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Studies on Haloalkaliphilic Bacteria Isolated from Lonar Meteorite Crater in India

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Abstract

Haloalkaliphilic bacteria were isolated from water samples collected from alkaline Lonar meteorite crater, (MS) India having pH 10.5. Among them one of the bacterial strains was identified by 16S rRNA sequencing showed 97% alignment match with *Bacillus fusiformis*. The optimum growth of such organism was at 12.5 pH and 18% salt concentration. Chemical analysis of water showed higher amount of metal ions and salts concentrations present in lonar crater. The *Bacillus fusiformis* have potential to produce hydrolytic enzyme such as amylases. The amylase was partially purified by 80% ammonium sulphate and obtained 2 fold increase amylase activity. The optimum enzyme activity was at 10 pH and 30°C temperature of. The enzyme was incubated in presence of different metal ions and showed its stability at 1mM concentration of $MgSO_4$.

Introduction

In recent years, there has been more interest in studying the microbiological flora of soda lakes since these naturally occurring alkaline hypersaline environments are the potential source of assorted microorganisms [7]. Halophiles, alkaliphiles and haloalkaliphiles are useful for the development of new bioprocesses and microbial products of commercial interest, may be isolated from these environments [10]. These studies have mainly relied on classical isolation techniques that have probably not detected all the types of microorganisms present at these sites [8]. It is now well known that only a small proportion of the microorganisms from an environmental sample can be isolated and cultured in laboratory conditions [13]. PCR amplification followed by cloning of the 16S rRNA genes derived from the total DNA extracted from environmental samples and subsequent phylogenetic analysis of the cloned sequences have enhanced our ability to assess naturally occurring biodiversity in many environments [1]. Contrary to other soda lakes, which are mainly non-polluted due to their inaccessibility, the former alkaline lake of Lonar crater is situated in Maharashtra, suffered many disturbances because of human activities. The Lonar crater, the third largest meteoritic lake in the world, is the only crater in basaltic rock. The lake has a circular periphery and is situated in a hollow, 0.14 km below the ground level with an amphitheatre of vertical cliffs. The diameter of Lonar Crater from top of the banks is about 2 km, while at bottom is 1.2 km [5]. In the present investigation, isolation of microorganism from Lonar Lake water sample and identified by 16S rRNA cataloging. The isolates were applied to production of biotechnologically and pharmaceutically important enzyme such as amylase. Alpha (α)-amylase, an extracellular enzyme degrades α, 1-4 glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin [4]. This enzyme is extensively used in many industries including starch liquification, brewing, food, paper, textile and pharmaceuticals [12].

Materials and Methods

Sample collection and chemical analysis: Water samples were directly collected in sterile bottles from different sites of crater. The chemical analysis of water was performed in triplicate by using standard method. The alkalinity of sample was estimated by potentiometric titration in terms of CaCO3, Ca, Mg, Cl as well as Na and K analyzed by Flame Photometer (Elico, India) [1 and 3].

Isolation and identification of bacterial strain: Water sample was used to isolate the different microflora from lonar crater. Enrichment of water samples was carried out in various growth liquid media, such as Horikoshi I, Horikoshi II [6] and Nutrient broth incubated at 37°C on rotary shaker (120 rpm) for 24 hrs. After enrichment, the samples were streaked on agar plates and incubate at 37°C for 24 hrs. [7]. Identification of bacterial strain was done by 16S rRNA sequencing [9].

Effect of sodium chloride and Hydrogen ions on growth of bacterial strain: The various concentrations of NaCl (1%, 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22% and 24%) and pH (9-13) was altered in growth medium and obtained isolate was studied for further investigation [1].

Inoculum preparation: The isolated colony of *B. fusiformis* SSDL-1 was transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of pre-sterilized inoculum medium

(nutrient broth). The flask was kept on rotary shaker (120 rpm) at 37°C for 24 h. The homogenous bacterial suspension was used as inoculum.

Production medium: Inoculate 4 ml inoculum in fermentation medium contained (g l^{-1}) 6.0 Peptone; 0.5 $MgSO_4$ .7H O; 0.5 KCl; 1.0 Starch and pH 10. Incubate the flask at 37°C for 24 hrs having 120 rpm on rotary shaker [2].

