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ISOLATION AND CHARACTERIZATION OF A NOVEL AMYLASE FROM *Bacillus Pseudofirmus* DW4(1)

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ABSTRACT

Culture dependent phenotypic characterization analyses were applied to study the amylase producing bacteria from Indian Soda (Lonar crater). The uniqueness of the Lonar Lake water is its salinity and alkalinity. A total of 29 bacilli strains were isolated from the water and sediment samples collected from the hyperalkalinesaline environment of Lonar crater. The fifteen bacterial strains were found amylase producing bacterial strains, out of them strain DW4(1) was selected for 16S rDNA sequencing, production and, partial characterizations of amylase on the basis of their maximum amylolytic activity. The phylogenetic position indicated the strain was related to phylum Firmicutes. Optimum enzyme activity was found to be at 40°C (1.2 unit/mL), pH 10.0 (5.9 units/mL), and 4% NaCl. The amylase was highly stable over broad temperature from 40 to 100°C, pH 6.0-12.0, and NaCl concentration 0.5-10% ranges, showing excellent thermostablity, and haloalkaline tolerant nature. A Lineweaver-Burk plot indicates that enzyme has a Km of 2.94 mg/mL and a Vmax of 90.90 mg/mL/ min. The enzyme activity has enhanced by BaCl2 (7.4 units/mL/min) but highly inhibited by KCl, indicating it was a metalloenzyme. Among the organic nitrogen sources, optimum amylase production was found to be in presence of yeast extract. This is a valuable information for enzyme production and optimization of amylase from Bacillus pseudofirmus DW4(1) has bright future towards the improvement and production of novel enzymes for entirely new areas of industrial and biotechnological applications involving molecular enzymology. The developing novel techniques in genetic engineering combined with better knowledge of structure and function allow fulfillment of industrial needs and exploration of novel applications in future.

KEYWORDS : Haloalkaliphiles, Lonar Lake, Bacillus, Enzyme, Amylase

Now a day, in modern biotechnology microbial enzymes occupy a prominent position. However, only a few of microbial strains has been biotechnological potentially explored, although the only few bacterial strains are often could suitable for harsh stipulation required in industrial processes due to delicate, denaturation and unstable nature. Because of these restrictions, researchers are concentrated towards extrmozymes that function under extreme conditions. The unusual properties of these metabolites offer a potential opportunity for their utilization in processes demanding such extreme conditions. Haloalkaliphiles, representing most diverse group of holophiles could grow optimally at high salt concentration along with grow optimally at pH values at or above 10 (Horikoshii, 1999). Disregards the outstanding stability of haloalkaliphilic enzymes, only few of them have been explored for their properties and industrial application. The various detail factors for optimization developing large scale production has not been performed due to very few documentation on the metabolic studies of these organism. The several attempts were studied for their physiology of these strains by maximum production stipulation or bioprocess that develop or designing bioreactor and bioprocesses that improve growth conditions and resulted into their productivity (Joshi et al. 2007). Among the extracelluar enzymes, alkaline amylases are among the most important classes of industrial hydrolytic enzymes. So far, alkaline amylase from haloalkaliphiles has been relatively less explored compared to other enzyme. However, as the amylase is wide spread among the haloalkaliphiles and many of them possess the potential that can be novel for biotechnological applications. With increasing importance on the environmental protection with decreasing pollution, the use of enzymes particularly from extremophiles, gained considerable attention from the last several years. In order to obtain commercially viable production, it is important to optimize fermentation media for the growth and amylase production. Moreover, most of the studies on haloalkaliphilic bacteria in past have largely focused on hyper saline environments and the exploration of relatively moderate saline and alkaline environments is only the beginning.

