INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Partial characterization and optimization of alkaline Amaylase from Bacillus *spp.* from Lonar Crater

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ABSTRACT

Alkaline Lonar Lake situated in the Buldhana district of Maharashtra state, India having a unique ecosystem and harbors various unidentified, unique haloalkaliphilic bacterial species which produces thermo-alkaliphilic enzyme with great importance in detergent and textile industries. The present study deals with isolation, production and partial characterization of amylase from bacterial strain isolated from the alkaline Lonar Lake. Isolation of bacteria was done by using Horikoshi medium and screened for production and partial characterizations of amylase on the basis of their optimum starch hydrolysis. Total seven cultures were isolated and two isolates DW1 (1) and OBW3 (2) were selected for further studies. Isolates were characterized by cultural, morphological and 16S rRNA gene sequencing. The results of 16S rRNA sequencing showed *B. pseudofirmus* DW1 (1) and *Bacillus* spp. OBW3 (2). The study reports production of amylase from bacteria isolates from alkaline Lonar Lake having haloalkaliphilic behavior. Amylase from *Bacillus pseudofirmus* and *Bacillus sp.* is active at high temperature, 55^{0}C and pH 10 and finds potential applications in food, pharmaceutical and detergent industries.

Key words: Lonar Lake amylase, haloalkaliphiles, *Bacillus*.

INTRODUCTION

Alkaline Lonar Lake situated in the Buldhana district of Maharashtra state, India having a unique ecosystem and harbors various unidentified, unique haloalkaliphilic bacterial species which produces thermo-alkaliphilic enzyme with great importance in detergent and textile industries[1,2,3]. The uniqueness of Lonar Lake is its salinity and alkalinity which is harbors various unidentified, unique haloalkaliphilic bacterial species which can be produces industrially important enzymes[4]. These enzymes are thermostable, resistance to alkali and most of the denaturing chemicals[5]. Alkaline amylase producing bacteria are of great importance in detergent and textile industry due to its high thermo-stability, pH stability and it is most important industrial enzymes, accounting for about 60% of total enzyme market[6,7]. Very less study has been done on amylase from Bacilli of Lonar Lake which can withstand high temperature as well as high pH and has wide application in different industries. As there is large demand of amylase, isolation and production of amylase enzyme is most important to fulfill this demand[8]. Therefore, attempt was made to study deals with isolation, production and partial characterization of amylase from bacterial strain isolated from the alkaline Lonar Lake which is very much useful in the food, pharmaceutical, detergent and textile industry.

MATERIALS AND METHODS

Isolation of Alkaliphiles: A total of four sediment and eight water samples were collected in year 2013 from alkaline Lonar Lake. The 1.0 g of soil or 10 ml water sample was transferred to 100 mL sterilized distilled water in 250 mL conical flask and agitated (200 rpm) at 37°C for 15 min in shaker. The suspension was then diluted to 10^{-7} dilutions. One mL of each diluted sample was lawn into Petri Plates

containing nutrient agar medium (pH 10) and incubated at 37°C for 24 h.

Screening of bacterial alkaliphiles: Individual bacterial colonies were screened for amylolytic activities on Starch agar medium (Starch 1.0, Peptone 5.0, Yeast Extract 1.5, Beef extract 1.5, Sodium Chloride 5.0, Agar 20.0, pH 10). The pH of the medium was adjusted to pH 10 with 1N NaOH before and after sterilization. The inoculated plates were incubated at 37°C for 48 h, floods the iodine solution into the plate. The halo zone was observed for amylolytic activity of isolates.

Identification of bacterial isolates: The bacterial identifications were performed on morphological, cultural and biochemical characteristics and its growth was tested at different temperatures, pH and NaCl concentration[9]. These isolates were identified in accordance with Bergey's Manual of Systematic Bacteriology[10]. The identified strains were maintained on nutrient agar slants having pH 10 at 4.0°C. The selected strains were then analyzed by 16S rRNA at NCCS, Pune and BLAST identification was made. The phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of Mega 4 program package[11].

Preparation of crude enzyme extracts: The 100 mL Starch nutrient medium was inoculated with culture and incubated for 48h at 37°C in incubator. After 48 h incubation, centrifuged the broth at 5000 rpm for 15 min. The supernatant served as crude enzyme source.

