

PRODUCTION AND COMPARATIVE STUDY OF α - AMYLASE FROM WILD AND MUTANT HOT SPRING *Bacillus subtilis*

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ABSTRACT

α -amylase, a catalytic protein produced by moderately thermophilic bacteria, *Bacillus subtilis* was isolated from hot spring at Unkeshwar-[Dist.Nanded(M.S)]. Effect of various kinetic factors on amylase produced by wild and mutant *Bacillus subtilis* were studied and compared in the present study. pH and temperature optima for α -amylase from isolated wild *B. subtilis* were found to be 8.5, and 65°C respectively. The amylase from ultraviolet(UV) mutant and 5-Bromouracil(BU) mutant demonstrated higher activity at same pH whereas the optimum temperature was found to be raised by about 5 °C to a temperature of 70°C for amylase from mutant as compared to amylase from wild *B.subtilis* , indicating its thermostable nature. Optimum time course of reaction and optimum substrate concentration for all three strains of *B.subtilis* α -amylase was found to be 15 minutes and 4mg/ml respectively. This optimum time and concentration of substrate was found to be similar for amylase from mutant though with higher enzyme activity.

KEYWORDS: Wild and mutant *Bacillus subtilis*, hot water spring, alkaline thermostable α -amylase.

INTRODUCTION

α -amylase (1,4 α -D glucanohydrolase) (E.C.3.2.1.1) is an hydrolytic enzyme catalyzing the hydrolytic splitting of glucosidic linkages in starch.. Bacteria producing α -amylases are *Bacillus subtilis*, *Bacillus cereus*, *B.polymyxa*, *B.megaterium*, *B.macerans*. Amylases are industrially important enzymes and its important areas of application include starch, alcohol and brewing industry, paper, textiles and feed industry, sugar, laundry and detergent industry. In 1894, first enzyme produced industrially was α -amylase, used as a pharmaceutical aid for the treatment of digestive disorders.^[1] Microbial amylases have successfully replaced the chemical hydrolysis of starch in starch-processing industries. They would be potentially useful in the pharmaceutical and fine chemicals industries if enzymes with suitable properties could be prepared.^[2] Most of the bacteria belonging of family "Bacillaceae" produce α -amylase extracellularly.^[3] Bacterial α -amylases are secreted proteins that hydrolyze α -1,4 glycosidic linkages, allowing for growth when starch is the sole carbon source.^[4]

A thermo acidophilic amylase from *Bacillus acidocaldarius* was characterized with optimum pH of 4.5 and temperature of 60-63°C.^[5] An alkalophilic *Bacillus* GM 801 when grown at pH 10.5 at 50°C produced alkaline amylases in culture broth.^[6] *Bacillus subtilis* was found to be a safe host for production of

harmless protein products especially hydrolytic enzymes such as amylases and proteases.^[7]

Taking into consideration importance of α -amylase and its commercial exploitation potential, a study of biotechnological aspects of α -amylase extracted from hot spring bacillus was carried out.

MATERIALS AND METHODS

Collection of water sample: - Water sample (pH-8.0 and temperature-50°C for the study was collected from hot water spring at Unkeshwar located 150 Km from Nanded (M.S).

Media

Nutrient broth and nutrient agar plates were used isolation of bacteria from water sample. The amylase cultivation media (composition:-Soluble starch-10.0g,Peptone - 10.0g,KCl-1.0g,MgCl₂.2H₂O - 0.2g CaCl₂.2H₂O-0.25g,FeSO₄.7H₂O - 0.001g MnSO₄.5H₂O - 0.001g, NaH₂PO₄ -5.4g, Na₂H₂PO₄ -5.26g, Distilled water -1000ml, pH - 8.0) was used for amylase production whereas starch agar was used for identification of potential amylase producer.

Identification of isolated bacteria

For identification of the isolated bacterial colonies various biochemical tests were performed. Isolation of potential α -amylase producing bacterial culture was done

by using starch agar plate. A loop full of each bacterial culture was spot inoculated in center of starch agar plate. Plate was incubated at 50°C for 24 to 48 hours and then flooded with gram iodine to check for zone of clearance around colony indicating hydrolysis of starch by α -amylase.

Production of α -amylase

Pure culture of *Bacillus subtilis* was inoculated in α -amylase production medium and kept for incubation on a rotatory shaker incubator at 50°C for 24 hrs.

Cells were removed by centrifugation (10,000 rpm for 20 min) and supernatant was collected and used as crude α -amylase enzyme. Extract obtained was subjected to purification procedure of precipitation with ammonium sulphate. The solution was left standing overnight at 40°C and the precipitate was collected by centrifugation at 10,000 rpm for 20 min. The precipitated enzyme was redissolved in minimum quantity of distilled water containing 0.01 M phosphate buffer and further subjected to dialysis.