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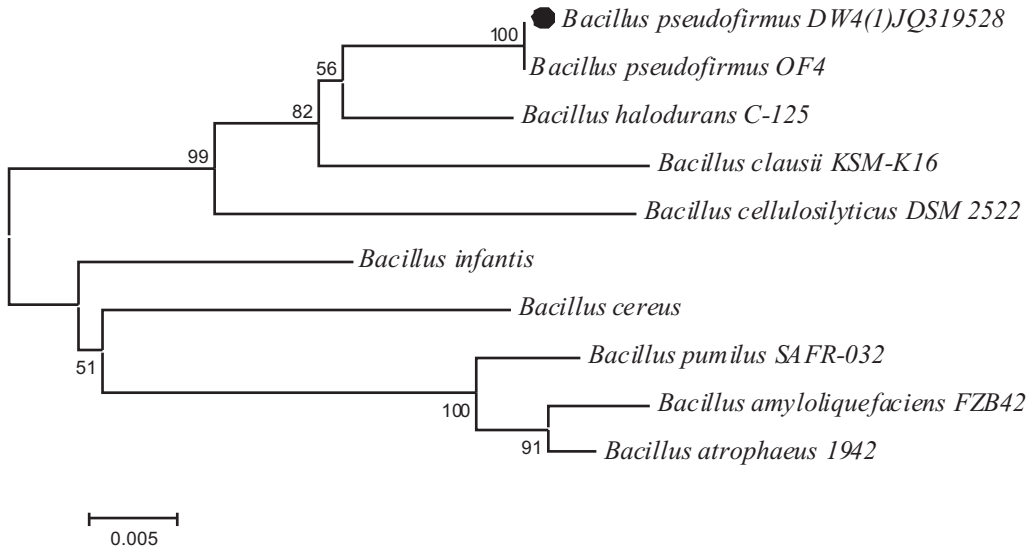


Figure. 1: Phylogenetic Tree Based on a Comparison of The 16s Ribosomal DNA Sequences of Lonar Lake Isolates and Some of Their Closest Phylogenetic Relatives. The Numbers on The Tree Indicates The Percentages of Bootstrap Sampling Derived From 1,000 Replications.

The microbial diversity of saline lakes has been studied primararily acting on the isolation and characterization of organisms with biotechnological potential (Horikoshi 1999; Jones et al. 1998). Martin et al. (2001) were found both alkalitolerant and obligate alkaliphiles were found and identified by phylogenetic analysis as the microbial species found in Ethiopian soda lake microbial population and known for being good amylase producers. As far as Indian soda lakes are concerned, a culture-dependent approach has not been yet applied to analyze bacterial diversityand alkaliphilic protease producing microorganisms isolated from Lonar Lake (Joshi et al. 2007). In view of the above facts, the present study focused on the optimization of alkaline amylase production from a haloalkaliphilic bacterial strain isolated from Indian Soda Lake.

MATERIALS AND METHODS

Collection and Isolation

Enrichment and isolation of microorganisms 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 chloride.

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, floods the iodine solution into the plate. The halo zone was observed for amylolytic activity of the isolates (Tambekar and Dhundale 2013).

Identification of the Bacterial Culture

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

16S rDNA Sequencesing

DNA was extracted from Bacilli culture using standard phenol chloroform protocol (Sambrook et al. 1989). 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 sequence (Foster, California USA).

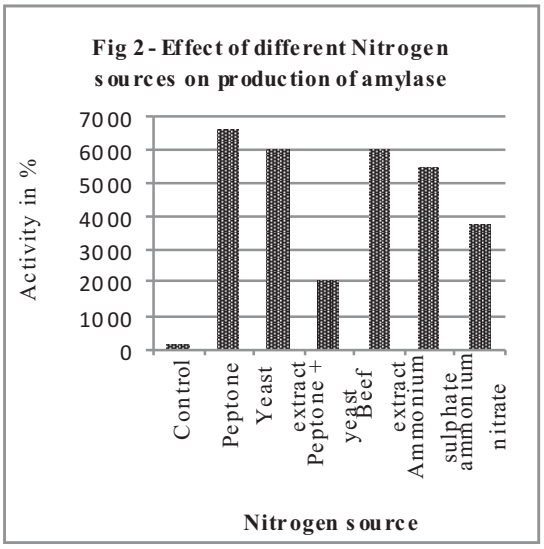


Fig 2 - Effect of different Nitrogen sources on production of amylase
Activity in %
7000
6000
5000
4000
3000
2000
1000
0
Control
Peptone
Yeast extract
Peptone + yeast
Beef extract
Ammonium sulphate
ammonium nitrate
Nitrogen source

