Isolation of Organic Solvent Tolerant Bacteria Producing Amylase and Characterization of Its Amylase Activity

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Abstract- *Over the past few decades, the use of amylase enzymes to carry out reaction in organic media has achieved considerable interest in the research area of biotechnology research and development. Animal and plant sources of enzymes are unable to meet the industrial demand, and therefore, screening and purification of microbial organic solvent tolerant amylases are important. Organic solvent tolerant bacteria with an ability to produce extracellular organic solvent tolerant amylase were isolated form petrol contaminated area near Sewri, Mumbai, Maharashtra, India. These strains were screened for the amylolytic activity using starch agar plates and results indicated that it was amylase producer. Further, identification of bacterial strains was done using MALDI-TOF mass spectroscopy. The result of analysis indicated that all bacterial strains show highest degree of similarities with Bacillus spp. Moreover, the activity of organic solvent tolerant amylases was checked to study the effect of various parameters like temperature, pH and salt concentration to obtain optimum enzyme activity. The stability of the enzyme amylase in the presence of different solvents and metal ions was studied. Observed results indicate that enzyme activity was optimum between pH 6-8 and temperature range from 20^{o} C to 70^{o} C. Further the strains also show high relative activity in presence of Ca^{++} metal ions in the presence of various organic solvents with low log_p activity. These characteristics of the enzyme in the presence of solvents are extremely favorable in lather industry, detergent industry and environmental remediation.*

Keywords- Soil, bacteria, amylase, organic solvents, solvent tolerance.

I. INTRODUCTION

Over the past few decades, the use of enzymes to carry out reaction in organic media has achieved considerable interest and has emerged as a major area of biotechnology research and development [1]. Because such enzymes naturally remain stable in the presence of organic solvents without the need for special stabilization, they are very useful for biotechnological applications in which such solvents are used. We know that enzymes are generally very labile catalysts and easy to lose their activities in organic solvent, several techniques such as solvent engineering, substrate engineering, and protein engineering have been employed to improve the stability of enzymes [2]. However, if enzymes were naturally stable and active in hostile environments, they would be excellent biocatalysts for application. Thus, the search for natural enzyme that shows high stability and organic solvent tolerance is more upfront.

Amylases are second most significant enzymes among all others and are of high significance in biotechnology field [19]. This class of industrial enzymes plays an important role in starch saccharification and at present constitute about 25-33% of the world range of application in many fields such as starch saccharification, textile, food, baking, brewing, distilling industries, and pharmaceutical sector [17].

Uses of non-aqueous medium provide advantages to improve the solubility of substrates and ease of product recovery in organic phase. It is known that when solvent tolerant enzymes show enhanced activity in the presence of solvents can be used in biodegradation in the coastal environments thus enhancing eco restoration. It was also observed that at lower concentrations these enzymes were more favorable for retaining and enhancing the enzyme activities [3]. Now a day, limitations of biotechnology has been exceeds, requirement of variety of organic solvent tolerant enzymes has enhanced. Demand of organic solvent tolerant amylase is greatly enhanced due to their significance in clinical, medical and analytical sectors. This present work focused on the isolation of organic solvent tolerant bacteria and its potential amylase catalytic activity in the presence of organic solvents.

II. AIM AND OBJECTIVES

The aim of this research was isolation of organic solvent tolerant bacteria producing amylase and

characterization of its amylase activity efficiency and objectives were as follows-

- To screen and isolate organic solvent tolerant amylase producing organisms.
- Identification of isolated culture.
- Optimization of various parameters on bacterial growth - substrate concentration, Carbon sources, Nitrogen sources, Salt concentration, pH, Temperature.
- To check the effects of various parameters on amylase like pH, temperature, NaCl concentration, metal ions, organic solvents.

III. MATERIALS AND METHODS

Collection and isolation

Enrichment and isolation of microorganisms carried by collecting soil sample from petrol contaminated area, Sewri, Mumbai, Maharashtra. Soil sample was collected in sterile polythene bags from defined sampling site. Enrichment of soil was carried out in starch broth at pH 7.00 with organic solvents such as toluene, benzene and alcohol in different flasks. The isolated strains from enrichment medium were checked for their colony characteristics and Gram nature.

