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Original Article

Molecular identification of amylase producing *Bacillus subtilis* and detection of optimal conditions

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ABSTRACT

Background: Amylase is of great significance in industrial applications like pharmaceutical, food, textile and paper industries. These enzymes represent approximately 25–33% of the world enzyme market.

Methods: Potential amylase producing bacterial strains were isolated using SAM from sago industries waste site samples and optimized for different parameters.

Results and diccussion: The maximum amylase production (538 U/ml) was achieved when the temperature was around 32 °C at pH 7 by the strain SSII2. The production of amylase was found to enhance when hydrolyzed sago starch was added as a sole carbon source, yeast extract as a nitrogen source and cysteine as an amino acid. After optimizing, the isolates scale up studies were carried out in 5 L fermentor with sago starch waste as a sole carbon source. The production of the enzyme reached 2.72 mg/L after 12 h. After partial purification the concentration of alpha amylase was found to be 54.54 mg/L. The maximum amylase enzyme activity was obtained at the beginning of the stationary growth phase.

Conclusion: The isolate was identified as *Bacillus subtilis* after 16S rDNA sequencing and it proved to be a potent amylase producing strain, which can be further used for potential applications in various biotechnological and industrial processes.

1. Introduction

Amylases hydrolyze starch molecules and yields various products like dextrins and smaller glucose units.[1] It is commonly accepted that, even though other amylolytic enzymes are involved in the process of starch breakdown, the contribution of α-amylase is a prerequisite for the initiation of this process. Starch degrading enzyme such as amylase are of great significance in industrial applications like pharmaceutical, food, textile and paper industries. The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders.[2] Amylase converts starch to sugar syrups and production of cyclodextrins for the pharmaceutical industry.[3] Starch is the second most important carbon and energy source among carbohydrates, followed by cellulose in biosynthesis.[4] Large scale production of α-amylase using various *Bacillus* sp. and *Aspergillus oryzae* has been reported.[5] *Bacillus* sp. is an industrial important microorganism because of its rapid growth rate, secretes enzyme into the extracellular medium and safe handling.[6] This study aims in isolation, molecular characterization of native amylase

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producing *Bacillus subtilis* from the soil samples collected from sago industry waste site and amylase production, optimization conditions and partial purification of α-amylases using cassava starch as carbon source also were studied. Nitrogen sources, pH, temperature, substrate concentration, amino acids, Inoculum concentration, incubation time and surfactants have been optimized for enhanced production and they play an incredible role in amylase production.

2. Materials and methods

2.1. Isolation of amylase producing bacterium and its identification

The soil samples were collected from a sago industrial waste site in Salem (11° 36′ N and 78° 39′ E) Tamil Nadu, India. The collected samples were stored at 4 °C. Starch degrading microbes were isolated using Strach Agar Medium (SAM). The isolates showing maximum clear halo zone were subcultured.[7] Selective isolates with maximum starch degrading activities were identified up to species level.[8,9] The most potent isolates were finally chosen for further studies.

2.2. Inoculum preparation for production of extracellular enzyme

The inoculum for further enzyme modulation and other studies was prepared using Luria Broth (LB) medium. The fresh overnight culture was used as an inoculum for the production of amylase.[10] The inoculated medium was incubated at 37 °C for 48 h by shake flask fermentation method at 200 rpm. The culture broth was then centrifuge at 8000 × *g* 10 min at 4 °C. The free cell supernatant was used as an extracellular crude enzyme.[11]

2.3. Total protein concentration determination

Total protein concentrations were determined by Bradford's method using Bovine Serum Albumin (BSA) as the protein standard.[12]

2.4. Starch hydrolyzed assay

α-Amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The amount of liberated reducing sugar was determined by Dinitrosalicylic acid (DNS) method. Glucose was used to construct the standard curve.[4]