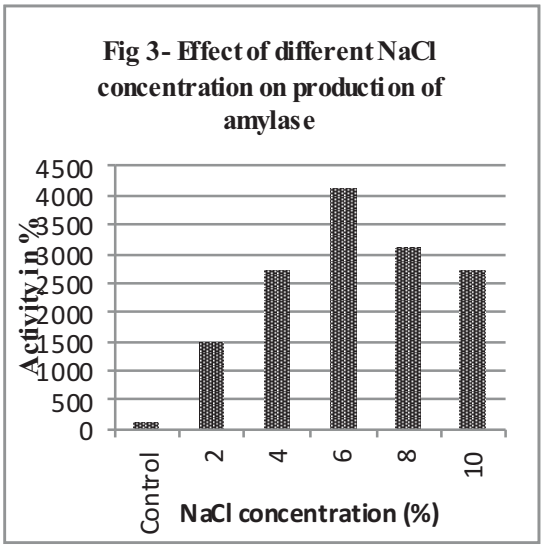


Fig 3- Effect of different NaCl concentration on production of amylase
Activity in %
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
2
4
6
8
10
NaCl concentration (%)

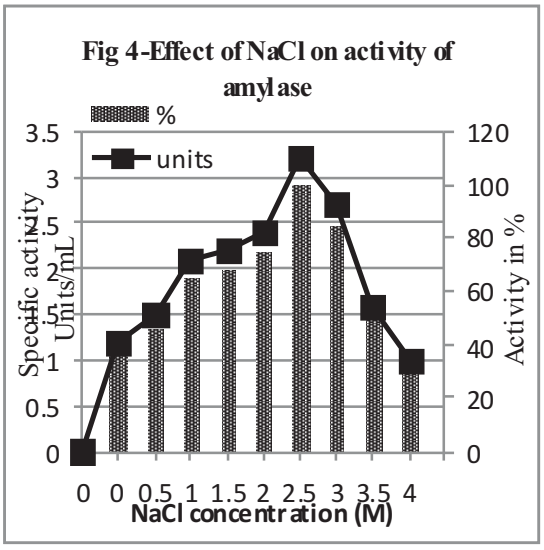


Fig 4-Effect of NaCl on activity of amylase
%
units
Specific activity Units/mL
Activity in %
NaCl concentration (M)

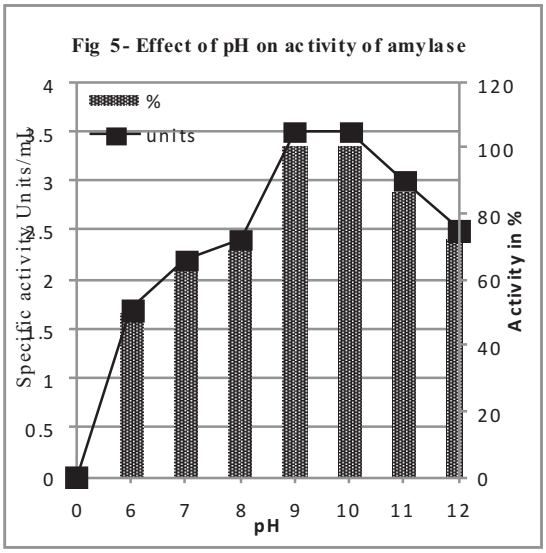


Fig 5- Effect of pH on activity of amylase
%
units
Specific activity Units/mL
Activity in %
pH

