

**CHARACTERIZATION OF ORGANIC SOLVENT TOLERANT HALO-ALKALI-THERMOSTABLE AMYLASE FROM *BACILLUS CEREUS* OCW 3(1)****VISHAL R DHUNDALE***Post Graduate Department of Microbiology New Arts Commerce and Science College Ahmednagar, Maharashtra (India)***ABSTRACT**

A bacterium-OCW 3(1) with an ability to produce extracellular halophilic, alkali-tolerant, organic solvent stable, and moderately thermostable amylase was isolated from Lonar soda lake India. Identification of the bacterium was done based upon biochemical tests and 16S rDNA sequence. The result of gene sequence analysis indicated that bacterium strain OCW3(1) shows highest degree of similarities with *Bacillus cereus*. Further this strain was screened for the amylolytic activity using starch agar plates and results indicated that it was amylase producer. The optimum amylase production was achieved at pH 10 and 70°C temperature in the medium containing 3% NaCl and 1% (w/v) starch. The enzyme was active over a range of 2–10% sodium chloride examined in culture broth. The optimum amylase activity was observed at 2% sodium chloride. The results showed that fructose was the best carbon source and yeast extract and peptone was the best nitrogen source. The activity of amylase was optimum when 2% NaCl and starch was used in media for production. There was highest enzyme activity at temperature of 70°C, pH 11, 1.5 mL enzyme concentration and 35 min of incubation period. Kinetic properties such as K_m and V_{max} were 10 mg/mL and 58.82 mol/min/mL, respectively. Enzyme activity strongly inhibited by heavy metals such as $BaCl_2$, $CuCl_2$, $CaCl_2$, $MnSO_4$, $ZnSO_4$ and KCl. Amylase was found to be stable and enhance by organic solvents such as Methanol, Acetone, 1-Butanol, Benzene, Toluene and Chloroform which has showed demonstrating the unique properties of amylase enzymes. From the results it was concluded that the enzyme is a haloalkaline, thermostable and organic solvent tolerant in nature. The determination in the present study, an enzyme production analysis was conducted to address this *Bacillus cereus* OCW3(1) as a potential for biotechnology application. To our knowledge, this study represents the first report of considering that, amylase enzymes could be further explored in their differential functionality for industrial application and for genetic breeding leading to higher production levels.

KEYWORDS: Amylase, *Bacillus cereus*, Organic Solvents, Metal Ion tolerant**VISHAL R DHUNDALE**Post Graduate Department of Microbiology New Arts Commerce and
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INTRODUCTION

Screening of new origin of novel and industrially beneficial enzymes is a key systematic investigation and pursuit in enzyme biotechnology. For relevance in industrial course of proceeding, the enzymes should be established at high temperature, pH, presence of salts, solvents, toxicants etc¹. In this relevant circumstance, the haloalkaliphiles have rising as a huge receptacle of new enzymes in recent years. Enzymes derived from haloalkaliphiles are endowed with remarkable structural features and catalytic ability to encourage the metabolic and physiological processes under high salt, alkaline and organic solvent conditions. Some of these enzymes have been reported to be active and stable under polyextreme condition²⁻⁴. Enzymes from these bacterial strains can work optimally under extreme conditions, making them vigorous biocatalysts with potential suitable in harsh industrial processes⁵. The fact of using enzymes in organic solvents being considerate several beneficial when compared to traditional enzymology, such as high solubility of hydrophobic substrates and reduced water pursuit which change the hydrolytic equilibrium and remove the microbial contamination⁶. Lipase, esterase and amylase are widely used as biocatalysis due to their ability to catalyze not only the hydrolysis of lipid, triacylglycerides and starch in aqueous solutions respectively, but also enantioselective synthetic reactions in organic media. Therefore, these enzymes that remains operative and establish in the presence of organic solvents might be very beneficial for biotechnological applications in which such solvents are used. Since salt and alkaline tends to greatly reduce water activity like organic solvents, enzymes from haloalkalitolerant bacterial strains may become the selective for biocatalytic processes execute in low water activity environments⁷. So far, numerous organic solvent-tolerant microbial esterases, lipase and protease have been reported⁸⁻¹¹. Since many substrates for various enzymes are weakly dissolved in water, so replacing aqueous buffers by organic solvent-containing solutions reinforced the concerns in organic solvent-tolerant enzymes, however,

documentation on the enzymatic behavior from haloalkalitolerant bacteria in non-aqueous media are limited¹². Salt-tolerant alkaliphilic *Streptomyces clavuligerus* were isolated and identified as organic solvent tolerance alkaline protease producer. Actinomycetes in particular are exceptionally explored for their tolerance under organic solvents¹³. But *Bacillus* is rarely explored for the organic solvent tolerant amylase producer. Thus purpose of this study is to isolate a novel haloalkali thermostable amylase producing bacteria in the presence of organic solvent. Simultaneous optimization and characterization of different parameters were also performed in this study.

