Identification, Characterization of Novel Halophilic Bacillus Cereus Ms6: a Source for Extra Cellular A-Amylase

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ABSTRACT

Bacterial enzymes are the ones that are having wide variety of industrial applications. Among these enzymes, a-amylase having high demand in industries such as pharmaceuticals, textiles, food processing etc; so, in this study we have identified novel Bacillus cereus MS6 bacteria, which can produces extra cellular a-amylase. Bacteria were isolated from sewage water of Mysore paper mill Bhadravathi, India. Bacterium was identified based on polyphasic taxonomic approach using phenotypic and genotypic methods. The bacteria are gram positive rod shaped, motile, Central Spore position and no swelling. The identification of the bacteria was confirmed by 16S rRNA gene sequencing studies. The phylogenetic tree revealed that the cells fit in to an evolutionary cluster comprising members of Bacillus cereus with 99% similarity. Further, this bacterium was exploited to produce a-amylase, which was purified by DEAE-Cellulose anion exchange and sepharose gel filtration chromatography, resulted in high yield of enhanced purity. Purified alpha amylase was shown approximate molecular weight of about 27 kDa and the optimum activity of amylase was observed at 45°C and pH of 7.0

Key words: Bacillus cereus MS6, 16S rRNA, Halophiles, a-Amylase, DEAE-Cellulose and Sephrose gel filtration chromatography.

Introduction

A- Amylases (E.C.3.2.1.1) from Bacillus species have potential worthwhile applications in many industries such as pharmaceutical, textile etc; therefore, these enzymes account for about 30% of the world’s enzyme production [7]. The major three classes of amylase have been identified in many microorganisms, namely a- amylase, β- amylases and glucoamylases [17]. All these enzymes are belongs to Glycosides hydrolases, although some a amylase was classified in the family 57. In this study, we described the isolation of a novel halophilic strain identified as Bacillus cereus MS6. The purification and biochemical characterization of the extracellular a- amylase was also investigated. These enzymes were isolated from many microbial sources including bacteria, fungi and some actonomiotics that are resistive to words high salt concentration, temperature and pH [15, 7].

In present investigation, the novel Halophilic Bacillus cereus MS6 is involved in the production of a amylase in starch and mineral containing media. The production was observed on different temperature and pH profile. The media was optimized for high yield in the production of enzyme.

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The identification and characterization of isolates was performed on the basis of biochemical tests and 16S rRNA gene sequencing method. The novel halophilic Bacillus species was isolated from many sources like industrial effluents, salt lakes and extreme environments are able to meet the industrial harsh condition. The α-amylase was purified using ammonium sulfate precipitation, ion exchange and gel filtration chromatography. The purified enzyme was characterized by SDS-PAGE.

Among bacteria, the Halophiles in comparison to the extensive uses of extremozymes from thermophiles and alkaliphiles, very few Halophilic enzymes have thus far found applications in industries and biotechnology [5, 7]. These bacteria are metabolically more versatile than the archaea and their enzymatic activity is more diverse [2,21]. In industries, Halophilic bacteria were used in the production of exopolysaccharides, biofuels, in the treatment of waste water, glycerol production, bioelectronic materials such as bacteriarhodopsin and enzymes like amylases, esterase, protease and xylanase [14,15]. Bacillus diposauri, Halobacillus sp, Halofexar mediterranei, Halothermothrix sp are known to be good producer of α-amylase. However, the production of α-amylases depends on the strain, composition of media, metal ions, pH, temperature, moisture and oxidative stress [7,17,21]. Due to extensive industrial uses which withstand the high load of salt concentration about 10-25%, the enzymes were preferred from halophilic bacteria. Most of the amylase enzymes were metalloenzymes, which require calcium ions (Ca²⁺) for these activity, structural integrity and stability. The application in the conversion of starch to sugar syrup, production of cyclodextrin, food, paper and textile industries etc, have gain the suitable advantage from α-amylase [5, 14,21]. Due to, increase in the demand for these enzymes in various industries, provided the up limit to industrial harsh condition.

Materials and methods

Sampling and Isolation of Bacteria:

The effluent Samples were harvested from the Mysore Paper Industry (MPM) Bhadravathi town, Shimoga district, Karnataka, India. The effluent was collected in different 30 ml sterile plastic tubes and stored in cold condition until use.