Dialysis was carried out in dialysis bags. Enzyme extract after precipitation was added in dialysis bag, whose sides were secured tightly to prevent leakage. It was suspended in beaker containing dilute buffer (0.01 M of phosphate buffer pH-7.0) and the contents were kept in cold condition overnight in refrigerator. Excess of water that entered the bag is removed by keeping the bag in a jar containing sucrose. The purified α -amylase enzyme thus obtained was used for further studies.

Strain improvement by mutagenesis

Production of UV (Ultra violet) and 5-BU (Bromouracil) mutant of *Bacillus subtilis* was done as a part of strain improvement programme. 0.1ml of active culture *Bacillus subtilis* was spreaded on nutrient agar plates. Plates were exposed to ultraviolet light at a distance of 15cm for various time intervals and kept for incubation at 37°C for 24 hours. Plates with 99% of kill were used for selection of ultraviolet i.e. UV mutant of *Bacillus subtilis*. Along with UV mutant, 5-Bromouracil (5-BU) mutant of *Bacillus subtilis* was prepared and used for amylase production and kinetic studies. A comparative study of amylase from wild, Ultraviolet and 5-Bromouracil treated *Bacillus subtilis* was made in the present study. Amylase production, effect of pH, temperature, time course, substrate concentration, V_{max} and K_m of the enzyme were studied and compared to amylase from wild *Bacillus subtilis*.

Studies on kinetics of α -amylase

Assay of amylase

Standard maltose graph was prepared with ten different dilutions of maltose ranging from 0.5mg/ml, 1 mg/ml, and 1.5 mg/ml.....up to 5mg/ml prepared from standard maltose stock solution (5mg/ml) Dilutions were subjected to estimation of sugar by summer's method. 1 ml of each dilution was taken in separate test tube and to

it 1ml of DNSA (dinitrosalicylic acid reagent) was added. Tube was kept in boiling water bath for 5 min. After cooling 10ml of distilled water was added to the reaction mixture, mixed together on cyclomixer and optical density was estimated at 540 nm. The optical density values obtained for the above ten dilutions were used to plot a standard graph of maltose. Similar treatment was done for 1 ml of extracted amylase enzyme. Optical density value obtained was plotted on standard graph of maltose to obtain the amount of maltose formed. The extracted α -amylase activity was expressed in enzyme units. Enzyme activity is expressed in Units (U). One amylase unit is equivalent to amount of enzyme required to release one μ mole of maltose per minute from respective substrate i.e. starch.

Effect of pH on α -amylase activity

1ml of starch and 1ml of buffer of a particular pH was taken in a test tube. In a separate tube 1ml of enzyme was taken and kept for equilibration at 50°C for 15 minutes. After equilibration enzyme was added in tube with starch and buffer. Similar steps were performed, for various buffers with different pH. Tubes were again kept at 50°C for 15 minutes. Reaction was terminated by addition of 1ml of DNSA. Tubes were treated for 5 minutes in boiling water bath, cooled to room temperature and volume of tube was made up to 12ml with distilled water. Absorbance was read at 540 nm on spectrophotometer.

Effect of temperature on α -amylase activity

1ml of starch and 1ml of buffer (pH-7.0) were added in one test tube. 1ml of α -amylase was taken in a separate tube. Blank was prepared by mixing 1ml of starch and 1ml of α -amylase. Tubes were kept at 40°C for 10 minutes. Same procedure was followed for different temperature of 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, and 75°C. After 15 minutes contents of the tube with enzyme was mixed with tube containing starch and buffer and again incubated at respective temperatures for 15 minutes. Reaction was terminated by addition of 1ml of 3, 5 dinitrosalicylic acid reagent. All tubes were kept in boiling water bath for 5 minutes and then cooled to room temperature. Volume of each tube was made up to 12ml with distilled water. Contents of the tube were mixed and absorbance was read at 540 nm against respective blank.

Effect of time course of reaction on α -amylase

1ml of starch and 1ml of buffer was added in a test tube and in a separate tube 1ml of α -amylase was taken. Tubes were kept at 50°C for 15 minutes, content of the tube with enzyme were mixed with that of tube with starch and buffer. Tubes were further kept for incubation at 50°C for 5 minutes. Similar sets were prepared and kept for incubation for varying time period of time i.e. 10, 15, 20,25,30,35 and 40 minutes. Blank was prepared by mixing 1ml of starch with 1ml of buffer. Reaction was terminated in each case by addition of 1ml of 3, 5 dinitrosalicylic acid reagent. Tubes were heated in boiling water bath for 5 minutes and cooled to room

temperature. Volume of the tube was made up to 12 ml with distilled water. Contents of tube were mixed in cyclomixer and optical density was determined at 540 nm against respective blank.