MATERIALS AND METHODS

Collection and isolation

Enrichment and isolation of microorganisms from Lonar lake, water and sediment sample were collected in sterile bottles and polythene bags respectively, from defined sampling site. Enrichment of water samples and sediment samples were carried out in nutrient agar at pH 10.0 with 30 g l⁻¹ sodium chloride¹⁴⁻¹⁵.

Screening for Amylase production

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 35.0, Agar 20.0, pH 10). The pH of medium was adjusted to pH 10 with 1N NaOH before and after sterilization. The inoculated plates were incubated at 37°C for 48 h, flooded with the iodine solution on the plate halos were observed for the amylolytic activity of the isolates.

Identification of the bacterial culture

The Gram positive amylase producing bacterial cultures were examined for their colony, morphological character, and biochemical characters according to Bergey's Manual of systematic bacteriology.

16S rDNA sequencing

DNA was extracted from *Bacilli* culture using standard phenol chloroform protocol. The partial sequence of the 16S rRNA gene was amplified by using polymerase chain reaction.

The amplified 16S rRNA gene PCR products purified by precipitation with polyethylene glycol and NaCl procedure and directly sequenced on the Applied Biosystems Model 3730 DNA sequencer (Foster, California USA). The 16S rDNA sequences were analyzed using BLAST program.

Preparation of enzyme extracts

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

Assay of enzyme activity and protein concentration

Characterization of amylase was carried out as described earlier by Tambekar *et al.*,¹⁶. The effect of temperature, pH and substrate concentration on α -amylase activity was studied and K_m and V_{max} values of the enzyme were calculated from Lineweaver-Burk (double-reciprocal) plot.

RESULTS AND DISCUSSION

Screening of bacterial isolates

Different bacterial species were isolated from water sample of Lonar Lake, out of which 15 isolates were amylase producer. OCW3(1) bacterial strains were selected for the further production and characterizations on the basis of maximum amylolytic activity.

Identifications of bacterial isolates

The selected isolates were studied morphologically and biochemical characteristics. According to Bergey's Manual of Systematic Bacteriology, the isolates were identified as *Bacillus* sp.

16S rDNA sequencing and Phylogenetic analysis

According to the phylogenetic tree the isolates shows homology with *Bacillus* sp. The isolates OCW3(1) shows sequence homology with *Bacillus cereus* (Fig 1).

Standard graph of maltose

As per the protocol which is mentioned above, the assay was estimated and standard graph of maltose was prepared. The graph of absorbance versus concentration of maltose

formed was plotted. One enzyme unit (unit/ml) is defined as the amount of enzyme which releases 1 μ mole of maltose. Characterization of amylase was carried out as described earlier by Tambekar *et al.*¹⁶

The estimation of amylase activity

The estimation of amylase activity was performed by following the assay conditions. The activity of amylase from *Bacillus cereus* OCW3(1) after 15 min of incubation was found to be 0.4 Units/mL. Similar study was conducted by Deb *et al.*¹⁷ by using *Bacillus amyloliquefaciens* for amylase production and Hashim *et al.*¹⁸ studied the *Bacillus halodurans* for amylase production which was isolated from kenyan soda lake.

Effect of different Carbon Sources on Amylase production

The influence of different carbon sources on amylase production by the alkaliphilic *Bacillus cereus* OCW3(1) was studied. In the present study, soluble starch was the best carbon source followed by the fructose (where activity was 8.8 Units/mL) for production of amylase and the production of amylase was intensely decreased with galactose by 87%. *Bacillus cereus* OCW3(1) showed comparatively low enzyme production when lactose and mannitol was used as carbon source. Sorbital was found to be the best carbon source for amylase production by *Bacillus subtilis*¹⁹. Dextrose was found to enhance the amylase production by *Bacillus megaterium* among the other carbon sources tested. Other carbon sources showed a repressive effect on enzyme synthesis in the studies of Aqeel and Umar²⁰. Effect of different carbon sources on α -amylase indicated that the growth of *Bacillus cereus* OCW3(1) production vary depending upon the carbon source used in the medium (Fig 2). Sreekanth *et al.*²¹ studied the different carbon source on the production of amylase and PHA with statistically maximum production observed on the starch, sucrose and glucose as a carbon source while Thippeswamy *et al.*²² revealed the maltose as a carbon sources for the production amylase and Sudharhsan *et al.*²³ examine the optimum production of amylase along with lactose as a carbon source. The hyperthermophilic archaeon *Sulfolobus solfataricus* and *Geobacillus* sp. IPTN in which glucose

repressed production of α -amylase²⁴. All the carbon and nitrogen sources would act as enhancer for the production of enzyme Negi and Banerjee²⁵.

Effects of Different Nitrogen Sources on Amylase Production

The nitrogen sources have a noticeable influence on the production of amylase of *Bacillus cereus* OCW3(1). Several inorganic and organic nitrogen sources were examined to optimize the source of nitrogen for amylase production. The result showed that there is maximum growth having activity of 1.6 Units/mL when yeast extract was used as a nitrogen source followed by peptone (1 Unit/mL). There is no growth in the presence of other nitrogen sources used except yeast extract and peptone (Fig 3). Hence, organic nitrogen sources were the best sources than inorganic nitrogen sources. Ogbonnaya *et al*¹⁹ also found that peptone was the best nitrogen source for production of amylase from *Bacillus subtilis* than other nitrogen sources. Yeast extract, an organic nitrogen source resulted in maximum production of amylase by *Bacillus* sp. CFR 67²¹.