The samples were serially diluted and each diluted samples were plated for nutrient rich media, containing 1% (w/v) starch as sole carbon source and incubated at 37°C for two days [5,6]. Large clear zone around the colony was observed when folded with 1% KI solution in to the media was selected for further identification and optimization for enzyme production.

Identification and Characterization of Bacterial Isolate:

Sub cultured isolate was used to inoculate medium having Nutrient Broth containing 1% (w/v) starch as carbon source. Flasks were incubated in a constant shaker (250 rpm) at 37°C for 24-36 h. The isolates were examined for their cell shape, Gram staining, spore formation, motility and pigmentation. Biochemical tests were performed according to Bergey’s Manual of systemic Bacteriology (1994).

Genomic DNA was isolated as described by Ausubal et al, with slight modification Madhusudan et al. Universal bacterial primers were used as a template for the amplification of 16S rRNA gene using PCR. The amplification pattern was analyzed by 2% agarose gel electrophoresis. The amplicon was eluted and purified from the gel using QIA quick PCR purification kit (Qiagen).

The purified amplicon was verified and sequenced by ABI automated DNA sequencer (Applied BioSystem, Sigma).

Partial 16S rRNA gene sequence was quired to NCBI-BLAST (http: // www.ncbi.nlm.nih.gov/Blast) and the nearest neighbor of the isolate was determined. The sequence was submitted to Genbank (NCBI) database to obtain the accession number for isolate. The construction of phylogenetic tree was performed by neighbor-joining tree algoritham using boot strap valve of the MEGA4. (http://www.megasoftware.net/) [20].

Growth Curve:

One volume of inoculum was incubated on the Leuria-Bertine broth (LB broth) supplemented with 0.25 % Ca²⁺ and incubated at 37°C.

At different intervals of time, the bacterial culture was measured optically at 660 nm. Each time the OD valves were recorded and plotted against number of viable bacterial count on Y axis and time of incubation on X-axis.

Optimization of A-amylase Production Parameters:

For the production of the enzyme, Starch (1%), yeast extract (5%), peptone (0.5%), MgSO₄·7H₂O (0.2%), CaCl₂ (0.25 %), NaCl (10%) and distilled water. The pH of the culture medium was adjusted to 7.0.

For the optimization of Bacillus cereus, 1 % of inoculum was added to the culture and incubated the flasks in a rotary shaker at 37°C for 24 to 48 hours at 250 rpm.

After specific intervals of time, samples were taken out to determine enzyme activity and protein concentration.
Enzyme Assay:

Amylase assay was performed according to the method as described by Miller et al., One unit is defined as the amount of enzyme required to liberate one mmol of reducing sugar per minute by applying the following formula [8].

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\text{Activity of enzyme} \times 1000 \quad \frac{\text{IU/ml/min}}{\text{Molecular weight of Maltose} \times \text{Incubation time.}}
\]

Estimation of Protein:

Total amount of protein throughout the experiment was measured according the method Lowry et al., using Bovine serum albumin (BSA) as standard.

Purification of Bacillus Cereus MS6 α Amylase:

Bacillus cereus MS6 was cultured for 24-48 hours in optimized starch broth at 40°C. After incubation, the culture was centrifuged at 10,000 rpm for 15 min at 4°C.

The supernatant was separated from the pellet containing cell debris. The ammonium per sulfate was added to the culture supernatant to get 75% saturation level from initial zero concentration. Then the precipitated solution was recentrifuged at 6000 rpm for 10-15 min at 4°C. The pellet obtained after centrifugation was dissolved in minimum volume of 50mM Phosphate buffer, pH 7.0. Overnight dialysis was performed using 10,000 MW cutoff dialysis bag for the precipitate against 0.001 phosphate buffer with respective three changes of same buffer, dialysate was centrifuged to 10,000 rpm at 4°C. Pellet was separated and freeze-dried.

The lyophilized enzyme was dissolved in minimum amount of 0.001 M phosphate buffer and applied on to the sephrose column(1.5 X 25 cm) previously equilibrated with 20mM Phosphate buffer. The fractions were collected by eluting the enzyme buffer with respective three changes of same buffer, the active fractions were collected by measuring the optical density at 280nm. The active fractions were collected and small aliquots were tested for enzyme activity by iodine method using micro plate method. Fractions showing amylase activity were pooled and again applied to (1 X 20 cm) DEAE-Cellulose matrix column (Bangalore genei).