Screening for Amylase production

Screening of organic solvent tolerant individual bacterial colonies were screened for amylolytic activities on Starch agar medium (1% Starch, 0.5% Yeast extracts, 1% Peptone, 7.5% NaCl, 1.5% agar and pH- 7). The enriched medium were first serially diluted by using 10-fold serial dilution method and isolated by spread plate technique. After inoculation plates were incubated at 35°C for 48 h, flooded with the iodine solution on the plate and halos were observed for the amylolytic activity of the isolates.

Identification of the bacterial culture

The selected Gram positive amylase producing organic solvent tolerant bacterial cultures were identified by MALDI-TOF analysis at National Centre for Microbial Resource lab, Pune, India. MALDI-TOF MS (Matrix-assisted laser desorption ionization-time of flight mass spectrometry) is emerging today as a test and cost effective reliable method of microbial identification. The mass spectra of microbial test strains are compared to the Bruker biotyper database (containing 6091 Mean Spectrum Profile (MSP) and NCMR in-house database [19].

Production of crude enzyme

The bacterial isolates were grown in 100 ml starch nutrient broth and incubated at 35°C for 48 h. After incubation, cell-free supernatant containing the extracellular amylase was harvested form broth by centrifugation at 8,000x g for 8 min at 27°C. The supernatant (crude enzyme) was used for enzyme activity estimation.

Amylase assay

Amylase activity was assayed using DNSA method by estimating reducing groups released from starch, by the reduction of 3,5-dinitrosalicylic acid (DNS). Enzyme sample (0.5 ml) was added to 2-ml (1%, w/v) starch prepared in Acetate buffer (20 mM, pH 6.5) and 1ml of 0.5% NaCl and was incubated at 35°C for 15 min. The reaction was stopped by addition of di nitro-salicylic acid (DNSA) solution. After that, amount of the reducing sugar was determined by colorimetrically at 540 nm. One unit of amylase activity is defined as the amount of enzyme that releases 1 µmol of reducing sugar as maltose per minute under the assay conditions [16].

Effects of different fermentation conditions on amylase production:

Effect of carbon and nitrogen sources

The effect of carbon sources (at 0.5% w/v) such as Mannitol, Galactose, Fructose and lactose were investigated for their effects on amylase production by isolates and effect of nitrogen sources (at 0.5% w/v) such as peptone, gelatin, yeast extract and soybean meal were also studied for their effects on amylase production by isolates. Amylase production was measured at the end of the cultivation period to determine the best carbon and nitrogen sources that support high yield of amylase.

Effect of pH and temperature

The effect of pH on amylase production was measured by growing the isolates at fixed media concentration and temperature 35°C with varied pH of 6.0 to 11.0 for 48 h using the appropriate buffers at concentrationof 50 mM solutions (Acetate buffer from pH 6.0 - 7.0 and phosphate buffer from pH 8.0 – 11.0) under standard assay conditions. The optimum temperature for enzyme production was determined by growing the isolates at fixed media concentration and pH with varied temperature from 15°C to 70°C. Amylase production was measured at the end of the cultivation period to determine the best pH and temperature that support high yield of amylase.

Determination of parameters for OST amylase from different isolates:

Effect of pH, temperature, and NaCl

The effect of pH on amylase activity was measured in the pH range of 4 to 12, using the appropriate buffers at concentration of 100 mM (Acetate buffer from pH 2.0- 7.0 and phosphate buffer from pH 8.0 – 10.0) under standard assay conditions. The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures ranging from 4°C to 90°C. To determine the effect of NaCl concentrations on enzyme activity, the enzyme assay was performed in the presence of 0–4 M NaCl.

Effect of different metal ions and organic solvents

The effect of metal ions ($CaCl_2.H_2O$, $MgSo_4$, $MnSo_4$, $ZnSo_4.7H_2O$, $Fe_3(So_4)_3.7H_2O$ and $EDTA.2H_2O$) on amylase activity were investigated by adding 10mM of each metallic ions to the reaction mixture.

Effect of different organic solvents (n-propyl alcohol, toluene, acetone, benzene and benzoyl chloride) on crude amylase activity were studied by introducing the selected organic solvent into the reaction and amylase activity was determined according to the standard assay procedure.

