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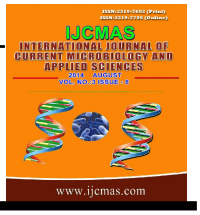

Original Research Article

Characterization of Amylase gene in Bacillus species isolated from different soil samples

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Keywords

Bacillus, Molecular characterization, Amylase enzyme, Primer

A B S T R A C T

Starch is an abundant carbon source in nature, and a-amylase (1, 4-a-D-glucanohydrolase; EC 3.2.1.1), which hydrolyzes a-1, 4-glucosidic linkage in starch-related molecules, is one of several enzymes involved in starch degradation. Alpha amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries. In the present study soil samples that contain kitchen waste soil samples rich in proteins were processed for isolation of bacteria capable of extra-cellular amylase production. A total of 12 bacillus species were isolated from the soil samples. The bacterial isolates obtained were screened using the starch hydrolysis plate assay and the isolates showing maximum zone of clearance were morphologically and biochemically characterized according to the Bergey's manual of determinative bacteriology. PCR amplification of the amylase gene (Amy α) for the selected bacterial isolates was performed using specific primers Amy F and Amy R. The bands observed under UV-transilluminator confirmed the presence of amylase gene in all the five bacterial isolates.

Introduction

Amylases contribute as a major class of industrial enzymes constituting approximately 25% of the enzyme market Sindhu M.K. et al., (1997) and Rao M. et al., (1998). Microbial production of amylase is more beneficial than other sources because it is economical; its production rate is high and can be engineered to obtain enzymes of desired characteristics. The most widely used thermostable amylases in the starch industry are produced from *B. licheniformis* Morgan, (1981**)**.

The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries, paper industries, etc. Moreover, microbial amylases have a broad spectrum of industrial applications as they are more stable with great genetic diversity, high enzymatic activity in a wide range of conditions (extreme pH, temperature, osmolarity, pressure etc.), simple and cost effective production and easily standarized to obtain enzymes of desired characteristics Tanyildizi et al., (2005), Vidyalakshmi et

al., (2009). With the emergence of biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. These increased uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes Aiyer, (2005). The major advantages of using microorganisms for production of amylases are their ability to produce in bulk and the ease at which it can be processed for desired products Lonsane, (1990).

Amylases are known to be produced by a variety of bacteria and fungi and their applications at industrial level have stimulated the interest to explore their amylolytic activity in several microbes to be used as bio-resources Akpan et al., (1999), Oliveriea et al., (2007). The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermo stability Pandey et al., (2000). Such industrially important microorganisms are infact found within the genus *Bacillus* and can be exploited commercially for their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the extracellular medium and safe handling. *Bacillus* is endowed with the production of thermostable α-amylase and also large quantities of other enzymes. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus* i.e. *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* Burhan et al., (2003). Some *Bacillus* strains produce enzyme in the exponential phase, whereas some others in the mid-stationary phase. Though, different *Bacillus* species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain.

The aim of the present study is to isolate amylase-producing *Bacillus sp.* from the soil samples receiving kitchen waste and its molecular characterization using gene-specific primers Amy F and Amy R.

Materials and Methods

Sample collection

Soil samples from various apartment dumping sites were collected from areas located in Bangalore. The samples were collected from a depth of 5-6 cm after scraping the top layer. The samples were brought to the laboratory in sterile zip lock covers and stored in refrigerated conditions if not used immediately.

Isolation of bacterial cultures

Isolation of soil bacteria was performed by serial dilution. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to nutrient agar plates. The sample was spread uniformly. The plates were incubated at 37°C for 24 hr. Individual colonies were isolated and maintained on nutrient agar slants. Only isolates which were Gram's positive bacillus were selected for the present study.

Screening of potent amylase producing bacteria was carried out by starch hydrolysis test. Bacterial isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate Aneja, (2003). The microbial isolates were streaked on the starch agar plate and incubated at 37°C for 48 hours. After incubation iodine solution was flooded with dropper for 30 seconds on

the starch agar plate. Presence of blue colour around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The isolates that produced clear zones of hydrolysis were considered as amylase producers and were further investigated.

The positive cultures were characterized by morphological, biochemical characterization as per Bergye's Manual of Determinative bacteriology.