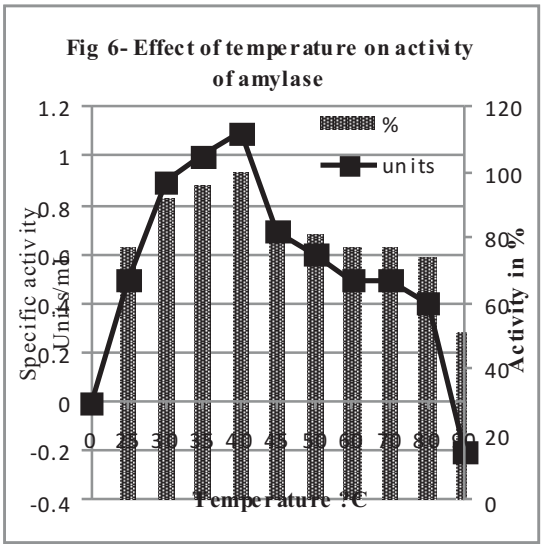


Fig 6- Effect of temperature on activity of amylase
%
units
Specific activity Units/mL
Activity in %
Temperature °C

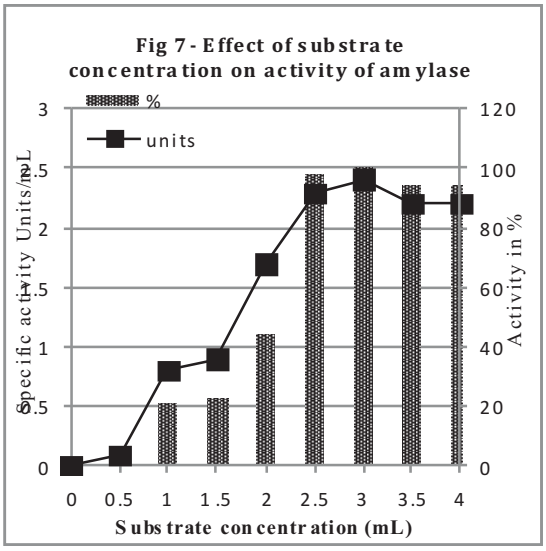


Fig 7 - Effect of substrate concentration on activity of amylase
%
units
Specific activity Units/mL
Activity in %
Substrate concentration (mL)

Phylogenetic Analysis

The 16S rDNA sequences were analyzed using BLAST program. Multiple Sequence Alignment of approximately 900 bp sequence was performed by using Clustal X version 1.8. The phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of Mega 4 program package.

Preparation of Enzyme Extracts

The 100 mL Starch nutrient broth 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 enzyme source.

Assay of Enzyme Activity and Protein Concentration

Characterization of amylase was carried out as described earlier by Tambekar et al, (2013). The effect of temperature, pH and substrate concentration on α-amylase activity was studied and Km and Vmax values of the enzyme were calculated from Lineweaver-Burk (double-reciprocal) plot.

RESULTS AND DISCUSSION

Identification of the Strain on Traditional and Molecular-Genetic Analysis

On the basis of the observed traditional morphological and phenotypic characteristics the DW4(1) was belong to genus Bacillus. Biochemical and physiological tests, growth properties and 16S rDNA sequencing indicated that the bacterial isolate obtained from the Lonar Lake sediment sample was Bacillus pseudofirmus. The 16S rDNA sequence was submitted to NCBI Genebank Database and the accession number as JQ319528