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The column was previously equilibrated with 50mM phosphate buffer pH 7.0. The enzyme bound to the column was eluted with a linear gradient of 25 mM phosphate buffer containing 0-0.3 M NaCl at a flow rate was 10 ml/hour, 2ml fractions were collected and checked for enzyme activity.

Based on activity profile, the active fraction was lyophilized for further characterization.

Characterization of Purified A-amylase:

Electrophoresis:

SDS-PAGE was performed using 12% polyacrylamide gel under non-reducing conditions [10]. The protein bands were visualized by staining coomassie brilliant blue. The molecular weight of the purified enzyme was determined by comparing with Rf valves of standard molecular weight markers. (Bangalore genei)

Effect of Ph and Temperature Stability:

The activity of the purified α amylase was characterized by monitoring the activity at different pH ranges from 7-12 and stability of the pH range for maximum activity was also determined. Effect of the temperature on the activity of purified α amylase was monitored in the range from 37°C to 100°C and its stability to that temperature is determined.

Results:

Screening and Characterization of Amylase Producing B. Cereus MS6:

Morphological and chemotaxonomic feature of amylase producing bacterial isolates was performed according to the Bergey’s Manual of Systematic Bacteriology [19]. Nutrient agar with 5% salt concentration was used for growth and maintenance of the isolate [16]. Isolate was aerobic gram positive rod shaped bacteria, showing irregular marginal surface. It is a motile and grows in the bunch of chains with central spore forming position.

A growth feature of the isolates was determined in different temperature and salt concentration showing highest viability of propagation is about 50°C with the 10% NaCl concentration. (Fig. 1) The isolate involved in the utilization of sugars like glucose, xylose, galactose, salicin, also showed positive towards citrate utilization, vogous praskus, nitrite utilization and starch hydrolysis. The detailed tests were performed in the identification and biochemical behaviors are summarized in the table 1. Based on the morphology and biochemical property of the isolates could belong to bacillus species.

The amplicon of 16S rRNA gene was sequenced and aligned using Clustal W, NCBI. Based on the sequence alignment on BLAST revealed the isolate belongs to bacillus species with the maximum evolutionary relation to Bacillus cereus with 99% sequence similarities.

The sequence was deposited in NCBI using Bankit program and assigned with the accession number HM245778.
The phylogenetic tree was constructed using the neighbor-joining tree making algorithm of the MEGA 4 package (Fig. 2)

Purification of a Amylase:

α- Amylase from Bacillus cereus was purified by three steps: ammonium sulfate precipitation (75%) and desalted using Sepharose 4B gel filtration column and DEAE ion exchange chromatography and; (Fig. 3) and (Fig.4) the detailed purification steps are summarized in the table 2. The purity of the enzyme after DEAE cellulose chromatography was more than 27 fold, when compared to the crude extract of enzyme supernatant. The specific activity of the enzyme after complete chromatographic steps was shown 154.7 U/mg. Where as, the fold purity and specific activity in initial crude extract and Ion exchange chromatography was 1.0, 7.4U/mg and 20.9, 28.36 U/mg respectively.

Molecular Weight of the Purified Alpha Amylase:

The purified a amylase was characterized for its apparent molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The resulted single protein band on the 12% gel showed the molecular weight of 27 kDa. The molecular weight of purified enzyme was calculated according to the RF valves of the standards used from 94 to 14 kDa.

Properties of a Amylase:

Influence of Temperature and Ph on the Activity of Enzyme:

The optimum temperature maximum was determined by performing activity changes in the range of 37 ⁰ to 100 ⁰C. Soluble starch was used as a substrate in the reaction condition. The optimum activity was shown to be at temperature 45 ⁰C and activity was stable up to 55 ⁰C for 1 hour with the addition of substrate (Fig 6) at pH 7.0. At a temperature of the 45 ⁰C, the enzyme showed the maximum activity. However, enzyme activity was retained at the pH 9.0 for 3 hours at 45 ⁰C. (Fig 7)

Effect of Substrate on the Activity of a Amylase:

The effect of different concentration of starch (0.2 -3.0 ml) for amylase activity was determined and the data showed maximum enzyme activity in 1 to 1.5 % of starch concentration. Progress in the activity of enzyme was evident upon increase in the substrate concentration up to 3%. (Fig. 8).The maximum activity was arrested upon increase in the starch level but, the relative activity was stable up to 3% and ceases above this level.