Extraction of amylase: After incubation, the production medium was centrifuged at 12000 rpm for 20 minutes at 4°C by using cooling shaker centrifuge. The supernatant was collected and used as crude source of amylase [2 and16].

Assay of amylase: The amylase activity was determined following the method of Bernfeld. An assay mixture containing enzyme extract, starch as substrate and DNS as coupling reagent was used. One unit of amylase activity was defined as the number of µ moles of maltose liberated by 1 mL of enzyme solution per minute [2 and 16].

Estimation of extra cellular protein. The protein concentration was estimated by the method of Lowry et al. (1951). BSA was used as a standard protein [1, 11 and 14].

Purification of amylase by ammonium sulphate precipitation: The 200 ml of crude amylase enzyme was brought by 80% (w/v) saturation with solid ammonium sulfate. The precipitated proteins were regimented by centrifugation for 15 min at 000 rpm. The resulted pellet was dissolved in 5 ml of 0.2 M phosphate buffer having pH 7.0 [11].

Effect of temperature: To study the effect of temperature on amylase activity was carried out at different temperatures such as 25°C, 30°C, 40°C, 50°C and 60° C [11].

Effect of pH on activity and stability of enzyme: The amylase activity was analyzed by varying different buffer pH values as phosphate buffer having pH 6.0, 7.0, 8.0 and glycine-NaOH buffer having pH 9.0, 10.0 and 10.6 [3 and 9]. Stability of the amylase was analyzed after 24hrs. [2].

Effect of metal ions on activity and stability of enzyme: The effect of metal ions at 1 mM concentration such as NaCl, $MgSO_4$, $CuCl_2$, KCl, $FeCl_2$ and $CaCo_3$ was assayed on amylase activity and stability was determined after 12 hrs. [11, 14, 15].

Results and Discussion

Isolation and identification of bacterial strain:

The sample was collected from different sites of the Lonar lake having different micro fauna were analyzed. The pH of the collected water sample was 10. The number of colonies was obtained on the surface of Horikoshi I and II agar medium, taking into consideration that 60 colony forming units were observed on the plate as well as the same collected water sample was checked on nutrient agar medium having variable concentrations of salt and pH. The growth of the haloalkaliphilic organisms was directly proportional to the increasing pH and salt concentration at 12.5 and 18% respectively. At lower concentration of pH and salt showed decreasing growth of the isolates. The isolated bacterial strain was morphologically analyzed of its colony character having, gram positive short rods, round colonies, translucent, convex, and white color. The isolated strain was analyzed by 16S rRNA cataloguing and showed 97% resemblance with *Bacillus fusiformis*. The phylogenetic position of the strain showed in fig: 1 Total genomic DNA was isolated using gene elute genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl2; 1x PCR buffer (Bangalore Genei, Bangalore, India); 200 µM each dCTP, dGTP, dATP, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a final volume of 20 µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene,as described previously. The PCR was performed using the Eppendorf Gradient Master cycler system with a cycle of 94°C for 5 min; 30 cycles of 94°, 60°, and 72°C for 1 min each and final extension at 72°C for 10 min, and the mixture was held at 4°C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice-using 70% ethanol and dissolved in Tris-HCl (10mM, pH 8.0).

The chemical analysis of water sample was analyzed according to American Public Health Organization (APHA). The six parameters were analyzed by potentiometric titration method and showed total hardness 292 mg/l, concentration of Ca, Mg and Cl was 88.1, 203.8, 3912 mg/l respectively. The sodium and potassium was analyzed by flame photometer (Elico India) having concentration 1512 and 16.9 mg/l respectively. Partial purification of amylase carried out by using different concentration of ammonium sulphate; among them the 80% was the most effective and got 2 fold increases its activity.