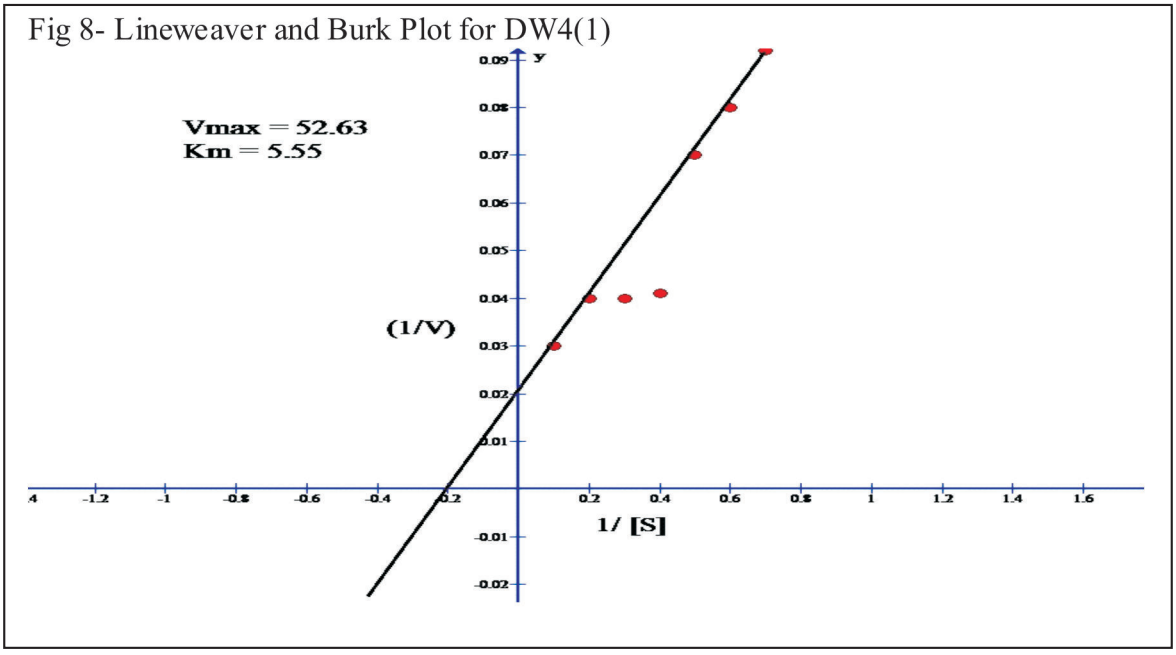
The estimation of Amylase Activity

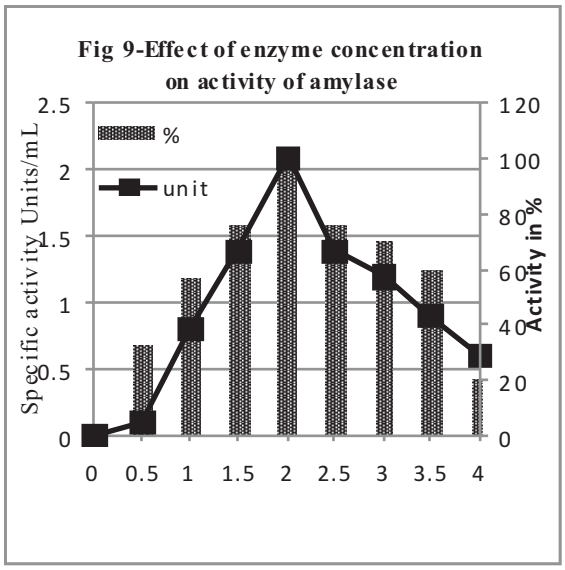
The results are particularly important in view of the fact that only few enzymes have been purified and biochemically characterized from Haloalkaliphilic bacteria. The estimation of amylase activity was performed by standard assay conditions. The activity of amylase from Bacillus pseudofirmus DW4(1) after 15 min of incubation was found to be 0.933 Units/mL.

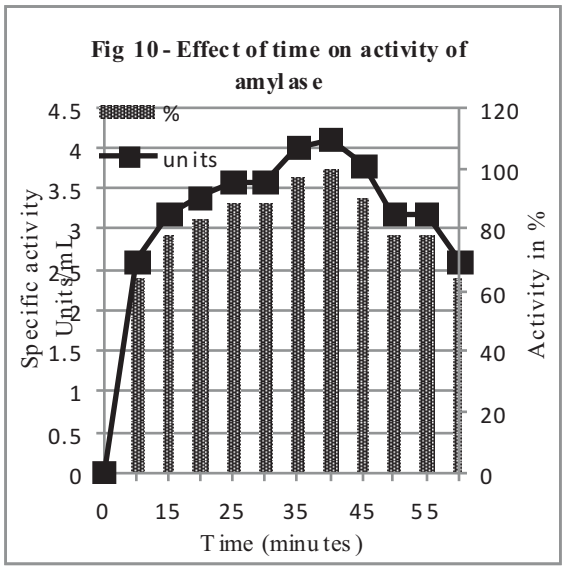
Effects of Different Nitrogen Sources on Amylase Production