Amylase Assay: The standard graph of maltose was prepared by adding different concentration of standard maltose (1 mg/mL) and 2mL DNS solution into a series of test tubes and incubated all tubes in boiling water bath for 5 min and then addition of 1mL NaK tartarete to stop the reaction. Estimation of amylase was carried out with 2.5 mL of (1%) starch solution; 2.5 ml of PO_4 buffer, 1mL of NaCl and 1 mL of enzyme source in a test tube and incubated in boiling water bath for 5 min and then addition of 1 mL of Nak^+ tartarate to stop the reaction.

C**haracterization of Amylase:** The effect of pH on alkaline amylase was determined by assaying the enzyme activity at different pH ranging from 6.0 to 12, effect of temperature by incubating from 30°C to 80°C using the PO_4 buffer systems (0.2 M). The effect of substrate concentration on alkaline amylase activity was determined by incubating the reaction mixture for 15 minutes with different substrate concentration, ranging from 0.5 mg / mL to 4 mg/mL. The effect of enzyme concentration on alkaline amylase activity was determined by incubating the reaction mixture (pH 10) for 15 minutes at different enzyme concentration ranging from 0.5mL to 4mL. The activity of the amylase was then measured as per assay procedure.

RESULTS AND DISCUSSION

In the present study, a total seven cultures were isolated from water and sediment sample of alkaline Lonar Lake, screened for amylolytic activity on starch agar medium and two prominent amylase producer bacterial strain DW1(1) and OBW3(2) were selected for further morphological, cultural, biochemical characteristics and 16s rRNA gene sequencing and partial characterization and optimization of amylase production from these isolates. In the present studies the phenotypic analysis of representative isolates indicated that all the bacterial isolates were related to the *Bacillus* genus and species were *Bacillus. pseudofirmus* (DW1(1) and *Bacillus sp*. (OBW3(2) (Table 1). The Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates OBW3(2) and DW1(1) some of their closest phylogenetic relatives was created by the neighbor-joining method. The numbers on the tree indicates the percentages of bootstrap sampling (Fig. 1)

The alkaline amylase producing *Bacilli* are great importance in industries due to its high thermo-stability and pH stability. The isolated *Bacillus pseudofirmus* and *Bacillus sp*. alkaline amylase production was maximum at pH 9-10. The effect of enzyme concentration on the activity amylase for the given strain *Bacillus pesudofirmus* was observed 2.5mg/mL and *Bacillus sp*. was observed 2.0mg/mL. As the concentration of enzyme is increases the velocity of the enzymatic reaction also increases but upto certain limit (Fig.2). The effect of substrate concentration on enzyme activity of amylase, the Michalies Menten constant (Km) and maximum velocity (Vmax) was found to be by *Bacillus pesudofirmus* the substrate concentration of amylase 1.45mg/mL and the optimum concentration of amylase was observed 0.8mg/mL and *Bacillus sp*. the substrate concentration of amylase was observed 0.5mg/mL and optimum substrate concentration of amylase was observed 2mg/mL. The activity of enzyme increases the substrate concentration but this increases up to a certain limit (fig. 3). Production of amylase by *Bacillus pesudofirmus* was optimum at 50^0C temperature and the production of amylase by *Bacillus sp*. was optimum at 55^0C. When the temperature was increased or decreased, was gradual decrement in enzyme activity was recorded. At 60^0C

temperature the production of amylase was low (fig. 4)

Optimum pH for the amylase production by *Bacillus pseudofirmus* was found to be optimum at pH 10 and *Bacillus sp*. was found to be optimum at pH 9. As pH was increased or decreased from 9 or 10, there was gradual decrease in growth of the organism. The organism did not grow in production medium below ph 6 and above pH 12 (fig. 5). The isolated bacterial *Bacillus pesudofirmus* and *Bacillus sp* produces the amylase enzyme which has thermophilic, alkalophilic and has potential to be used in industry.