IV. RESULTS AND DISCUSSION

Screening of bacterial isolates

Different bacterial species were isolated from soil sample of the Sewri, docks near Bharat petroleum, out of which 18 isolates were amylase producer. Best four bacterial isolates (*B. spizizenii DSM 618*, *B. flexus 100331*, *B. mojavensis DSM 9205T* and *B. subtilis DSM 5611*) were selected for the further production and characterizations on the basis of maximum amylolytic activity (Fig.1).

Identifications of bacterial isolates

The selected isolates were studied morphologically by Gram staining in that all isolates were stained with Gram positive stains and the isolates were identified by MALDI-TOF MS analysis.

Analyte (PRN)	Sample name	Organism (best match)	Score	Organism (second match)	Score
M-MAR-18-163	S7	*Bacillus* sp. DMVJ16CS (MCC 2505)	2.091	*Bacillus subtilis* DSM 5611 DSM	1.931
M-MAR-18-162	S2	*Bacillus vallismortis* DSM 11031T DSM	1.944	*Bacillus mojavenzis* DSM 9205T DSM	1.91
M-MAR-18-161	I5	*Bacillus* sp. DMVM_15SBW (MCC 2398)	2.22	*Bacillus flexus* 100331_30 USP	2.082
M-MAR-18-160	I1	*Bacillus* sp. DMVJ16CS (MCC 2505)	2.2	*Bacillus subtilis* ssp *spizizenii* DSM 618 DSM	1.805

MALDI Biotyper Classification Results

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 1µ mole of maltose. Characterization of amylase was carried out as described above.

The estimation of amylase activity

The estimation of amylase activity was performed by following the assay conditions for different time intervals. The activity of amylase of *B. spizizenii DSM 618*, *B. flexus 100331*, *B. mojavensis DSM 9205T* and *B. subtilis DSM 5611* were found to be 23.25µmole/ml, 25.89µmole/ml, 23.25µmole/ml and 25.89µmole/ml respectively after 60 hrs of incubation period (Fig.2). Similar study was conducted by Deb *et al* [9] by using *Bacillus amyloliquefaciens* for amylase production and Hashim *et al.* [10] studied the *Bacillus halodurans* for amylase production which was isolated from kenyan soda lake.

Effect of different Carbon Sources on production of amylase

The influence of different carbon sources on amylase production by isolates was studied. Here a number of carbohydrates (fructose, galactose, lactose, maltose, and soluble starch at 0.1 % w/v concentration) were tested as carbon sources for amylase production. In the present study, fructose was the best carbon source for isolates *B. flexus 100331* (65.4µmole/ml) and *B. mojavensis DSM 9205T* (60.13µmole/ml) followed by galactose whereas *B. spizizenii DSM 618* (44.33µmole/ml) and *B. subtilis DSM 5611* (52.23µmole/ml) isolates used galactose more for amylase production than fructose as carbon source (Fig.3) and this result was relatively similar to the work done by *Vishal R. Dhundale 2015* [1].

Effect of Different Nitrogen Sources on production of amylase

The nitrogen sources have a noticeable influence on the production of amylase. Several inorganic and organic nitrogen sources were examined to optimize the source of nitrogen for amylase production. When gelatin was used as nitrogen source, the determined activity for *B. spizizenii DSM 618* was 17.98µmole/ml and for *B. subtilis DSM 5611* was 28.52µmole/ml followed by soyabean and peptone. Whereas for *B. flexus 100331* and *B. mojavensis DSM 9205T* showed activity was calculated to be 15.35µmole/ml, 23.25µmole/ml respectively when soyabean used as nitrogen source. *B. spizizenii DSM 618* displayed 10.08µmole/ml activity by using gelatin. . Hence, organic nitrogen sources were the best sources thaninorganic nitrogen sources. But by the study of *Ogbonnayaet al* [11], it was found that peptone was the best nitrogensource for production of amylase from *Bacillus spp*. than other nitrogen sources. Gelatin and soyabean, the organic nitrogen sources resulted in maximum production of amylase by all four bacterial isolates (*B. spizizenii DSM 618, B. flexus 100331, B. mojavensis DSM 9205T* and *B. subtilis DSM 5611*) (Fig.4).