Effect of different Starch concentration on production of amylase

Maximum growth of *Bacillus cereus* OCW3(1) and amylase production occurred at 2% of substrate concentration (Fig 4). Similar results also reported from Lily *et al*²⁷ also found that at 2% of starch concentration there is maximum growth of strain *Bacillus subtilis* BMT4i and maximum amylase yields was obtained at 1% of soluble starch during the studies performed by Swain *et al*²⁸.

Effect of different NaCl concentration on production of amylase

The strain *Bacillus cereus* OCW3(1) grew well at various concentrations of NaCl ranging from 2-10%. The optimum growth was at 2% NaCl and no growth was observed in the absence of NaCl. The amylase retained 37 and 50 % of activity in the presence of 4 and 6% NaCl, respectively. However, more than 64 % of the enzyme activity could be detected even at 10 % NaCl concentration. Similar results were revealed with strain *Bacillus pseudofirmus* DW4(1) grew well at various concentrations of NaCl ranging from 2-10% (Fig 5). The

optimum growth was at 6% NaCl and no growth was observed in the absence of NaCl²⁹. Martins *et al*³⁰ isolated different starch hydrolyzing bacteria from Ethiopian soda lakes. From that most of the isolates were able to grow in presence of 10% NaCl implying their tolerance to the salinity usually associated with soda lakes and also some strains like LS-6C and LS-9C showed growth at salt concentration up to 5%. Hashim *et al*¹⁸ found that *Bacillus halodurans* can tolerate salinity upto 12% NaCl. In contradictory to our work Dutta *et al*³¹ studied amylase stable at NaCl solution and found 30% loss the activity from 2M to 5M concentration and below the 0.5 M, the enzyme activity showed 100% for 24h incubation at experimental condition. In the present study, the increasing salt concentration the activity was also increasing up to specific and then decrease. Such fact clearly known that the enzyme was halotolerant³²⁻³³.

Effect of pH on activity of enzyme amylase

The effect of pH on amylase activity of *Bacillus cereus* OCW3(1) was determined by incubating the enzyme in different pH buffers ranging from 6-12 for 10 minutes at 37° C. The optimal pH of *Bacillus cereus* OCW3(1) amylase were found to be 11. The enzyme was active between pH ranging from 9-12. Amylase activity was relatively low at pH 6 (0.8 Units/mL). At pH 8, 9 and 10, the enzyme has relative activities of 36, 42 and 52% respectively (Fig 6). The activity decreased dramatically at pH 12 (2.3 Units/mL) while the effect of pH on α -amylase activity was produced by thermophilic *Bacillus* sp. studied by Cordeiro *et al*³⁴ and revealed optimum pH was found to be 7.5 (100%). Effect of pH on enzyme activity was decreased in both acidic stipulation at pH 5.5 and alkaline 10.0 were 73% and 55% respectively. Andualem³⁵ were found the effect of pH on the enzyme activity of the isolates. The amylase activity of A2 strain was increased from 97.6% at acidic to 99.25% at neutral pH while A3 strain increased amylase activity from 97.3% at pH 5 to 100% slightly alkaline pH. Dhundale *et al*²⁹ were studied the effect of pH on amylase activity of *Bacillus pseudofirmus* DW4(1) was determined activity in different pH buffers ranging from 6-12 and revealed effect of pH on amylase activity was relatively low at pH 6 (1.7

Units/mL) while optimum activities of this enzyme were at pH 9 and 10 with 3.5 units/mL. The enzyme activity decreased dramatically at pH 11 (3 Units/mL) and 12 (2.5 Units/mL). In the present studies the optimum activity of amylase from *Bacillus cereus* OCW3(1) was found to be at pH 11 with 3.7 enzyme units/mL (100%).

Effect of temperature on activity of enzyme amylase

Influence of temperature on *Bacillus cereus* OCW3(1) amylase activity was observed by incubating the enzyme at different temperature ranging from 25-100°C and residual activity were determined under enzyme assay condition. The temperature profile of amylase activity of *Bacillus cereus* OCW3(1) showed maximal enzymatic activity of 1.1 Units/mL (100%) at 70°C, which indicated that the enzyme was thermostable at high temperature. The amylase retained more than 86-97% of the highest activity between 50-60°C. Subsequently, the enzyme activity progressively decreased at 80° C (0.8 units/mL) and when the temperature was 100°C the enzyme activity was as poor as 0.3 Units/mL (Fig 7) while similar results also found to be amylase production from the *Chromohalobacter* sp. TVSP 101³⁶. The optimum temperature for activity of amylase isolated from *Bacillus subtilis* was from 37° C and there was a slow decrease in the activity above 70°C³⁷. Also maximum amylase activity was observed at 55° C from *Bacillus* sp. WA21 by Asad *et al*³⁸. Quadan *et al*³⁹ in their studies found that the alkali tolerant amylase from hyperthermophilic *Bacillus* strain HUTBS71 has maximum enzyme activity of 72 U/mL at a temperature of 100° C after 10 min of incubation period.