2.5. Effect of pH, temperature and incubation time on amylase production

Five percent bacterial inoculum was added aseptically to 500 ml of sterile growth medium and incubated at 37 °C at 150 rpm. Twenty ml of culture was taken periodically for 48 h at every 6 h intervals. The amylase activity was determined in the culture filtrate. The effect of pH on amylase activity was determined at different pH (6.5, 7, 7.5, 8, 8.5 and 9) and the effect of temperature on enzyme activity was determined using different temperature (26 °C, 29 °C, 32 °C, 35 °C, 38 °C and 41 °C).[11]

2.6. Optimization of different carbon sources, nitrogen sources and amino acids for amylase production

Different carbon and nitrogen sources (both at concentration of 10 g/L) were used in minimal medium, pH 7 and incubated at 32 °C for 24 h. Similarly different amino acids like glycine, alanine, aspartic acid and cysteine were used in the medium for optimization.[13] The culture filtrates were assayed for total protein content and amylase activity.

2.7. Partial purification of amylase enzyme

The culture filtrate was precipitated using 80% w/v Ammonium sulfate precipitation method.[14] Then the precipitate was separated by centrifugation at around 6700 × *g* for 10 min. The pretreatment of the dialysis membrane was done Ashwini et al, 2011.

2.8. Phylogenetic analysis

Genomic DNA was extracted using phenol–chloroform extraction method. The PCR parameters for the amplification of 16S ribosomal DNA were optimized. 50 μl of PCR master mix contained universal primer set 27 F- (5′-AG AGT TTG ATC MTG GCT CAG-3′)/1492 R- (5′-G GYT ACC TTG TTA CGA CTT-3′), 10 mM dNTPS, 10× PCR Buffer, 1 U Taq DNA polymerase, 2 mM Mg^{+} and (100–200 ng) template DNA. PCR steps included initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 2 min, elongation at 72 °C for 1 min and final extension at 72 °C for 10 min. Approximately 1.5 kb amplicons were generated. The PCR product was purified using Gene JET PCR purification kit (Fermentas) using manufacturer's instructions. Purified PCR products were sequenced and sequence search similarities were conducted using BLAST.[4,15] Phylogenetic analysis of sequence data of bacteria under study was aligned with reference sequence homology from the NCBI database using the multiple sequence alignment of MEGA 5.0 Program.[16]

2.9. Laboratory scale fermentation studies

Scale up studies were carried out in a 5 L glass fermentor (Model: Bio Spin-05A, Bio-Age) with a working volume of 3.5 L containing [Sago starch – 10 g, Yeast Extract – 20 g, KH_2PO_4 – 0.05 g, $MnCl_2 \cdot 4H_2O$ – 0.015 g, $MgSO_4 \cdot 7H_2O$ – 0.25 g, $CaCl_2 \cdot 2H_2O$ – 0.05 g, $FeSO_4 \cdot 7H_2O$ – 0.01 g, Cysteine 1 g (g/L)] at pH – 7.0. Fermentor glass vessel containing 3.5 L of fermentation medium was sterilized in an autoclave for 20 min at 15 lbs pressure (at 121 °C) and cooled to room temperature. 350 ml of 10% inoculum was transferred to the fermentor vessel through a port at the top plate under aseptic conditions. The incubation temperature was 32 °C, while the aeration and agitation rates were maintained at 0.8 L/L/min (DO) and 95 rpm respectively throughout the fermentation period. The air to be supplied was sterilized by passing through Millipore membrane filters (0.2 μm pore size). Sterilized solution of 1 N HCl/NaOH was used for pH adjustment. Sterilized

polypropylene glycol (0.01% (v/v) of 50%) was used to control foam, formed during the fermentation process. After incubation, the fermented broth was filtered. The filtrate was used for the estimation of alpha amylase.[17]

3. Result and discussion

3.1. *Screening of amylase producing bacterial isolate from sago industrial waste*

Sago industrial waste soil samples were used for isolation of amylase producing bacteria on SAM. Totally 30 different soil samples were collected from sago starch industry waste sites. Among that 22 isolates showed amylase activity upon primary screening using SAM supplemented with cassava starch as a carbon source. Only two out of 22 isolates showed high amylase activity. One potential isolate (SSII2) was identified by standard morphological and biochemical characterization and it was confirmed to be *Bacillus* sp.