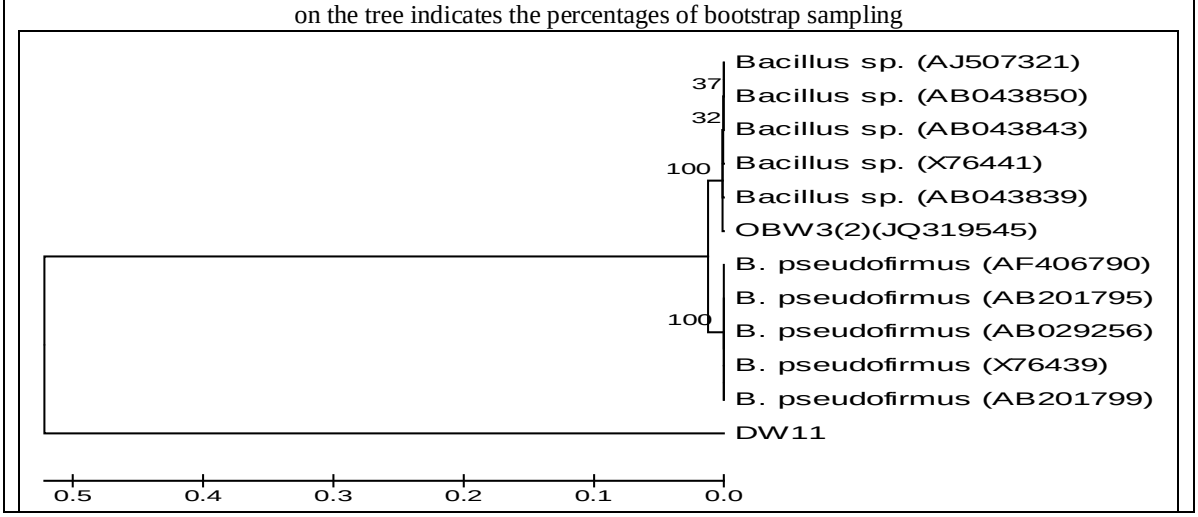
CONCLUSION

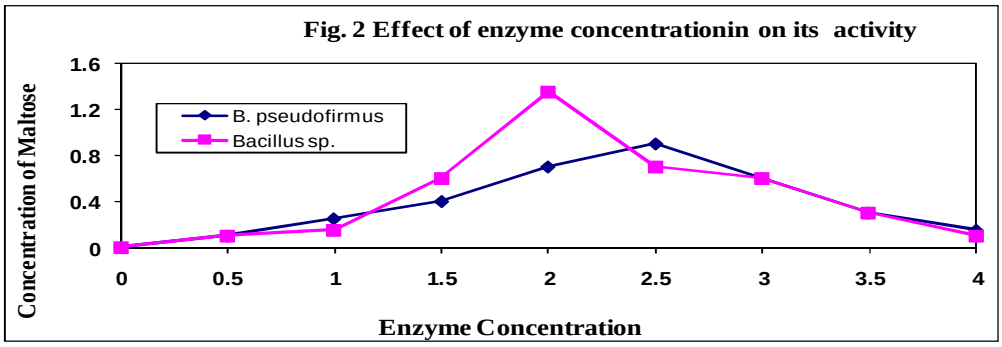
Amylase is one of the most important industrial enzymes known and is of great significance having approximately 25% of enzyme market and finds potential applications in food, pharmaceutical and detergent industries. This study reports production of amylase isolated from alkaline Lonar Lake. Amylase from *Bacillus pseudofirmus* and *Bacillus sp*. is active at high temperature, 55^0C and pH 10 and finds potential applications in food, pharmaceutical and detergent industries.

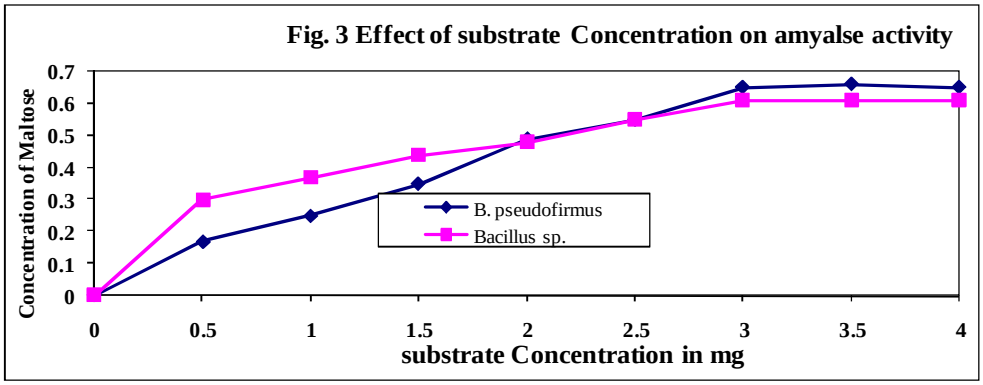
Table 1
Morphological, cultural and biochemical characteristics of amylase producing bacteria from Lonar lake