Effect of substrate concentration on α -amylase

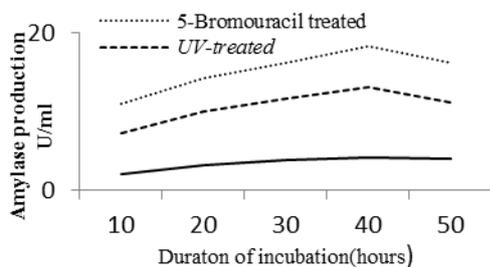
Different dilutions of starch ranging from 1mg/ml, 2mg/ml, up to 5mg/ml were used. 1ml of each dilution was taken in a test tube. 1ml of extracted α -amylase enzyme was taken in a separate tube. Tubes were kept at optimum temperature obtained in the experiment (65°C) for 15 minutes. Then enzyme was added in starch containing tubes and tubes were again kept at 65°C for 15 minutes. Reaction was terminated by addition of 1ml of 3, 5 dinitrosalicylic acid. Tubes were treated in boiling water bath for 5 minutes and cooled to room temperature. Volume of each tube was made up to 12 ml by adding distilled water. Contents of the tube were mixed and absorbance was read at 540 nm.

Determination of V_{max} and K_m for extracted α -amylase

The optical density values for different substrate concentration obtained in the earlier experiment of effect of substrate concentration on α -amylase were used for determination of V_{max} and K_m . $1/[S]$ values were obtained and a graph of $1/[S]$ on X-axis and $1/[V]$ on Y axis known as Lineweaver-Burk plot was plotted. The graph being linear can be extrapolated and values of K_m and V_{max} can be determined. Large K_m denotes low enzyme substrate affinity and small K_m indicates high enzyme substrate affinity.

RESULT AND DISCUSSION

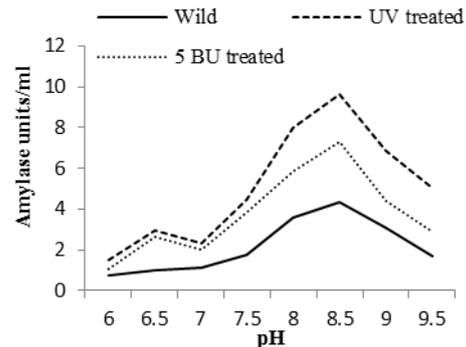
Amylase production by *Bacillus subtilis* was studied with respect to amylase activity (unit/ml) at various time intervals. Amylase production began after 10 hours and increased exponentially up to 40 hours. The maximum amylase production (4.3 U/ml) was at 40 hours for all the three types of *Bacillus subtilis* i.e wild, UV (ultraviolet) mutant and 5-Bromouracil (BU) mutant [Graph 1].



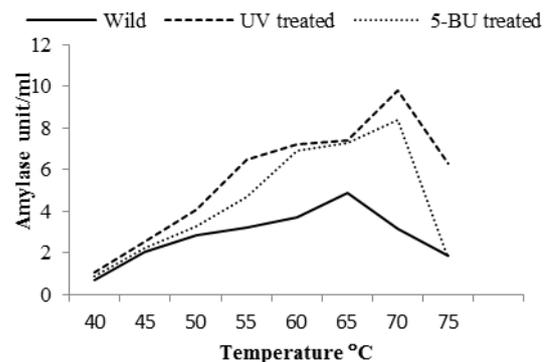
Graph 1: Production of amylase by *Bacillus subtilis*.

Amylase from wild, UV mutant and 5-Bromouracil mutant *B. subtilis* when studied for their activity at various pH indicated same pH optima i.e. pH-8.5 for wild *B. subtilis* (4.351U/ml), UV mutant (9.628 U/ml) and 5-Bromouracil mutant *B. subtilis* (7.27 U/ml). Enhancement in enzyme activity (Strain improvement) was observed in both mutants as compared to wild type of bacteria [Graph 2]. It could be concluded that α -

amylase produced was alkaline in nature. Such alkaline amylases from *Bacillus subtilis* with optimum pH of 8.5 could have potential applications for hydrolyzing starch under high pH condition in the starch and textile industry and as an ingredient in detergents.

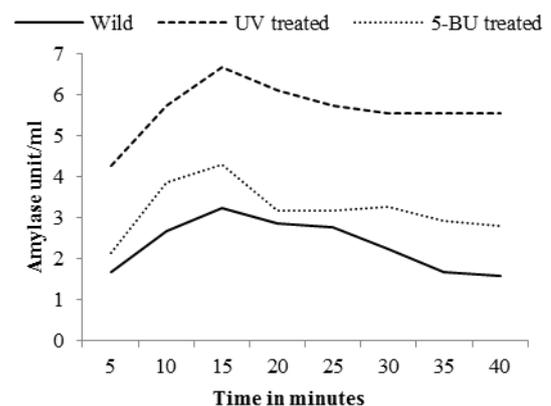


Graph 2: Effect of pH on amylase activity.



