Optimization of Production Conditions and Partial Characterization of Extracellular Amylase from *Bacillus Subtilis* under Submerged Condition

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α-amylases are one of the most important enzymes used in industries. Bacterial amylases have potential application in food, fermentation, textiles and paper industries. Considering these facts the present study was designed to isolate and screen amylase producing bacteria from soil followed by optimizing physiological conditions for enhanced bacterial growth and amylase production. Garden soil was collected and grown on starch plates and the zone of starch hydrolysis was noted. Among 10 isolates, SS4 isolate showing maximum amylase activity was characterized microscopically and biochemically. Based on these results SS4 isolate was confirmed as *Bacillus subtilis*. Effect of various factors such as pH (4-10), temperature (20-45°C), incubation period (24-48 hours) and starch concentration (0.2-1.2%) on growth and amylase production was studied. *B. subtilis* showed maximum growth after 48 hours of incubation, at pH 8 and temperature 37°C in 1% starch containing liquid media. The optimum temperature for amylase production was found to be 37°C. *B. subtilis* showed maximum amylase activity (1.2±0.09 U/mg protein) when cultured at pH 8.0. Amylase production occurred in 0.2-1.2% of starch containing media with a maximum at 1% (1.32±0.09 U/mg protein) after 48 hours of incubation. Amylase was purified and 48 kDa protein was found in SDS polyacrylamide gel electrophoresis.

**Keywords**: Amylase, *B. subtilis*, optimization, partial characterization, submerged condition.

**Introduction**

α-amylase (α-1,4-glucan-4-glucanohydrolase) (EC 3.2.1.1) is the key enzyme hydrolyzing starch into polymers of glucose subunits. Amylases are among the most important enzymes and are of great significance in biotechnology as they constitute a class of industrial enzymes having approximately 25% of the world enzyme market. Being an extracellular enzyme, they have wide applications in starch processing, brewing and sugar production, in detergent industries and drug manufacturing processes. α-amylase is found in various microbes including bacteria and fungi as well as plants. However bacteria are the preferred source of amylase production due to cost-effectiveness, consistency, less time and space requirement, ability to produce amylase in bulk and ease at which it can be manipulated for desired product. *Bacillus* strains have predominantly been reported to produce industrially important enzymes and accounting for nearly 60% of commercially available enzymes. Amylase production from *Bacillus amyloliquefaciens*, *B. subtilis*, *Bacillus caldolyticus* DSM405, *Bacillus licheniformis* GCBU-8 and *Bacillus dipsosauri* DD1 have been reported. For bulk production, optimizations of factors like temperature, pH, incubation period and substrate concentration is the pre-requisite. The present work is undertaken for screening of amylase producing *B. subtilis* from soil and optimization of cultural conditions for efficient production of amylases.

**Materials and methods**

**Isolation of bacterial strains from soil sample**

Soil sample was collected from garden area aseptically in the zip-lock bag. One gram of soil was added to a glass tube containing 10 ml sterilized distilled water and mixed for one hour. Furthermore, serial dilution was done in phosphate buffer saline and was spread on nutrient agar plates containing 1% starch and incubated at 37°C for 48 h. After incubation, 1% iodine solution was overlayed on the agar plates and observation was made to note the substrate utilized zone around the colony. The colonies forming clear zones around them were picked up and streaked on nutrient agar plates to get

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pure culture and to confirm zone formation. The strain that formed a better zone was taken for further study.

Physical and biochemical characterization

The colony morphology of bacterial isolate was observed under the microscope with respect to color, shape and size.

The isolate was Gram and endospore stained. For biochemical characterization the isolate was tested for indole, methyl red, Voges-Proskauer and citrate (IMVIC) test and utilization of different sugars (lactose, dextrose and sucrose), urease and catalase tests. The procedures adopted for all the above physical and biochemical tests were taken from Cappuccino and Sherman. The isolate was identified according to Bergey’s manual of systematic bacteriology.

Determination of optimum growth conditions

For optimum growth of the B. subtilis isolate, pH, temperature, incubation period and starch concentration were considered. For determination of optimum temperature isolate was grown at 20°C, 30°C, 37°C and 45°C. After an incubation period of 24 h, their absorbance was taken at 600 nm using Spectrophotometer (Elico, India). For determination of optimum pH isolate was grown at 4, 5, 6, 7, 8, 9 and 10 pH for 24 h followed by their absorbance was taken at 600 nm. For determination of optimum incubation time isolate was grown at 24, 48 and 72 hours and their absorbance was taken at 600 nm. For determination of optimum starch concentration isolate was grown at 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2%. After an incubation period of 24 h, their absorbance was taken at 600 nm.

Amylase Production

B. subtilis was subjected to amylase production medium (starch 1.0%, yeast extract 0.04%, (NH4)2HPO4 0.4%, KCl 0.1% and MgSO47H2O 0.05%) and incubated in a shaking incubator for 48 hrs at 200 rpm and 37°C.