Effect of pHand temperature on production of amylase

The effect of pH and temperature on production of amylase was studied by growing all isolates in different pH (2 to 10) and at different temperature (from 4^{o}C to 70^{o}C) and determined its contribution in amylase production. All the isolates had showed activity till 50^{o}C and optimum temperature was found to be 35^{o}C but the work done by *S.P. Pandey et al, 2012 gave* maximum activity at 50^{o}C (Fig.5). pH conditions for *B. spizizenii DSM 618, B. flexus 100331, B. mojavensis DSM 9205T* and *B. subtilis DSM 5611* are in the range of 6 to 8 but the optimum was pH 6 (Fig.6) whereas *Bacillus cereus* required optimum pH 11 (*Vishal R. Dhundale 2015)* and *Lactobacillus yamanshensis* required optimum pH 7 to maintain the amylase activity in active condition [4].

Effect of NaCl on activity of enzyme amylase

When different molar concentrations of NaCl were used to check the activity of amylase, it was found that maximum amylase activity ranges between 23.25µmole/ml to 31.15µmole/ml. The strain *B. spizizenii DSM 618* and *B. subtilis DSM 5611* showed optimum activity at 3 Molar, *B. flexus 100331* at 3.5 Molar and *B. mojavensis DSM 9205T* at 2.5 Molar. Similar type of study was also done by with *Haloarcula spp*. [6] that showed salt tolerance upto 4.3 Molar (Fig.7).

Influence of various metal ions on activity of enzyme amylase

The influence of different metal ions on activity of all isolates was carried out under the assay condition. The enzyme activity was not affected by all the metal ions which were tested such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and EDTA. Amylase activity was enhanced in presence of Metal ions like Ca^{2+}, Zn^{2+} and Fe^{2+} (Fig.8). Similarlily, *B. subtilis* AF333249 strain had also showed enhanced effect in presence of Ca^{2+}, Zn^{2+}, Mg^{2+} and Fe^{2+} metal ions [4]and *Bacillus pseudofirmus*DW4(1)[12] showed enhanced enzyme activity by Ba^{2+} and Ca^{2+}. Interestingly, the amylase activity was decreased in the presence of Mg^{2+}. Metal ions have been reported to play vital role in maintaining the active site conformation of the enzyme [5].

Effect of pH on activity of enzyme amylase

The effect of pH on amylase activity of all strains (*B. spizizenii DSM 618, B. flexus 10033, B. mojavensis DSM 9205T* and *B. subtilis DSM 5611*) were determined by incubating the enzyme in different pH buffers between the range of 6 to 10 for 15 minutes at 35^{o}C. The optimal pH of *B. flexus 100331* and *B. mojavensis DSM 9205T* culture were found to be 8 and for *B. spizizenii DSM 618* and *B. mojavensis DSM 9205T*, the optimum pH was found to be 6. The enzyme was active between pH ranging from 2 to 10. Amylase activity was relatively low *B. mojavensis DSM 9205T* at pH 6 whereas by *B. spizizenii DSM 618* at pH 8 (Fig.9). The resultant amylase activity showed by crude enzyme between pH 6 to 8 has quite similar result with the amylase activity showed by *Haloarcula spp*. [6].

Effect of temperature on activity of enzyme amylase

Influence of temperature on amylase activity of all the isolated strains (*B. spizizenii DSM 618, B. flexus 100331, B. mojavensis DSM 9205T* and *B. subtilis DSM 5611*) were observed by incubating the enzyme at different temperature ranging from 4^{o}C - 90^{o}C and resultant activity was determined under enzyme assay condition. The temperature profile of amylase activity of stains (*B. spizizenii DSM 618, B. flexus 100331, B. mojavensis DSM 9205T* and *B. subtilis DSM 5611*) showed optimum activity of 25.89 µmole/ml, 31.15µmole/ml , 20.65µmole/ml at 50^{o}C, and 23.25 µmole/ml at 70 ^{o}C respectively which indicate that the enzyme was thermostable at high temperature (Fig.10). The resultant amylase activity showed by crude enzyme between temperatures 50^{o}C -70^{o}C has quite similar result with the activity showed by

Haloarcula spp. [6]. Also highest amylase activity was observed at 55° C from *Bacillus sp*. WA21 by Asad *et al* [14]. Quadan*et al* [15] in their studies found that the alkali tolerant amylase from hyper thermophilic *Bacillus* strain HUTBB. *B. subtilis DSM 5611* had concentrated enzyme activity of 72µmole/ml at a temperature of 90° C after 10 min of incubation period.