Effect of substrate concentration on activity of enzyme amylase

The kinetic parameters Km and Vmax of the enzyme were determined from the Lineweaver-Burk double reciprocal plots of the amylase. The influence of different concentrations of substrate was assayed ranging from 0.5-4 mL under constant assay conditions. Substrate concentration revealed that during the period of 10 minutes incubation at 37°C, 58% of substrate was utilized (0.7 Units/mL) but maximum substrate utilization

(1.2Units/mL) occurred at 1-2 mL of substrate concentration (Fig 9). The kinetic parameters Km and Vmax of the enzyme amylase produced from *Bacillus cereus* OCW3(1). This Lineweaver-Burk plot indicates that this enzyme has a Km of 10 mg of starch per millilitre and a Vmax of 58.82 mg of maltose per millilitre per min (Fig 8). The Km values obtained in the present study were found higher than those observed for the previous studies⁴⁰⁻⁴³.

Effect of Enzyme concentration on activity of enzyme amylase

The effects of different enzyme concentrations ranging from 0.5-4 mL was carried out under assay conditions. The enzyme shows maximum enzymatic activity (1.7 Units/mL) at 1.5 mL of enzyme concentration. The activity of amylase decreases as the enzyme concentration increase with and above 2. The enzyme retained about 78% of its activity (1.3 Units/mL) of enzyme concentration at 2 mL. There was very less activity at 4 mL of enzyme concentration revealed 0.3 Units/mL (Fig 10).

Time interval for hydrolysis of starch

The activity of enzyme was examined at different time intervals ranging from 15-60 minutes. As the time period goes on increasing the activity of amylase also goes on increasing. The highest activity with 1.1 Units/mL was shown at 37°C when the reaction mixture containing enzyme was incubated for 35 minutes. But as the incubation time goes on increasing the activity was decreasing from 40 min to 55 min. The lowest activity was shown when incubation period was of 55 minutes (0.1 Units/mL). There was no activity of amylase when incubation period was 60 minutes (Fig 11).

Influence of various organic solvents on activity of enzyme amylase

The effect of organic solvents on the activity of the amylase was determined. The data elucidate that the enzyme was highly active to all organic solvents tested as compared to control which was considered as 100%. There was no enzyme activity found in the presence of chloroform. Thus chloroform has no effect on activity of amylase. The highest activity was found in presence of Toluene (533%)

(Fig12). Therefore, all the solvents except the chloroform has an enhancing effect on the activity of amylase produced by *Bacillus cereus* OCW3(1) while Li and Yu⁴⁴ studied the effect of organic solvent on the amylase and protease, revealed that the enzyme were found to be tolerant to organic solvents. The enzyme more than 90% of the enzyme activity retained after treated with DMSO, acetonitrile, ethanol, and acetone while ethanol and acetone were found activator for amylase. Dhundale *et al*²⁹ data elucidate that the Benzene, have an enhanced effect on the activity of amylase produced by *Bacillus pseudofirmus* DW4(1). Tiwari *et al*⁴⁵ studied the amylase of *Bacillus tequilensis* RG-01 is extraordinarily stable in the presence of organic solvents n-dodecane, isooctane, n-decane, xylene, toluene, n-hexane, n-butanol, and cyclohexane enhance the amylase activity while benzene, methanol, and ethanol were found to be inhibitor for amylase. Contradictory to this increased activity in the presence of organic solvents which were water-immiscible acetone, ethanol and chloroform activity of the enzyme was significant enhanced of a halophilic α -amylase from *Nesterenkonia* sp. Therefore the different organic solvents were highly influenced on the amylase activity by different microorganism of genus with tolerant to different organic solvent. These organic solvent-tolerant enzymes are regarded to have potential for benefits in industrial chemical processes⁴⁶.

Influence of different metal ions on activity of enzyme amylase

The influence of different metal ions on activity of *Bacillus cereus* OCW3(1) amylase was carried out under the assay conditions. The