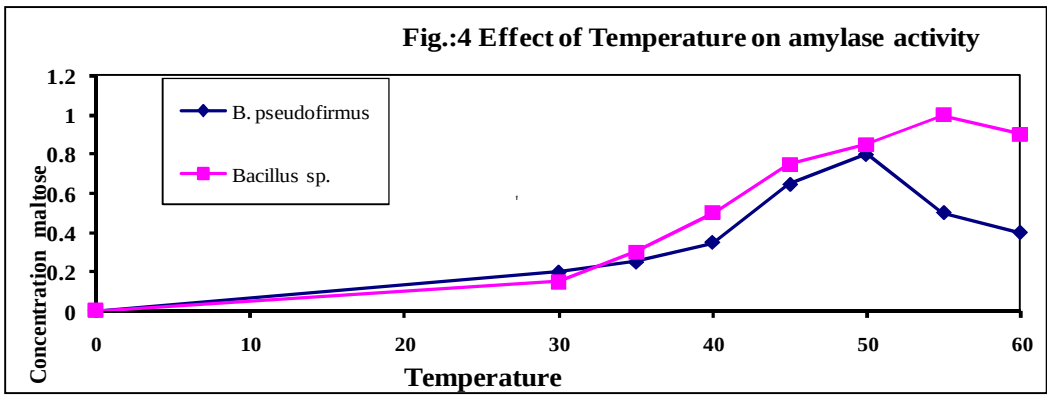
Bacterial Isolate	*Bacillus sp.* OBW3 (2)	*B. pseudofirmus* DW1 (1)	Bacterial Isolate	*Bacillus sp.* OBW3 (2)	*B. pseudofirmus* DW1 (1)
Morphological Character			**Biochemical characters**		
Gram character	+	+	Catalase	+	+
Shape	LR	LR	Oxidase	-	+
Arrangement	S	S	Indol	-	-
Spore bearing	+	+	MR	-	-
Position of Spore	C_1	C_1	VP	-	+
Shape of Spore	C_2	C_2	Citrate Utilization	-	-
Swollen Sporangia	-	+	Urea Hydrolysis	-	-
Capsule	-	+	Nitrate reduction	-	-
Motility	+	+	**Utilization**		
Growth at temperature			Glucose	+	-
37° C	+	+	Arabinose	-	-
45° C	+	+	Mannitol	+	-
50°C	+	+	Xylose	-	-
55° C	+	+	Lactose	-	-
Growth at pH			Trehose	-	-
pH7	+	+	Sucrose	-	-
pH8	+	+	Cellobiose	-	-
pH9	+	+	Galacrose	-	-
pH10	+	+	Maltose	-	-
pH12	+	+	Fructose	-	-
Growth at NaCl			Salicin	-	-
1% NaCl		+	Sorbitol	-	-
2% NaCl	+	+	Raffinose	-	-
3% NaCl	+	+	**Hydrolysis**		-
4% NaCl	+	+	Starch	+	+
5% NaCl	+	+	Lipid	+	+
6% NaCl	+	+	Casein	+	+
7% NaCl	+	+	**Note: LR- Long rod, S- Single, C- Chain, C_1- Central, C_2-Cylindrical, E- Ellipsoidal**		

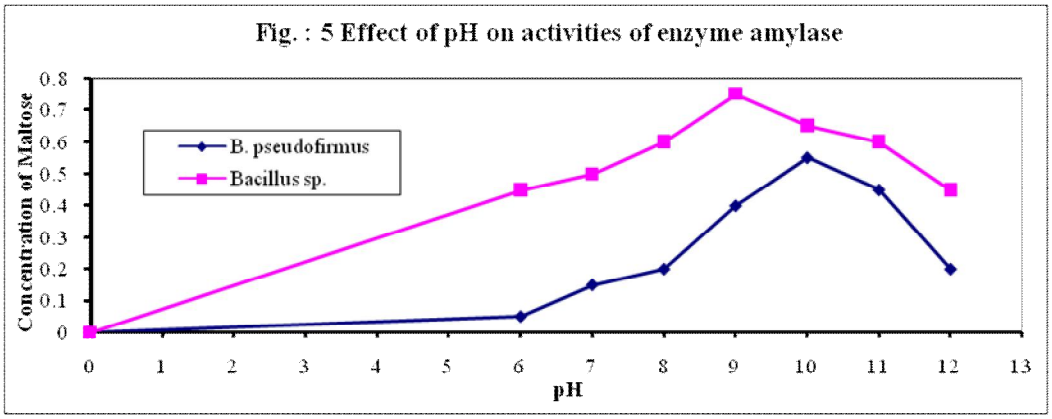
Fig. 1 Phylogenetic analysis of *Bacillus pseudofirmus* and *Bacillus sp*. Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates OBW3(2) and DW1(1) some of their closest phylogenetic relatives. The tree was created by the neighbor-joining method. The numbers on the tree indicates the percentages of bootstrap sampling











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