Graph 3: Effect of temperature on amylase activity.

The enzyme activity unit/ml at various different temperatures indicated maximum α -amylase activity (4.907unit/ml) at a temperature of 65°C. Temperature optima was found to be enhanced to 70°C for α -amylase from mutant *Bacillus subtilis* i.e UV-mutant *B. subtilis* (9.8 U/ml), and 5-Bromouracil mutant *B. subtilis* (8.41 U/ml) [Graph 3]. Increased temperature optima of α -amylase for both mutants could be the result of change in protein confirmation after mutation.

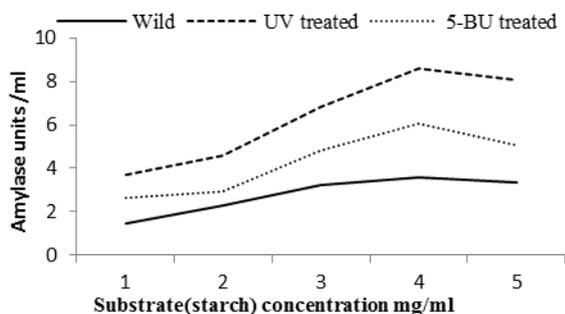


Graph 4: Effect of time course on α -amylase.

The amylase from wild *Bacillus subtilis* exhibited maximum enzyme activity (3.24 unit/ml) at 15 minutes reaction time. Optimum time course of reaction in all three strains was found to be 15 minutes, but the maximum amylase activity demonstrated was found to be varying with 6.66 U/ml for UV-mutant *B. subtilis* as compared to 4.37 U/ml and 3.24 U/ml for 5-Bromouracil mutant and wild *Bacillus subtilis* respectively [Graph 4].

Maximum activity of amylase was observed at a starch concentration of 4 mg/ml. Amylase from UV mutant bacteria demonstrated maximum enzyme activity (8.602 unit/ml) as compared to 5-BU mutant amylase (6.08 unit/ml) and wild amylase (3.611 unit/ml) respectively. Beyond 4 mg/ml of concentration of substrate i.e. starch, a reduction in enzyme activity was observed [Graph 5]. Such an observation is common during amylase synthesis by most bacilli at higher concentration of starch since most bacilli are susceptible to catabolite repression by glucose. A possible explanation for by observed relationship between starch concentration and α amylase production and activity is the repression of enzyme synthesis at higher starch concentration by starch hydrolysis products such as glucose or a difference in O_2 availability due to change in viscosity at higher starch concentration.

$1/[V]$ and $1/[S]$ values calculated for various different concentrations of starch when used for plotting of Lineweaver Burk plot indicated V_{max} and K_m as 0.0066 μ moles/min and 1.38×10^{-2} M respectively for wild α -amylase. V_{max} and K_m was found to be 0.0076 μ moles/minutes and 1.2×10^{-2} M for UV mutant and 0.906 μ moles/minutes and 1.32×10^{-2} M respectively for α -amylase from 5-BU mutant. K_m indicates affinity between enzyme and substrate. Lower is the K_m higher is the affinity between enzyme and the substrate, and faster is the catalytic action. Hence it could be concluded that amylase from UV mutant is having highest catalytic activity.



Graph 5: Effect of substrate concentration on amylase activity.

CONCLUSION

Amylase production profile of all three types of strains of *B. subtilis* demonstrated amylase production after 10 hours which increased exponentially up to 40 hour.

Amylase production which continued to be maintained for an extended period may possibly be due to long lived m-RNA specific for α -amylase.^[8] Probably post exponential phase cells contained sufficient preformed m-RNA to enable exocellular amylase synthesis to be maintained for an extended period of time in absence of RNA synthesis.

Study of the effect of pH on α -amylase of wild and both mutant strain of *Bacillus subtilis* was found to at pH 8.5 hence they are alkaline in nature.

Temperature optima of mutant *B. subtilis* were found to raised to 70°C as compared to 65°C for wild *B. subtilis*. Increased temperature optimum of α -amylase for both mutants could be the result of change in protein confirmation after mutation. Effect of substrate concentration indicated that amylase from UV mutant as having highest catalytic activity compared to wild and 5-BU mutant.

The above research investigation with *Bacillus subtilis* α -amylase produced by wild, UV mutant and 5-BU mutant can provide an insight into an efficient alternative to commercially available preparation. It has led to identification of alkalophilic thermostable α -amylase produced by *Bacillus subtilis* from exotic habitat of hot spring.

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