3.2. *Effect of incubation time, temperature and pH*

The maximum amount of amylase production was observed with 42 h incubation. The high protein content of 2.99 U/mg and the maximum enzyme activity of 456 U/ml was observed at 24 h (Fig. 1a). The main advantage of enzyme production by *Bacillus* sp. is a shorter incubation period which will reduce cost as well as autolysis of the enzyme created by protease itself during the fermentation process.[18] Previously amylase activity had been reported in *B. subtilis* (22.92 U/ml) after 72 h and *Bacillus amyloliquefaciens* after 72 h.[6] Maximum yield of 550 U/ml of enzyme and protein content 3.43 U/mg was observed at 32 °C (Fig. 1b). A decrease in enzyme yield was observed with further increases in temperature. This report is similar to work done by Ganiyu 2005 amylase activity gradually increased from 20 to 50 °C in waste water from cassava fermented with pure strains of *Saccharomyces cerevisiae* together with *Lactobacillus delbruckii* and *Lactobacillus coryneformis*.[19] The optimal temperature recorded for maximal growth and α-amylase production by *B. subtilis* in the present study 32 °C which is almost identical to the work by Unakal et al, 2012 reported maximum enzyme yield for *Bacillus licheniformis* grow on wheat bran, for *B. subtilis* grow on banana stalk.[20] The potent pH was found to be 7 which showed protein content 1.34 U/mg and maximum enzyme activity of 483 U/ml (Fig. 1c). The pH of 6 and 7 has been reported for normal growth and enzyme activity in *Bacillus* strain isolated from soil. Optimal pH at 32 °C for amylase production was reported using *Bacillus thermooleovorans* NP54, *Bacillus coagulans*, *B. licheniformis*, and *B. subtilis*.[6]

3.3. *Effect of media components on amylase production*

Various carbon sources, nitrogen sources and amino acids were used for the production of amylase by *B. subtilis*. Glucose in the basal medium was replaced by other carbon sources such as glycerol, soluble starch, glucose, mannitol, sago starch and maltose. Mannitol was found to be effective and showed higher protein content 1.34 U/mg and enzyme activity of