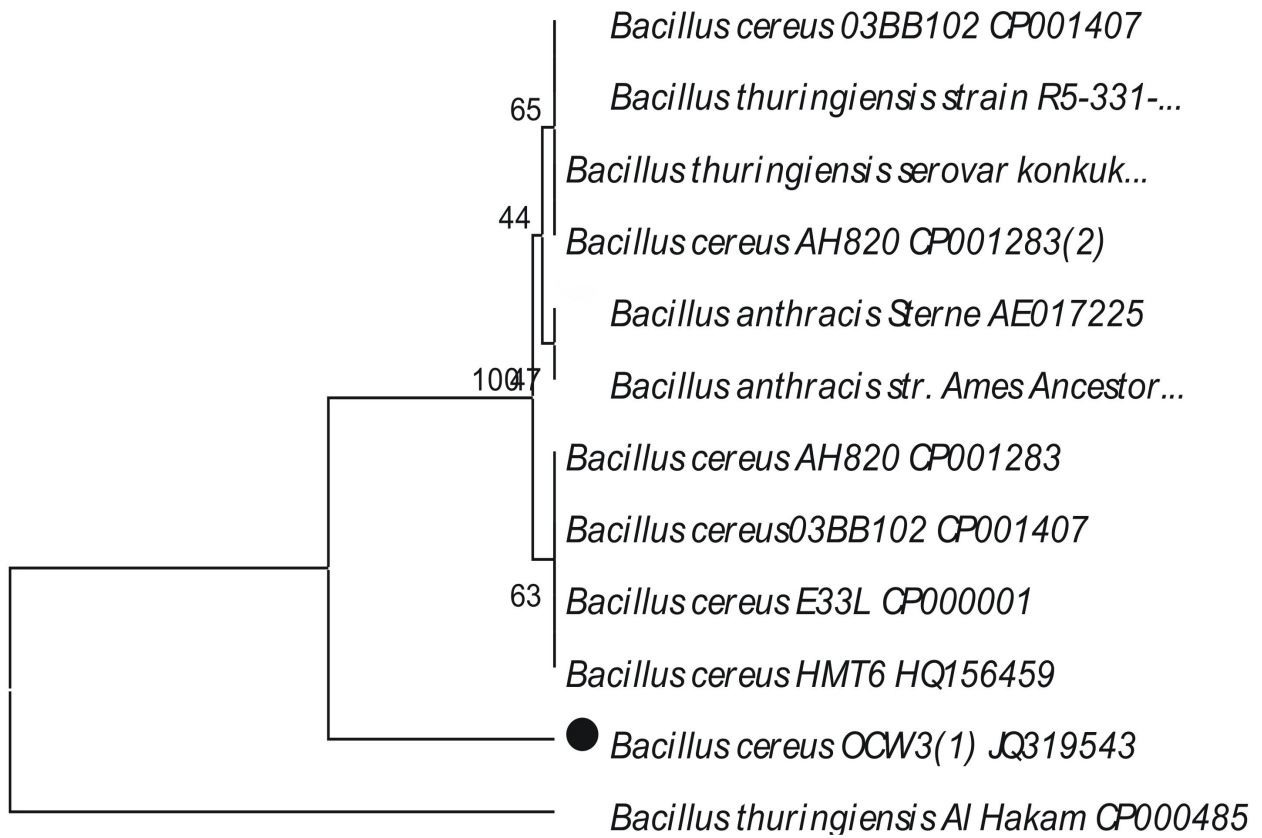
enzyme activity was inhibited by all the metal ions which were tested. Hence, all the metal ions tested have an inhibitory effect on activity of amylase. The contradictory results were revealed to *Bacillus pseudofirmus* DW4(1)²⁹. The enzyme activity was enhanced by BaCl_2 and CaCl_2 (Fig 13). However, the most α -amylases are known to be metalloenzymes, supplementation of metal ions improved the enzyme activity⁴⁷. Kundu and Das⁴⁸ also studied the effect of metal ions on the amylase from *A. oryzae* EI 212 and concluded that there was no significant difference between SSF and mSSF for α -amylase production in presence of different metal ions.