The physico-chemical parameters, composition and concentration of media and enrichment stipulation broad effect on the growth and production of extracellular enzymes from microorganisms. Therefore in the present study several inorganic and organic nitrogen sources were examined to optimize the source of nitrogen for amylase production. The result showed that the peptone was the best nitrogen sources having activity of 3.25 Units/mL followed by beef extract and yeast extract (90%). The enzyme activity without any additives was taken as 100%. The nitrogen sources have a noticeable influence on the production of amylase of Bacillus pseudofirmus DW4(1).

Fig 8- Lineweaver and Burk Plot for DW4(1)







Similar results were also reported by Bacillus sp. and Bacillus thermooleovorans for the peptone has been reported as a good nitrogen source for á-amylase production (Tanyildizi et al. 2005; Malhotra 2000). Aqeel and Umar perform the detail study about the various nitrogen sources with also quantative analysis of nitrogen source on the production of amylase from Bacillus megaterium. Different nitrogen which were source that enhance the production of amylase such as yeast extract, beef extract, peptone, ammonium sulphate, ammonium chloride, cysteine, urea, potassium nitrate and ammoium nitrate. Suribabu et al. (2014) studied on the submerged fermentation for optimization of various nitrogen sources for the production of amylase from Brevibacillus ortelnsis.

Effect of Different NaCl Concentration on Production of Amylase

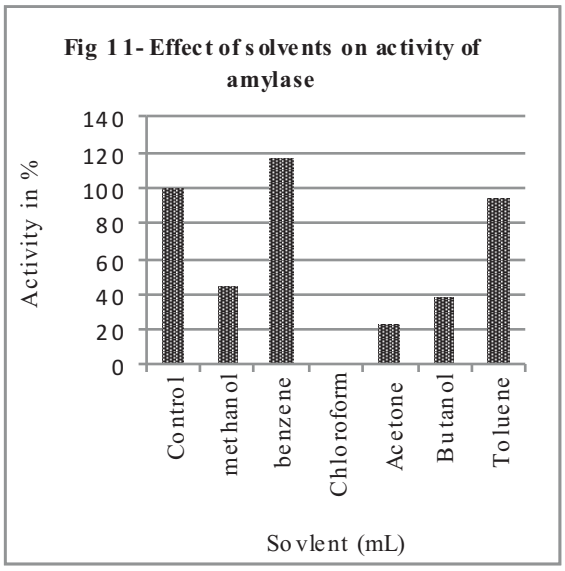
The strain Bacillus pseudofirmus DW4(1) grew well at various concentration of NaCl ranging from 2-10%. The optimum growth was at 6% NaCl and no growth was observed in the absence of NaCl. Since the NaCl were required for growth and production of amylase. The amylase retained 75 and 65% of activity in the presence of 8 and 10% NaCl, respectively. However, very less activity was observed in presence of 2% NaCl concentration. In the present studies, the high concentration of NaCl were required for optimum production of amylase. The enzyme characterizations in the present study focus on several features which were similar to those found in other halophilic enzymes, including salt-dependent activity (Pandey and Singh, 2012).