Influence of various organic solvents on activity of enzyme amylase

The effect of five different organic solvents on the activity of the amylase was studied. The data indicated that the enzymes collected from all organic solvent tolerant bacteria showed high activity in the presence of tested organic solvents as compared to control. Crude enzyme extracted from *B. flexus 100331* containing broth was showed highly tolerance to n-propyl alcohol, toluene, and benzene whereas crude enzyme extracted from *B. spizizenii DSM 618* culture containing broth was highly tolerant to acetone and benzyl chloride as compared to others. n- Propyl alcohol was tolerate more by *B. flexus 100331* then *B. mojavensis DSM 9205T* and *B. subtilis DSM 5611* and least by *B. spizizenii DSM 618*. The highest activity was found in the presence of toluene as 31.15µmole/ml by *B. flexus 100331 B. subtilis DSM 5611* (28.52µmole/ml), *B. mojavensis DSM 9205T* (25.89µmole/ml) (Fig.11). Acetone was maximumly tolerated by *B. spizizenii DSM 618* (25.89µmole/ml) then *B. flexus 100331, B. subtilis DSM* 5611 and *B. mojavensis DSM 9205T*. Benzene was tolerated maximum by *B. flexus 100331* (23.25 µmole/ml), *B. mojavensis DSM 9205T* and lastly by *B. spizizenii DSM 618* and *B. subtilis DSM 5611*. Benzyl chloride was highly tolerated by *B. spizizenii DSM 618* (23.25 µmole/ml) and least by *B. mojavensis DSM 9205T* (7.45 µmole/ml). Almost equivalent results had obtained which was shown by *Haliarcula spp*. [6], *Bacillus spp*, and *Pseudomonas spp*. [7].

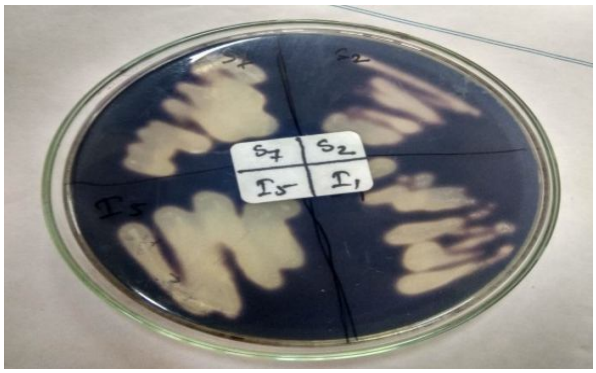
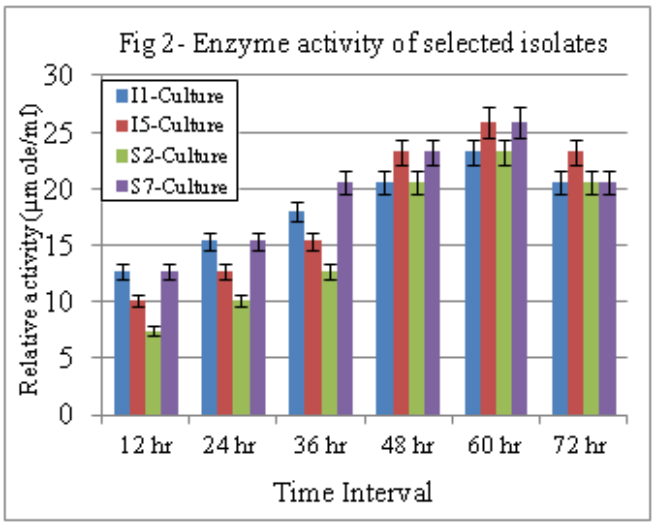
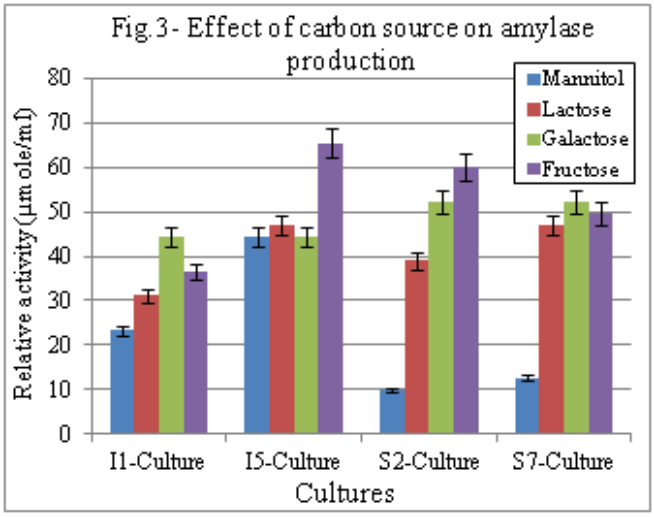
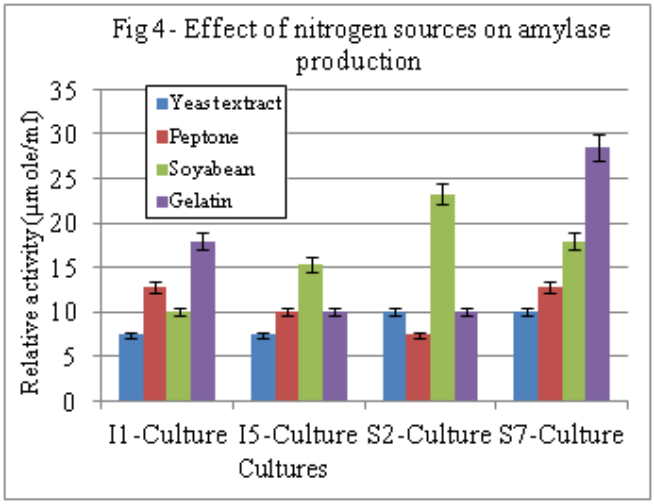