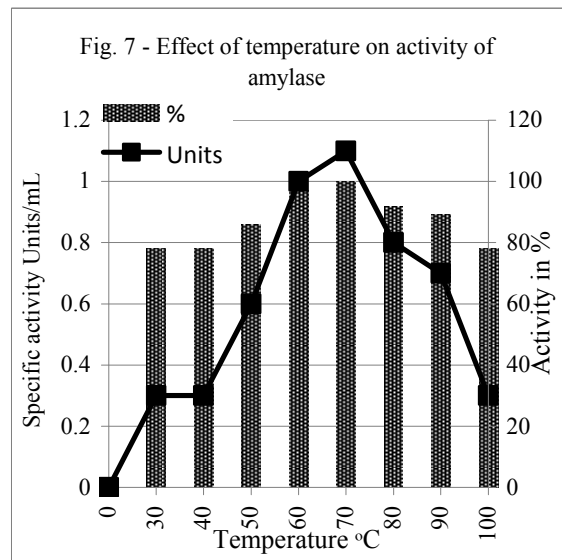
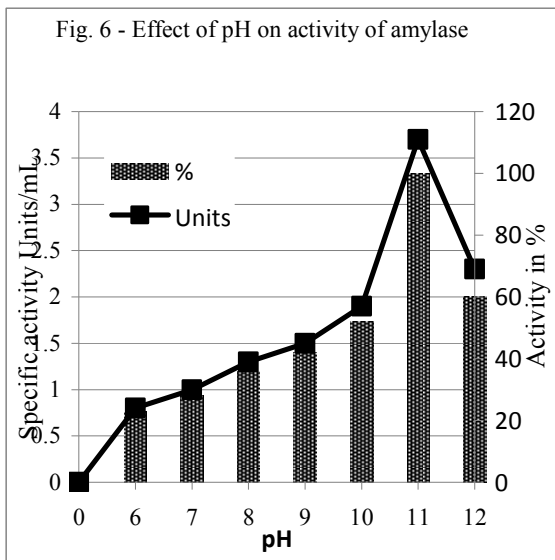
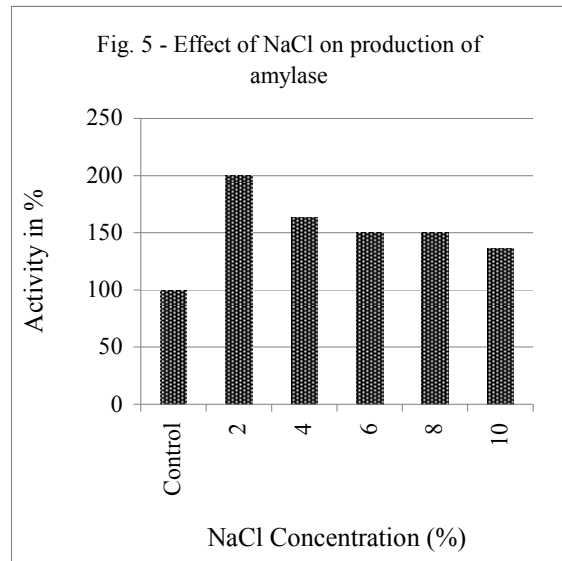
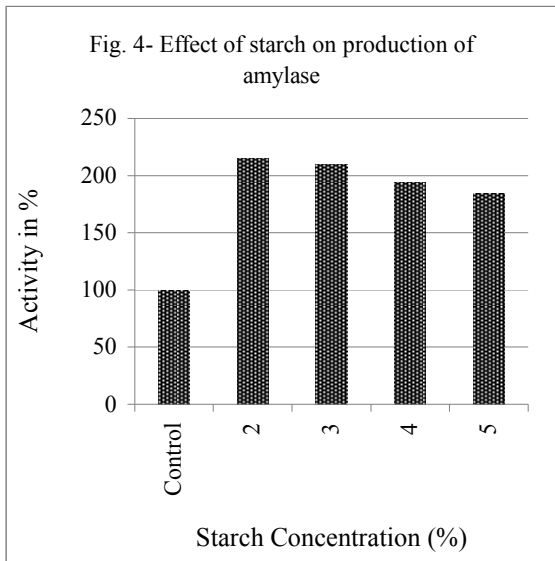
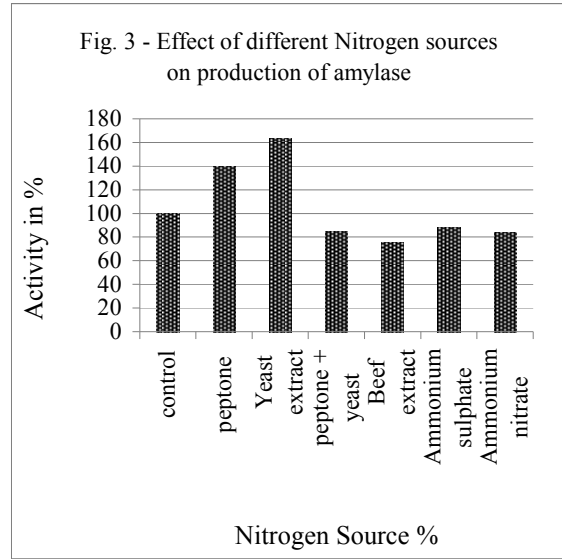
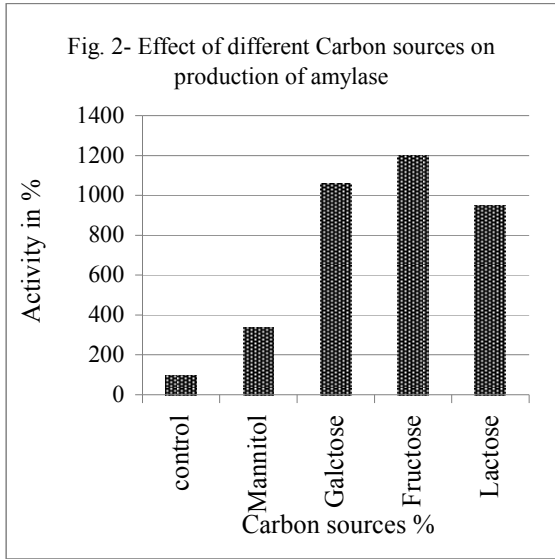
Effect of NaCl on activity of amylase

When different molar NaCl concentrations were used to check the activity of amylase, it was found that the highest activity (3.2 Units/mL) was found at 3 and 3.5 M of NaCl concentration which was considered as 100%. The lowest activity (1.5 Units/mL) was observed at 0 M concentration of NaCl concentration (46%) (Fig 14). Anupama and Jayaraman⁴⁹ assayed the amylase activity in presence of different NaCl concentrations ranging from 0-5 M. In their investigation they found that at 0-1 M salt concentration there was a significant amount of enzyme activity. However when NaCl concentration was increased up to 1 M, the activity decreased but was above 75% till 3 M salt concentration. Dhundale *et al*²⁹ was found that the highest activity (3.5 Units/mL) was found at 2.5M of NaCl and the lowest activity (1.2 and 1 Units/mL) was observed at 0 and 4 M concentration of NaCl concentration which was amylase produced from *Bacillus pseudofirmus* DW4(1).