Extraction of enzyme from bacteria (recovery of amylase)

B. subtilis was grown at 37°C for 24 h in 50 ml of 1% (w/v) of starch medium placed in 250 ml Erlenmeyer flasks and placed in a shaker incubator operated at 120 rpm at 37°C. After incubation period of 24 h grown bacteria was centrifuged at 5000 rpm for 20 min. The supernatant obtained was collected and used as enzyme source for enzyme assays in subsequent experiments.

Amylase activity assay

Amylase activity was determined as described by Okolo et al. In brief, 0.5 ml of crude enzyme, 1 ml of sodium phosphate buffer (pH 7.0) and 1% soluble starch were incubated at 37°C. After 10 min, the amount of reducing sugar liberated in the mixture was estimated by the addition of 2 ml of 3, 5 dinitrosalicylic acid (DNS) method followed by boiling for 10 min. The absorbance of the mixture was measured at 540 nm, and D-glucose was used to create a standard curve. One unit of amylase activity was defined as the amount of enzyme that releases 1μmol of glucose per minute under the assay conditions. Activity of the enzyme was expressed in units per mg protein. All analytical measurements were performed at least in triplicates.

Estimation of Protein Content

Total amount of protein throughout the experiment was measured according to method, using bovine serum albumin (BSA) as Standard.

Determination of optimum conditions for enzyme production

The optimal temperature for activity was determined by incubating the medium at different temperature ranges of 20, 30, 37 and 45°C followed by enzyme activity by DNS method. Furthermore, effect of pH on enzyme activity was investigated by incubating the medium at different pH ranges of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10. Moreover, effect of incubation period on enzyme activity was carried out by incubating for 24, 48, and 72 h, after which assay was determined by DNS method. The present study was carried out at different starch concentration such as 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2%, after which assay was determined by DNS method.

Determination of optimum conditions for enzyme activity

The optimal pH for enzyme activity was determined by measuring the amylase activity in different buffers: sodium acetate (pH 5.0), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 8), glycine–NaOH buffer (pH 9). The optimum temperature for the enzyme activity was evaluated by evaluating the amylase activity at different temperatures (20–60°C) in 0.1 M sodium phosphate buffer (pH 7.0) and 1% soluble starch.

Partial purification of amylase using ammonium sulphate precipitation and SDS-polyacrylamide gel electrophoresis

For partial purification of amylase enzyme, B. subtilis was grown in the presence or absence of 1%
starch. Following growth culture supernatant was precipitated with ammonium sulphate upto 50% saturation and incubated overnight at 4°C with constant stirring. Furthermore, the precipitate was collected by centrifugation at 10,000 g for 20 min at 4°C, re-dissolved in distilled water and electrophoresed onto a 12% SDS polyacrylamide gel\textsuperscript{18}. Gels were stained with coomassie brilliant blue R-250.

Statistical analysis
Experiments were run in triplicate and repeated at least three times. Each time three readings were taken, their mean, and standard error of the mean were calculated. Statistical analysis between two groups was performed using unpaired ‘t’ test and among multiple groups by one-way ANOVA followed by Newman–Keul’s post analysis test. Results were considered significant at p<0.05.

Results and Discussion

Identification and isolation of bacterial isolates
Screening, isolation and production of α-amylase from garden soil was examined in the present study. The collected garden soil was screened for amylase producing bacteria on nutrient agar containing 1% starch followed by iodine reaction. Ten isolates were selected on the basis of hollow zone formed followed by purification by sub-culturing. Furthermore, these 10 isolates were screened for the production of amylase in nutrient broth containing 1% starch. Of all the 10 cultures tested, bacterial isolate SS4 was the highest amylase producer and therefore was selected for further studies (Fig. 1A). Morphological feature of SS4 isolate revealed it Gram positive and rod shaped. SS4 isolate showed positive test for methyl red, voges proskauer, citrate test, dextrose and sucrose fermentation, starch hydrolysis and catalase test, whereas negative for indole test, lactose fermentation and urease test. The results obtained were confirmed with Bergey’s manual of systematic bacteriology\textsuperscript{13} and identified as \textit{B. subtilis}. Other investigators also reported \textit{B. subtilis} as amylase producer \textsuperscript{19,21} Ubalua. (2014) have isolated amylase producing \textit{B. subtilis} agricultural soil\textsuperscript{22}.

Optimum growth conditions
The optimum for the growth of \textit{B. subtilis} was found to be pH 8 (Fig. 1C) and the most suitable temperature for the growth of bacterium was found to be 37°C (Fig. 1D). The highest growth of the bacteria was found to be after 48 hours (Fig. 1E) at 1% starch concentration (Fig. 1F).