Isolation of genomic DNA

Genomic DNA was isolated using the phenol-chloroform isolation method as described by Sambrook and Russell, (2001). 2 ml of fresh bacterial cell suspension (18 h old bacterial cell suspension grown in Luria–Bertani broth) was asceptically transferred and centrifuged at 15,000 rpm for 10 mins at 4°C. The pellets obtained were resuspended in 500 µl lysis buffer containing 0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS. Further extraction was carried out by phenol-chloroform method The sample was deproteinated by adding equal volume of phenol (Tris-equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15,000 rpm for 15 mins at 4°C. The aqueous phase was carefully transferred into a fresh tube and the process was repeated once more. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15,000 rpm for 15 mins at 4°C to separate the aqueous phase which was then transferred to a fresh tube. Then the DNA was precipitated by incubation at -20°C overnight after adding equal volume of chilled absolute ethanol. The precipitated DNA was collected by centrifugation at 15,000 rpm for 15 mins at 4°C and the pellet washed with 70% ice cold ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes left open until the pellet dried. The DNA pellet was dissolved in autoclaved distilled water.
The isolated DNA was quantified using a Nanodrop spectrophotometer (Abs260) and the purity of DNA assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs260/Abs280). Agarose gel electrophoresis was performed to qualitatively determine the isolated DNA.

PCR amplification using gene specific primers

The isolated DNA samples were amplified using gene specific primers AmyF5'AGG CGC GCC AGT GCT GAA ACG GCG AAC AAA TCG AA 3' and 5'TTG CGG CCG CTC AAT GGG GAA GAG AAC CGC TTA AG 3'. The PCR mixture consisted of 2.5µl of PCR buffer, 2 µl dNTPs, 1µl each of forward and reverse primers, 1 µl Taq DNA polymerase and 1 µl of template DNA. The PCR conditions used were initial denaturation at 95°C for 5mins, followed by 35 cycles of 1min denaturation at 94°C, annealing at 54°C for 1min, extension at 72°C for 3mins and the final extension was extended for 15mins.

The PCR products were run on 1% agarose and viewed under the UV-transilluminator of the Alpha Imager Gel Documentation system for observation of the desired amplification of the gene of interest.

Results and Discussion

Isolation of the amylase producing organisms

The soil samples yielded different types of colonies on the agar plates with different

colony morphology. The isolated colonies on nutrient agar plates were analyzed for their colony characteristics and Gram's staining. Of the total twelve isolates which were gram positive bacilli were subjected to amylase activity, out of which five were found positive and further subjected to molecular work.

Screening for Amylase producing *Bacillus sp.*

The isolates were primarily screened for their amylase production by starch hydrolysis test. The five isolates showing maximum zone of inhibition as mentioned by Atlas et al., 1995 were selected and maintained in nutrient agar slants for further analysis.

Morphological and Biochemical characterization of the isolates

The amylase-positive isolates were further characterized morphologically and biochemically based on the Bergey's manual of systemic bacteriology. The results obtained ascertained the selected strains were *Bacillus sp.*

Qualitative estimation using Agarose gel electrophoresis

The DNA isolated was run on agarose gel to determine the presence of DNA (Figure-1).

Quantification of DNA using Nanodrop

DNA was isolated from the strains which showed maximum amylase production. These cultures were: X_7, X_{10}, PA_1, PA_2 and PB_2. Quantification of DNA was carried out to determine the purity of the DNA isolated from the different samples. The concentration of DNA (in μg/mL) for each sample was also determined from the NanoDrop spectrophotometer (Figure-2).

PCR amplification

PCR amplification of amylase gene was observed in the lanes using gene specific primers Amy F and Amy R (Figure-3).

The present study deals with the molecular characterization of amylase gene from *Bacillus* species. The novelty of this project lies in the use of specific primer, AmyF and AmyR for amplification of the amylase gene commonly found in the *Bacillus* sp, isolated from soil (kitchen area soil) with properties of meeting the changing industrial and economic requirements.

A total of 10 pure cultures were obtained which were checked for amylolytic activity of which five showed highly positive results. The five highly amylase positive cultures were selected and subjected to biochemical characterization. This was done for confirmation of the genus to which the organisms may belong to. These were then processed for DNA isolation and amplification by PCR. The DNA from the bacterial samples was isolated by Phenol-chloroform extraction method. The qualitative estimation of the DNA on 0.8% agarose gel gave single, sharp and distinct bands devoid of any smear. Thus, DNA of good quality without any degradation was successfully isolated from all the eleven samples.

Specific primers namely AmyF and AmyR which were 35 bp sequences were used for the amplification of the amylase gene in the DNA sequence of the isolated bacterial species. The bands of amplified gene sequence were visible after the PCR products were run on 1% agarose gel and viewed on the Gel Doc.Strains were isolated

from the soil sample near kitchen area which are considered to show high amylolytic activity. The nature of culture conditions, temperature and pH for the optimal growth of microbes and production of amylase by the isolated bacterial strains has been observed in this study. The study was carried out on a small scale to find out whether the isolated bacterial strains are amylase producing organisms.