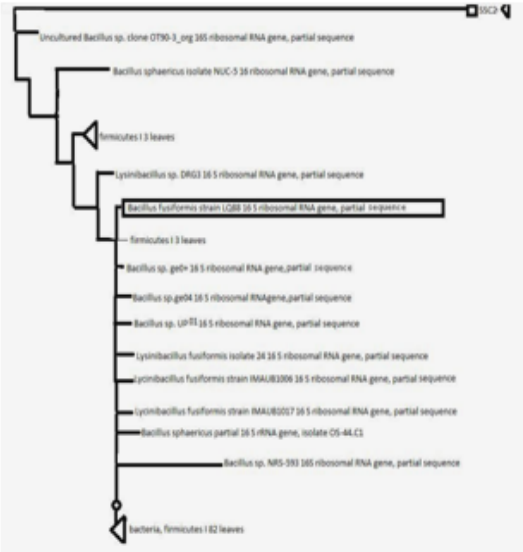

Fig: 1. Phylogenetic tree alignment of *B. fusiformis.*

Effect of temperature on amylase activity: The effect of temperature on amylase activity was analyzed at variable temperatures. Among the used temperature the optimum enzyme activity was found at 30°C showed 78.89 U/mg activity. At 40°C the amylase activity was slightly similar to the previous temperature and it was found that 71.55 U/mg

activity. According to Behal [2] the isolated amylase from *Bacillus sp.* AB 04 was showed its optimum activity at 40°C. At temperature within the range 50° to 60°C a decline enzyme activity was counted [2]. The similar results were obtained by Behal [2], the extracted amylase from *Bacillus sp.* AB 04 was decreased its activity at 50° C and 60°C. A 36.75 % reduction in enzyme activity was observed at temperatures 20°C [2]. The isolated amylase was unable to tolerate the higher temperature.

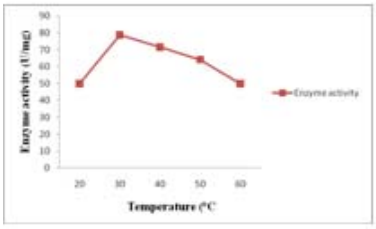

Fig: 2 Effect of temperature on amylase activity

Effect of pH on amylase activity and stability

The optimum pH was determined using five different buffer systems. The enzyme presented specific activity over the pH range of 6.0 to 10.6 with an optimum 10 having 100% activity, while at pH 10.6 about 94% of the maximum enzyme activity was obtained, increasing to 88, 83 % at pH 8 and 9 respectively (Fig 3). According to Ashabil [1] the extracted amylase from *Bacillus sp*. AB68 was showed optimum activity at 10.5 pH and at below and above 10.5 the enzyme activity occurs in decline condition [1]. The interference of pH on the stability of the enzyme was determined by incubating it at 37ºC for 24 hours. The obtained results revealed that the stability was at 10 pH. It has been reported that most alkaline amylases of different species of *Bacillus* have optimum pH around 10 to 11 [3 and 9].

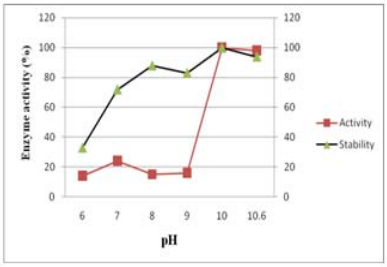

Fig: 3 Effect of pH on amylase activity

Effect of enzyme activators on amylase

The enzyme was incubated at 40ºC for 30 min at 1 mM concentration of different metal ions such as NaCl, $MgSO_4$, $CuCl_2$, KCl, $FeCl_2$ and $CaCo_3$. The activities measured were expressed as specific activity in percent. All the used metal ions showed its stability after 12 hrs. But at initially a stronger inhibitory effect was observed in the presence of Na^+, Cu^{2+} and Mg^{2+}. The inhibitory effect of heavy metal ions is well documented in the literature [15]. After incubation at 12 hrs the amylase activity was stimulated by Mg^{2+}. These results suggest that the applied metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at elevated temperatures [14]. The applied metal such as Ca^{++} showed inhibitory effect against enzyme activity after incubation as compared to other used metals. According to Natasa, in the presence of $CaCl_2$ enzyme retained 55% of its activity [11].

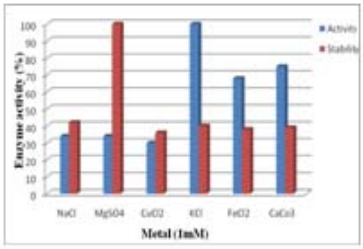

Fig: 4 Effect of metal ions on amylase activity

In conclusion, the studied alkaliphilic amylase enzyme found variable features showed haloalkaliphilic behavior. Amylase from *Bacillus fusiformis* SSDL-1 may be active at variable pH, temperature and metal ions are considered, but the enzyme demonstrates significant alkaliphilic properties.

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