Fig 1- Amylase production (colorless region) by isolates







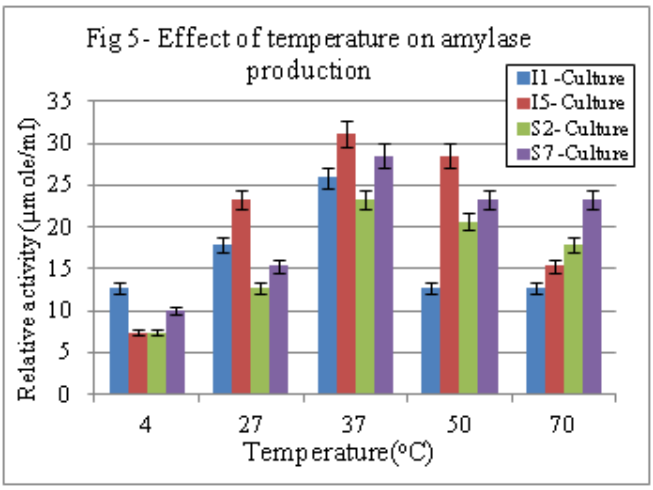


Fig 5- Effect of temperature on amylase production
I1 -Culture
I5- Culture
S2- Culture
S7 -Culture
Relative activity (µm ole/ml)
0
5
10
15
20
25
30
35
4
27
37
50
70
Temperature(°C)

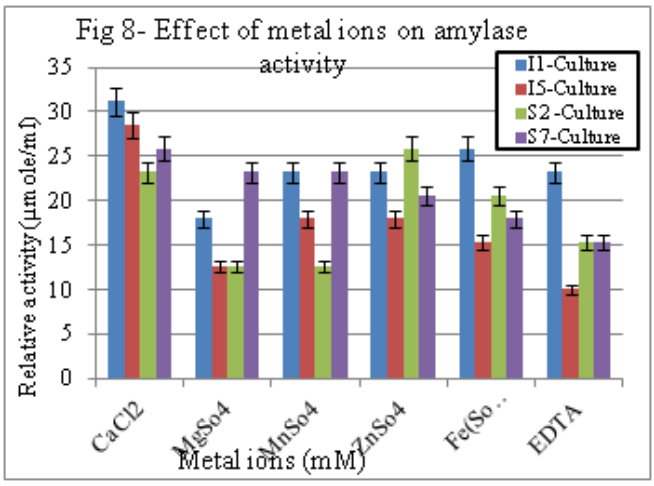


Fig 8- Effect of metal ions on amylase activity
I1-Culture
I5-Culture
S2 -Culture
S7-Culture
Relative activity (µm ole/ml)
0
5
10
15
20
25
30
35
CaCl2
MgSo4
MnSo4
ZnSo4
Fe(So…
EDTA
Metal ions (mM)