Figure 1

Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates OCW3(1) and 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





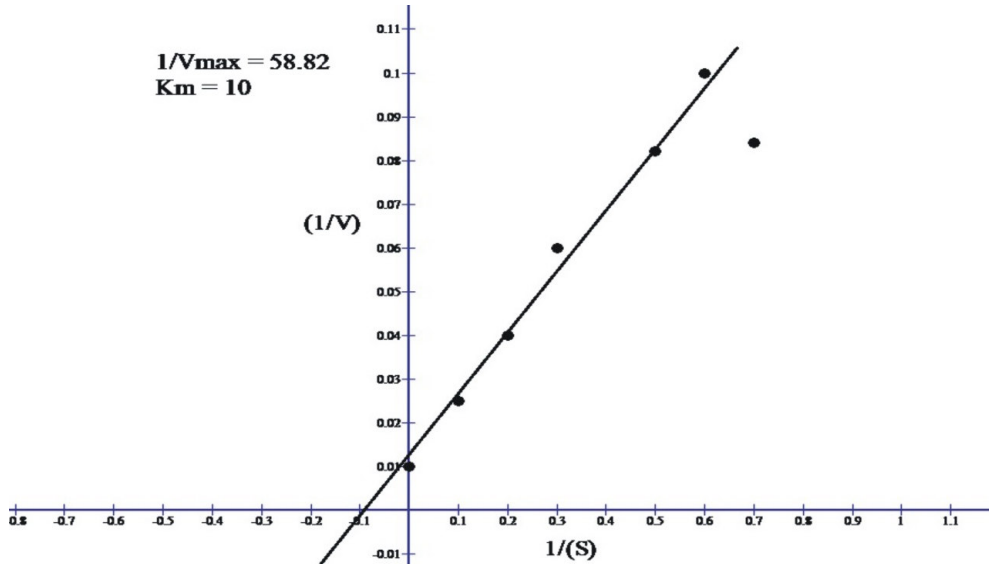
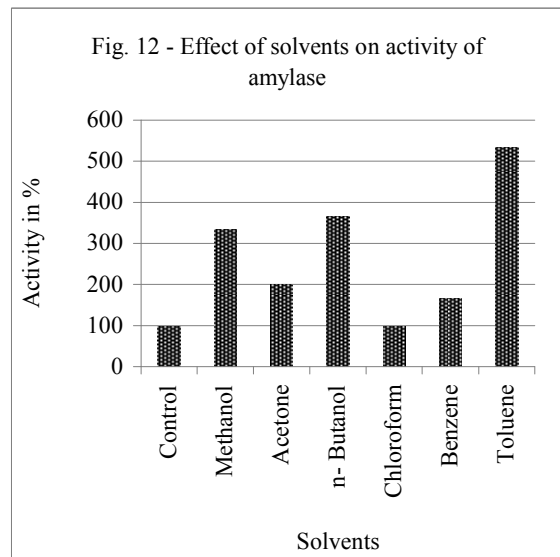
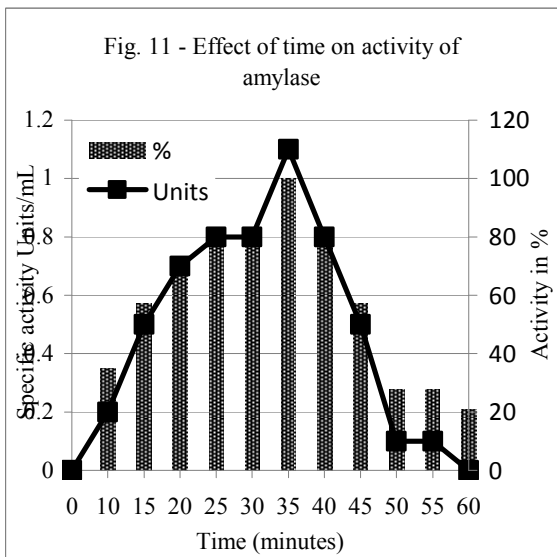
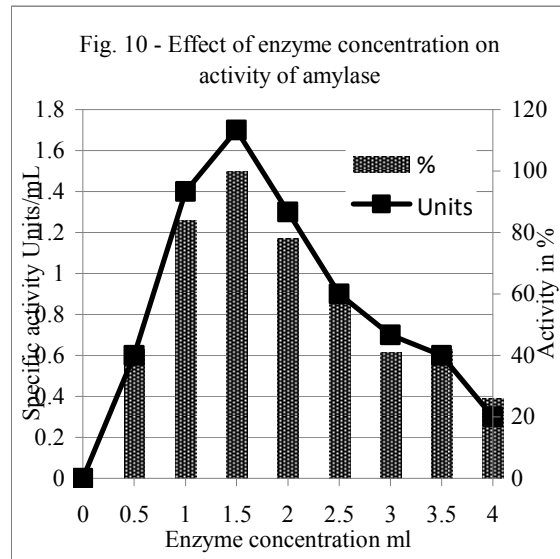
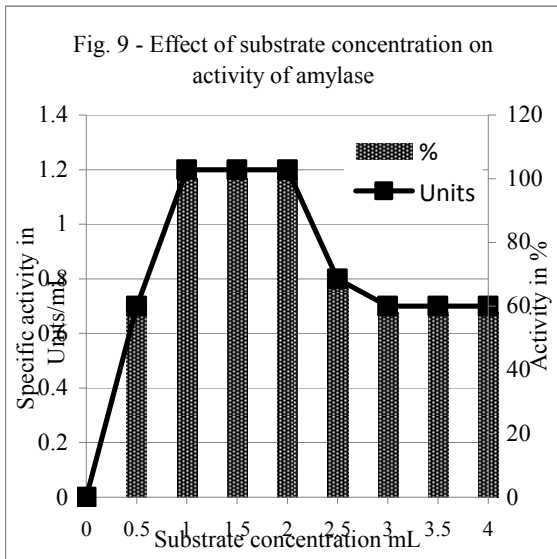
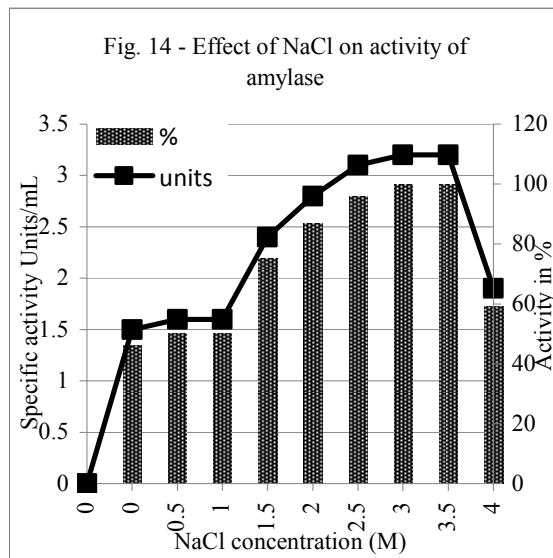
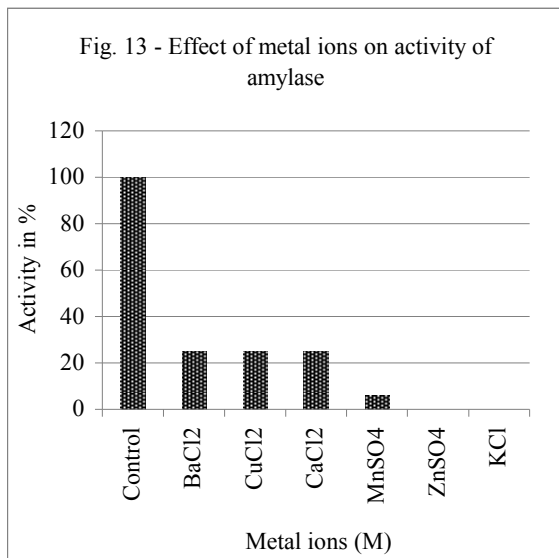