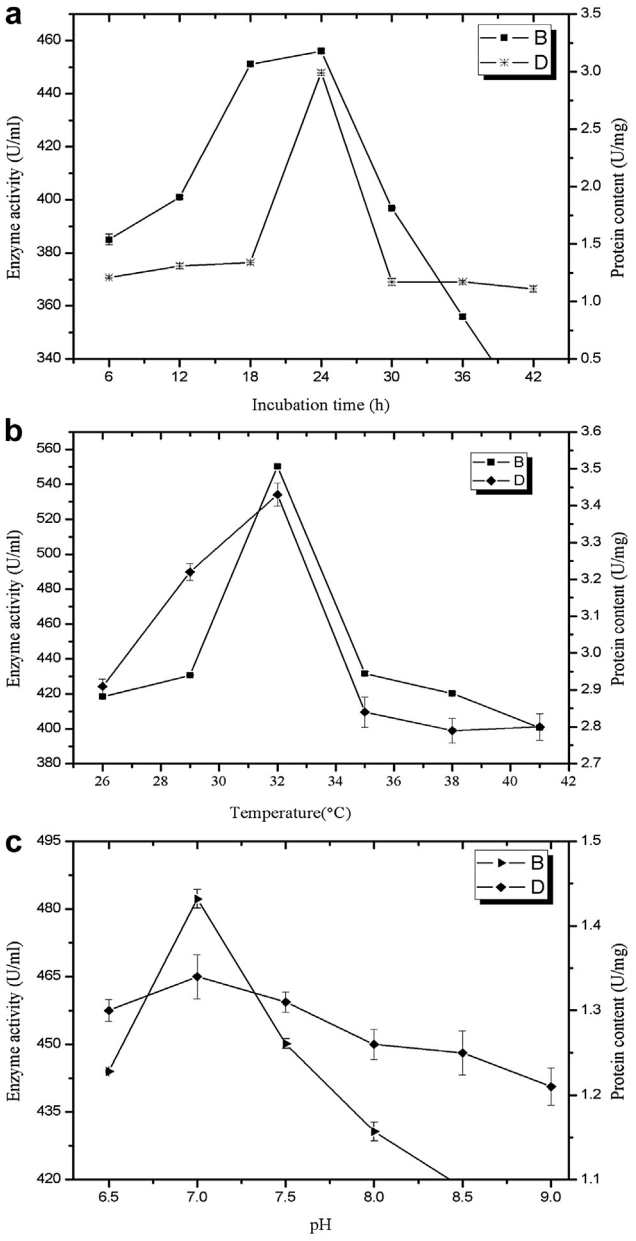

Fig. 1 – a: Effect of incubation period of amylase production by *B. subtilis* (B-Enzyme activity U/ml; D-Protein content U/mg). b: Effect of temperature on enzyme activity and protein content. c: Effect of pH on enzyme activity and protein content.

0.538 U/ml (Fig. 2a). The results were contradictory to the study conducted by Vijayalakshmi et al where six different carbon sources were used for amylase production and the maximum activity was observed with starch as the carbon source. Even though the maximum activity of amylase enzyme was observed in the presence of mannitol as a carbon source, sago starch is used for supplementation in the production process, because it acts as a cheap source as compared with mannitol. Enhanced extracellular α-amylase production using sago starch as the carbon source, provides a way to utilize the sago starch. Nitrogen is found to be playing a prominent role in the growth and development of the bacteria