Effect of pH on amylase production and activity
pH is one of the important factors that determine the growth and morphology of microorganisms as well as enzyme activity as it modulates substrate binding and catalysis. Aberrant pH results in poor microbial growth and reduction in enzyme production\textsuperscript{23}. The production of α-amylase was investigated at different pH values ranging from 4 to 10. At pH 4, \textit{B. subtilis} had amylase activity of 0.32 U/ mg protein. As the pH increased from 4 to 8, amylase activity was found to be increased consistently. Maximum production (Fig. 2A) and activity was recorded at pH 8. Further increase in pH led to a gradual decrease in amylase activity (Fig. 2E). These results suggest that the activity of enzyme is relatively higher in alkaline pH. This result is in agreement with Olajuyigbe and Ajele. (2005) who also reported optimum pH of 8.0 for \textit{Bacillus} species\textsuperscript{24}.

Effect of temperature on amylase production and activity
Temperature is the most important factor, which markedly influence growth of microorganism and enzyme activity as it could be denatured at high temperature and inactive at low temperature. The effect of temperature (20, 30, 37 and 45°C) on activity of amylase produced by \textit{B. subtilis} was studied. At 20°C \textit{B. subtilis} had amylase activity of 0.32 U/ mg protein, which increased upto 37°C (1.14 U/ mg protein) (Fig. 2B). Similar trend was observed in amylase activity where 37°C was found to be optimum incubation temperature. Further increase in temperature led to decrease in amylase activity (Fig. 2F). It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and enzyme formation was also inhibited. Similar observations were made by Raul \textit{et al.} (2014) who also found 37°C as optimum temperature for \textit{B. subtilis} amylase activity in solid state fermentation (SSF) using wheat bran as substrate\textsuperscript{25}. The influence of incubation temperature on amylase activity by \textit{B. subtilis} was also studied by several workers\textsuperscript{2,18,20}.

Effect of incubation time on amylase production
Incubation period is another parameter that affect the growth microbes and hence enzyme production. The effect of incubation period on the activity of amylase enzyme was demonstrated. \textit{B. subtilis} had amylase activity of 0.96 U/ mg protein at 24 h, this
was followed by an increase in amylase activity at 48 h (1.32 U/ mg protein), which recorded maximum amylase activity. After this hour, there was a decline in amylase activity at 72 h (0.69 U/ mg protein) (Fig. 2C). This may be due to excessive consumption of nutrients and production of other byproducts which inhibit enzyme activity by feedback inhibition\textsuperscript{26, 27}.

Similar observations were made by Oyeleke and Oduwole. (2009)\textsuperscript{28}.

**Effect of starch concentration on amylase production**

Starch has important role in the catalytic activity of amylase enzyme. Effect of different concentrations of starch (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2%) on amylase activity was examined. The maximum (1.3 U/ mg protein) and minimum (0.35 U/ mg protein) amylase activity was observed in 1.0 and 0.2% starch respectively with \textit{B. subtilis} (Fig. 2D). This may be due to higher substrate availability to the enzyme.
Effect of temperature (A), pH (B), incubation time (C) and starch concentration (D) on extracellular amylase production. Effect of pH (E) and temperature (F) on amylase activity. (G) SDS-PAGE pattern of extracellular ammonium sulfate precipitated proteins of B. subtilis. Lane 1 - Protein marker; 2,3 - Nutrient broth; 4 - Amylase production medium. The data is a representation of 5 independent experiments, and have been expressed as Mean ± SEM (†p<0.05 for pH5 Vs pH4, **p<0.01 for pH6 Vs pH4, ###p<0.01 for pH7 Vs pH4, ##p<0.01 for pH8 Vs pH4, *p<0.05 for 37°C Vs 20°C, ¤p<0.05 for 0.6 Vs 0.2, **p<0.01 for 0.8 Vs 0.2, ###p<0.01 for 1 Vs 0.2, &&p<0.01 for 1.2 Vs 0.2, **p<0.01 for pH8 Vs pH5, *p<0.05 for 40°C Vs 20°C).
Partial Purification of $\alpha$-Amylase

Partial purification of $\alpha$-amylase secreted from B. subtilis in the presence of amylase production medium was attained by ammonium sulphate precipitation. Amylase with molecular weight of 48 kDa was observed in SDS-PAGE in the presence of 1% starch (Fig. 2G, lane 4). However amylase was not detected in the absence of starch (Fig. 2G, lane 2 and 3). Shafaat et al. (2011) reported the purified thermo-stable, alkaline amylases of 45 and 15 kDa with molecular mass of 18 and 43 kDa in native SDS-PAGE. Moreover, Swain et al. (2006) reported the purified amylases from B. subtilis with molecular mass of 18 and 43 kDa in native SDS-PAGE. Yang and Liu. (2007) reported 65 kDa amylase protein by SDS PAGE.

Conclusion

Amylase producing isolate SS4 from the garden soil was isolated and identified as Bacillus subtilis. Optimization of the fermentation conditions (temperature, incubation time, pH and starch concentration) were optimized. Based on the results, maximum amylase production was obtained after 48 hours of incubation, at pH 8 and temperature 37°C in 1% starch containing liquid media. It can be concluded that, B. subtilis can be a potential producer of extracellular amylase which could find applications in industry.

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References