Fig. 8 - Lineweaver and Burk Plot for OCW3(1)





CONCLUSION

Bacterial enzymes are abundant and ubiquitous in the extremophilic environment, where they serve essential functions that enhance microbial survival. It was globally held that extremophilic microorganism would need unique cultivation methods and would not likely produce protein other than those essential for living in extreme environment. By culture dependent approach various types of the bacterial culture were cultivate in vitro and able to synthesize natural proteins. The present work report, sequencing of bacteria and biochemical characterization of amylase from *Bacillus cereus* OCW3(1). The described amylase was tolerant and stable in the presence of extreme conditions such as pH, temperature and high salt concentrations and organic solvent tolerant compared to other bacterial amylase. Therefore, the biotechnological exploitation of this enzyme could be of great importance in biotechnological potential. Besides, the characterization of the truncated variant revealed improved catalytic properties that are distinct to the wild type enzyme and should demonstrate the tremendous

advantage to further genetic engineer this amylase for biotechnological applications. Recently, solvent-tolerant bacteria as a relatively novel group of extremophilic microorganisms with unique ability to synthesize the enzyme which can be tolerated to organic solvents have attracted a great attention among researcher. Since extracellular enzymes which were organic-solvent tolerant microorganisms were possibly stable in the presence of organic solvents. To emphasize, the extracellular haloalkaliphili, thermostable enzymes with solvent tolerance can be used in several processes where high salt concentrations and hydrophobic organic solvents are present. The *Bacillus cereus* OCW3(1) amylase may also emerge as valuable for peptide synthesis under non-aqueous conditions, which otherwise may be thermodynamically unfavorable in water. This property could be exploited to carry out bioremediation and biocatalysis in organic phase. The role of solvent-stable enzymes in nonaqueous biocatalysis needs to be explored and could result in novel applications.

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