![Fig. 1: Growth kinetics of Bacillus cereus MS6 in the medium containing starch as a carbon source.](image-url)
Fig. 2: Phylogenetic position of *Bacillus cereus* MS6 generated MEGA 4 package. The numbers of nodes represent the percentage of bootstrap value obtained from 1000 sampling. Bar 0.5 nucleotide substitution per site.

Fig. 3: DEAE-Cellulose anion exchange chromatographic purification of MS6 α-amylase using 0.1 to 0.5 M NaCl gradient.
Fig. 4: Sephrose 2B gel filtration column purification of MS6 α-amylase.

Fig. 5: SDS-PAGE analysis of final purified α-amylase from *Bacillus cereus* MS6 showing single band (Right lane) of approximate molecular weight 27 kDa. Left lane represents the protein standards from 66 to 14 kDa.

Fig. 6: Effect of temperature on purified MS6 α-amylase activity showing the maximum activity at 45°C temperature.
Discussion:

Novel Halophilic strain was isolated from effluent of paper industry. The strain was characterized by physiological and chem. taxonomical approach revealed the strains belongs to Bacillus sp. The growth characteristic of strain showing the maximum stability at the temperature 40-45°C but, can able to grow when increase in the temperature to 55°C. The ability to withstand the salt concentration was determined using the liquid media with varied in salt concentration, ranging from 1% to 25%. The strain showed the maximum growth in 15% salt containing media. Further, 16S rRNA gene sequencing studies it was conformed that the Bacillus sp showed 99% sequence similarities to Bacillus cereus. Further the production of alpha amylase was optimized using mineral containing media. Alpha amylase was purified from culture supernatant by using ammonium per sulfate, sepharose gel filtration and DEAE cellulose chromatography. The purified alpha amylase showed the molecular weight approximately 27 kDa. The enzyme activity was shown optimum at 45°C and stable up to 55°C for 1 hour. PH 7 to 9 was required for the activity but shown maximum at neutral and able to shown activity even at pH 9 for 3 hours under optimized temperature.

Activity and specific activity of the final purified enzyme was shown 32.5mg/ml and 156.7 UI/mg respectively with 22.108 fold purity and 13.308% of yield. Similarly α-amylase from Penicillium olsonii is purified by gel filtration and ion chromatography with 25.14 fold purification and specific activity of 23.43 mg/ml of protein [1]. In comparison with P. Americana required 3 steps of purification gel filtration, ion exchange and affinity chromatography [18]. The purified α-amylase has a specific activity of 94.32 UI/mg, which corresponds to 38.42 fold purification, and a yield of 24.41%. The purification of P. Americana α-amylase was confirmed by the appearance of a single band after SDS/PAGE.
The molecular weight of the purified a-amylase, which is a nonnumeric enzyme, was estimated to be 60,000 (by SDS/PAGE) and 48,000 (by gel filtration on TSK column gel), SDS-PAGE 27 kDa.

The optimum hydrolysis pH of the purified a-amylase is 5.6. This acidic pH is within the optimum hydrolysis pH of glycosidase that generally spreads from 4.5 to 6.0 [6,9]. The optimum pH of P. Americana a-amylase was similar to those reported for other a-amylases from mushroom Scrytalidium thermopile [3] and optimum temperature of the purified a-amylose was 55°C. This enzyme was quite stable in an acidic range between pH 5.6 and 6. The Bacillus ceruse MS6 a-amylase observed more activated by using Ca2+ and K+ similarly, P. Americana a-amylase was more activated by Ca2+ and K+.

Thus, further studies on the thermal stability of a-amylase enzyme produced by Bacillus ceruse MS6: have to be carried out to confirm its applications for starch liquefaction. Presence of metal ions like Ca2+ and Mg2+ known to improve the thermal stability may show promising results.

References