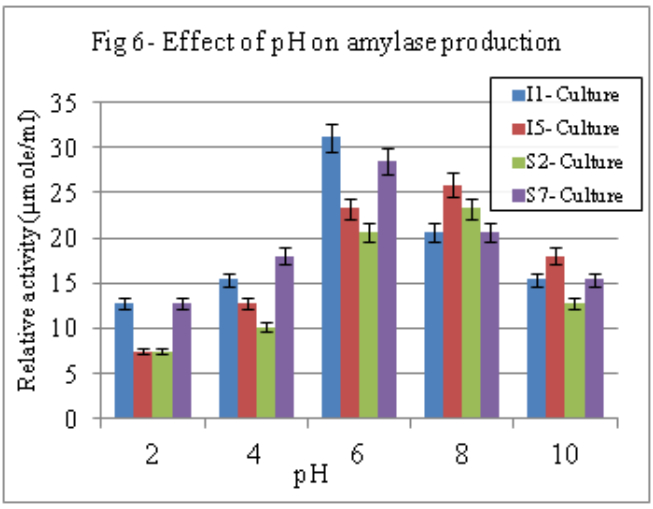


Fig 6- Effect of pH on amylase production
I1- Culture
I5- Culture
S2- Culture
S7- Culture
Relative activity (µm ole/ml)
0
5
10
15
20
25
30
35
2
4
6
8
10
pH

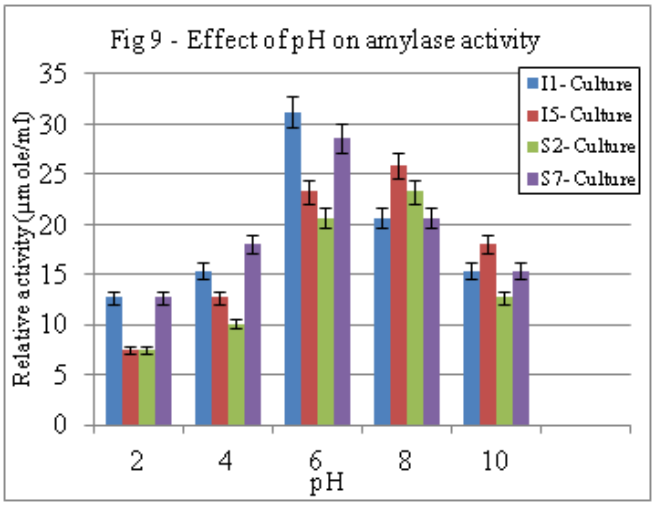


Fig 9 - Effect of pH on amylase activity
I1- Culture
I5- Culture
S2- Culture
S7- Culture
Relative activity (µm ole/ml)
0
5
10
15
20
25
30
35
2
4
6
8
10
pH

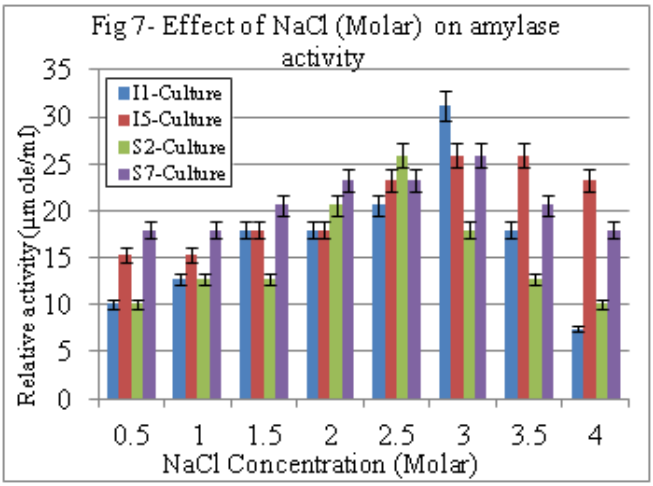


Fig 7- Effect of NaCl (Molar) on amylase activity
I1-Culture
I5-Culture
S2-Culture
S7-Culture
Relative activity (µm ole/ml)
0
5
10
15
20
25
30
35
0.5
1
1.5
2
2.5
3
3.5
4
NaCl Concentration (Molar)

