase [26]. Amylase is an inducible enzyme and is generally induced in the presence of specific concentrations of starch [27]. Among various carbon source, soluble starch (31.06 U/ml) appeared as best substrate for amylase production followed by corn flour (28.0 U/ml) > Wheat flour (22.89 U/ml)> rice flour (20.1 U/ml)> wheat bran (19.2 U/ml)> rice bran (10.36 U/ml) as shown in Fig. 7. In fact, it has been found that soluble starch used as carbon source leads to maximum amylase production [10, 23]. Hence, it was selected for optimization of its concentration, where 2 .5 % (w/v) of starch found to be appropriate one with activity of 48.21 U/ml (Fig. 8) below and above this concentration amylase production was low.

## CONCLUSION

Optimization of culture conditions have always been of great interest to the researchers because of its significance in the enzyme industry for higher enzyme production. In the present work, production medium was optimized for enhanced secretion of amylase by *Bacillus subtilis*RK6. Maximum amylase was produced at 48h incubation, pH 6.0 and 50 °C. Organic nitrogen source gave better results in terms of enzyme production than inorganic sources while 2.5% of soluble starch emerged as most appropriate carbon source among the experimented one.

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***Corresponding Author:**
***Shilpi Kiran****
*Email:* **shilpikpwc@gmail.com**