The study holds a variety of future applications in the industrial and research field. The microbial production of amylase is beneficial as it is economical, gives high yield and it can be engineered to produce enzymes with desired characteristics. Microbial amylases are used potentially in pharmaceuticals, fine chemical industries, paper industries, food and beverage industry, textiles etc (Kaur *et.al*, 2012). The gene for amylase production further be cloned into other organisms and the process of amylase production can be optimized. Thus, the present study holds a diverse range of future applications.

Fig.1 DNA bands visualized on Agarose gel electrophoresis

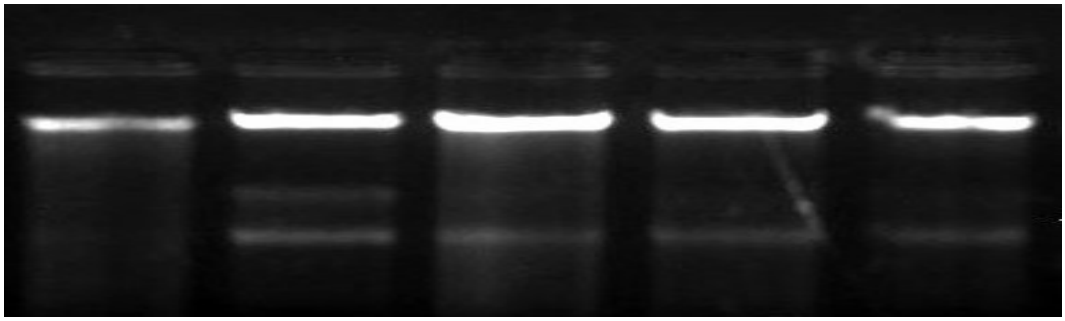


Fig.2 Quantification of DNA using NanoDrop

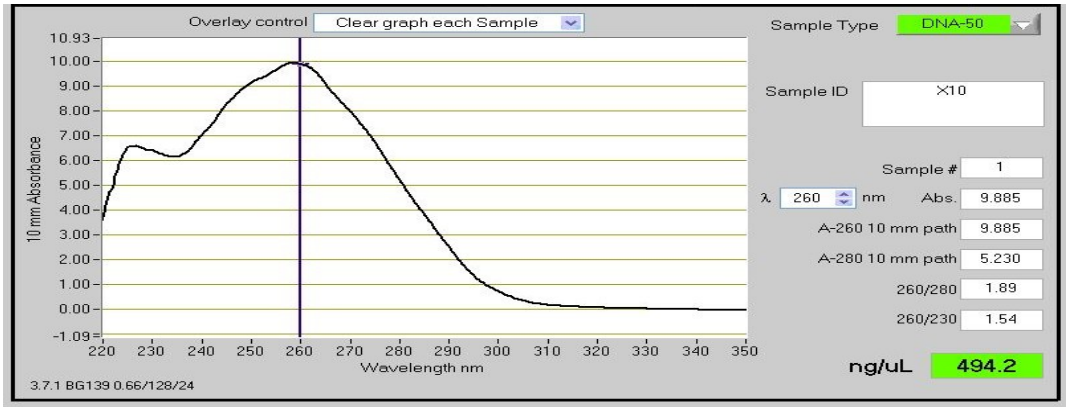
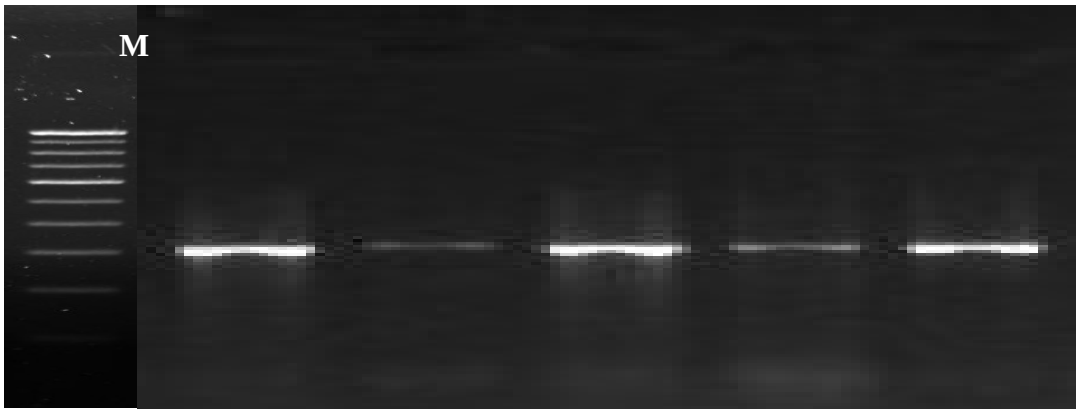


Fig.3 Amplification of the amylase gene using primers AmyF and Amy R



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