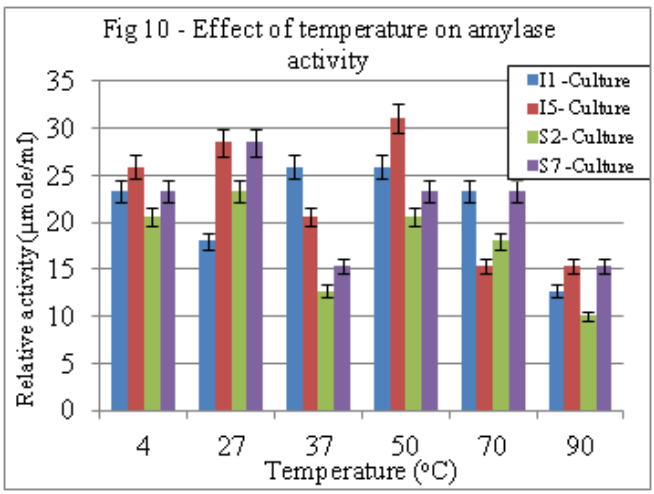
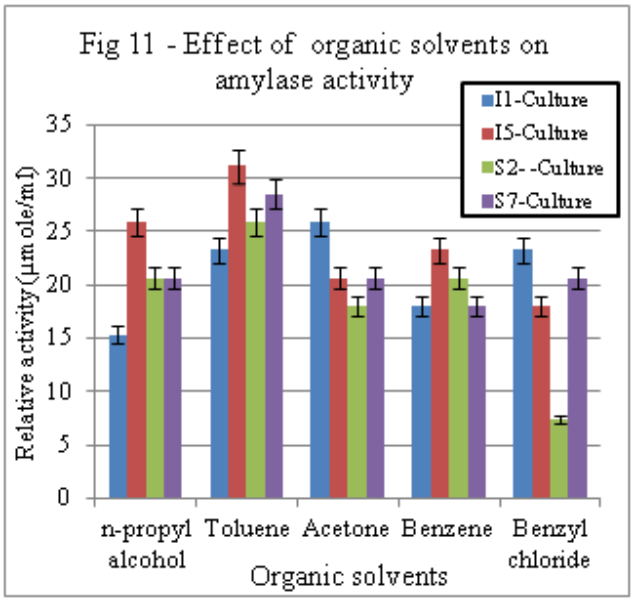


Fig 10 - Effect of temperature on amylase activity
I1 -Culture
I5- Culture
S2- Culture
S7 -Culture
Relative activity (µm ole/ml)
0
5
10
15
20
25
30
35
4
27
37
50
70
90
Temperature (°C)



V. CONCLUSION

The described amylases in the present study were highly tolerant and stable in the presence of solvents at various pH, temperature and salt concentrations and organic solvent tolerant which is quite similar to those found in other OST amylases. OST amylase producers were successfully isolated from oil contaminated soil. These organisms were able to produce good amount of amylase enzyme in the presence of organic solvents such as n-propyl alcohol, acetone, toluene, benzene and benzyl chloride.

Recently, solvent-tolerant bacteria are considered into a novel group of extremophilic microorganisms which has unique ability to synthesize amylase enzyme which can be tolerated to organic solvents. Since extracellular compounds which were natural solvent tolerant microorganisms were potentially steady within the sight of natural solvents. Therefore, the biotechnological exploitation of this enzyme could be of great importance in biotechnological potential.

As all enzymes were showed supreme activity in the presence of calcium ions and organic solvents. Thus, it can have major application in detergent industry for liquid soap preparation, in textile industry and pharmaceutical industry. The findings on the OST extracellular α-amylase with respect to its catalysis and enzymatic stability under unnatural environment such as salt, temperature, and organic solvents, would enrich the knowledge on non-aqueous enzymology, broadening the prospects of biocatalysis [2]. This property could be exploited to carry out bioremediation and biocatalysis in organic phase. The part of solvent stable compounds in non-polar biocatalysis should be investigated and could bring about novel applications [20].

In future, study could be done on purification of the crude OST amylase carried out in order to obtain purified form of OST amylase and SDS- PAGE analysis would be done to determine the molecular weight of OST amylase. Further, one can use this purified enzyme to treat starchy waste in the environment as well as in the industries where non aqueous parameters are dominant.

VI. AKNOWLEDGEMENT

We are thankful to the Microbiology department of K. J. Somaiya College of Science and Commerce, Mumbai, India, for financial support in the form of a search project.

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