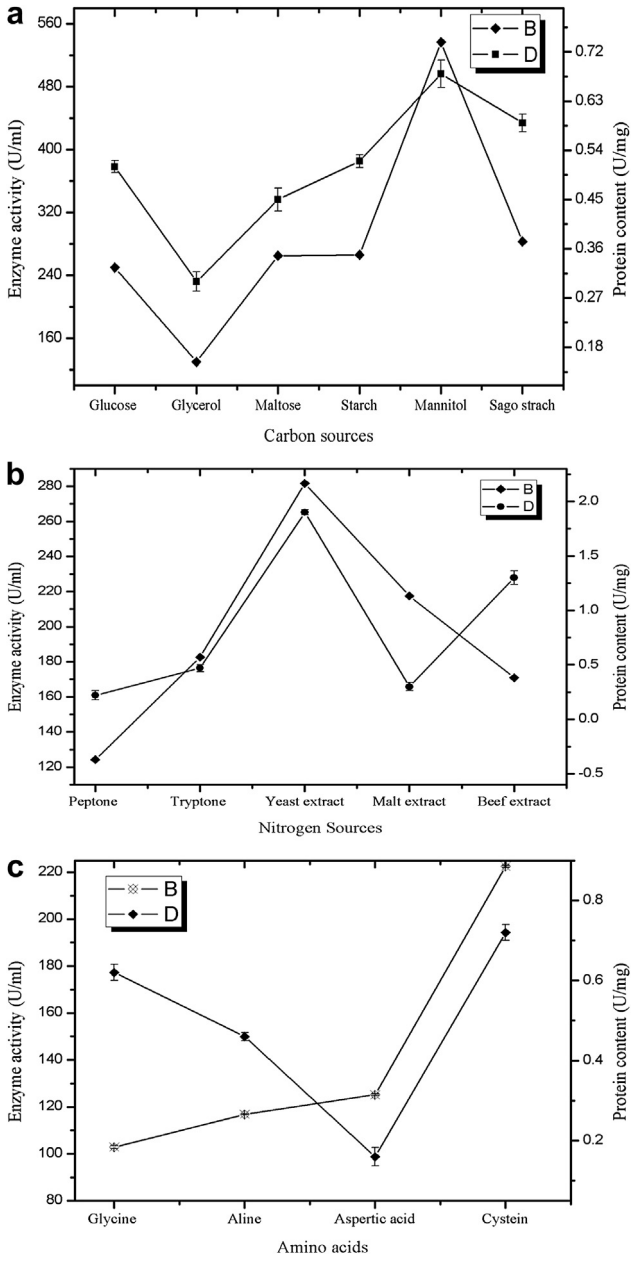

Fig. 2 – a: Amylase activity with additional supplementation of various carbon substrates. b: Amylase activity with additional supplementation of various nitrogen substrates. c: Effect of different amino acids on enzyme activity and protein content for amylase production.

in this study. Hence different nitrogen source is used and yeast show high protein content of 1.9 U/mg and maximum enzyme activity of 281 U/ml (Fig. 2b). Similar results were obtained by in the production of amylase by *Bacillus marini*.[21] In this study amino acid cysteine was found to be the better source for enhanced production. The high protein content of 0.72 U/mg and maximum enzyme activity of 222 U/ml was observed in the presence of cysteine (Fig. 2c). Our results are contradictory to previously reported[13] where aspartic acid showed higher amylase production.

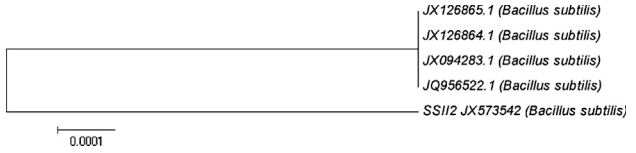

Fig. 3 – Phylogenetic tree based on homology sequence of 16S rDNA analysis. Each number on a branch indicates the booststrap confidence values (500 replicates). The scale bar indicates 0.0001 substitution per nucleotide position.

3.4. *16S ribosomal sequence detection*

The selected potential isolate were identified by 16S rDNA sequencing and PCR parameters were optimized for maximum amplification of 16S rDNA gene. BLAST was performed for obtained sequences in order to find out homology with the sequence in GenBank in which 99% similarity was found with *B. subtilis* JX573541. Following BLAST, the best five sequences were selected. All ambiguous position were removed for each sequence pair was assessed by using BOOTSTRAP program in sets of 100 re-samplings (MEGA-5). The phylogenetic tree was constructed from the sequence data by neighbor joining methods phylogenetic analysis, and was identified as *Bacillus* sp. scale bar indicates 0.0001 substitutions per nucleotide position (Fig. 3).

3.5. *Submerged fermentation (small scale)*

The fermentation rate of SSII2 (*B. subtilis*) strain for the alpha amylase production was investigated in 5 L submerge fermentor. The culture aliquots were withdrawn every 6 h, starting from 12 h aseptically and subjected to enzyme estimation up to 40 h of fermentation period. After submerged fermentation, the maximum activity of amylase was obtained in the enzyme extract harvested after 12 h at pH 7 and 32 °C temperature. During submerged fermentation process the production of amylase reached maximum of 4 U/ml at 10 h of incubation period. The enzyme production reached its maximum enzyme production 2.72 g/L at 12 h.[20] Partial purification of amylase enzyme by ammonium sulfate precipitation showed maximum protein content of 54.54, which is mg/L up to 80% purification fold.

4. Conclusion

Amylase assay showed maximum extracellular enzyme activity of 538 U/ml. Optimum parameters were identified in submerged fermentation which was carried out in a 5 L fermentor with a working volume of 3.5 L and the maximum protein content was estimated to be 2.72 mg/L. Ammonium sulfate precipitation was performed to partially purify the fermented product and it showed maximum protein content of 54.54 mg/L which is about 80% higher than non purified enzyme. The SSII2 isolate was characterized by 16S rDNA sequencing and found to be *B. subtilis*. The partially purified protein can be further characterized by SDS-PAGE analysis and column chromatography. By doing so, a stable amylase with higher enzyme activity can be identified which may have

wide industrial applications and high amylase producing potential.

Conflicts of interest

All authors have none to declare.

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