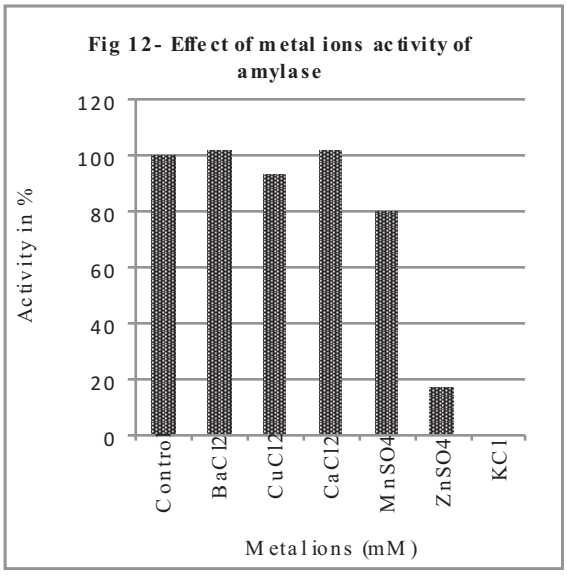
Effect of NaCl on Activity of Amylase

When different molar NaCl concentrations was used to check the activity of amylase, it was found that the highest activity (3.5 Units/mL) was found at 2.5M of NaCl concentration which was considered as 100%. The lowest activity (1.2 and 1 Units/mL) was observed at 0 and 4 M concentration of NaCl concentration (37% and 31%) respectively.

Effect of pH on Activity of Enzyme Amylase

The effect of pH on amylase activity of Bacillus pseudofirmus DW4(1) was determined by incubating the enzyme in different pH buffers ranging from 6-12 for 10 minutes at 37°C. The amylase was active in a wide pH range with optimum activity at pH 9-10 and was stable over a broad pH range 6-12 with retention of more than 50-70% of initial activity. The optimum activities of this enzyme were at pH 9 and 10 with 3.5 units/mL. Amylase activity was relatively low at pH 6 (1.7 Units/mL). At pH 8, 7 and 6, the enzyme has relative enzyme activities were decreases upto 69, 63 and 50% respectively. The enzyme activity decreased dramatically at pH 11 (3 Units/mL) and 12 (2.5 Units/mL). It was highly active over a broad pH range from 5 to 7 and stable in a wide pH range between 4 and 9. However, as the amylase is wide spread among the haloalkaliphiles and





many of them possess the potential that can be novel for biotechnological applications. Alkaline amylase from Bacillus pseudofirmus DW4(1) novel feature in the extremozyme which can produce the amylase at alkaline pH which was beneficial for industrial status.

Effect of Temperature on Activity of Enzyme Amylase

The starch solution and enzyme dilutions prepared in different buffers of pH ranging between 6 and 12 were used in the reaction mixtures and incubated at 40 °C. For assessing the effect of temperature on enzyme activity, the reaction mixtures were incubated at different temperatures 25°C-90°C and residual activity were determined under enzyme assay condition. The temperature profile of amylase from Bacillus pseudofirmus DW4(1) showed maximal enzymatic activity of 1.1 Units/mL at 40°C. The amylase retained more than 92-96% of the highest activity between 30-35°C. Subsequently, the enzyme activity progressively decreased from 45-80°C. About 15-49% of activity was retained at 45-80°C and there was no activity at 90°C. Hence the enzyme was inactive at 90°C. However many Bacillus strains were produce thermostable amylases, the most commonly used for their biotechnological potential such as Bacillus licheniformis, Bacillus amyloliquifaciens, Bacillus stearothermophilus, Bacillus subtilis and Aspergillus niger (Riaz et al. 2003). However the species of bacillus have been globally use for the commercial application of thermostable amylase with a higher operational stability and a longer shelf life. For such stabilization amylase was isolated from the thermophilic microorganism were used for the amylase production (Rao and Satyanarayana, 2007).

Effect of Substrate Concentration on Activity of Enzyme Amylase

The influence of different concentrations of substrate was assayed ranging from 0.5-4 mL under constant assay conditions. Substrate utilization revealed that 97% of substrate was utilized (2.3 Units/mL) but maximum substrate utilization (2.4Units/mL) occurred at 2.5 mL of substrate concentration. The kinetic parameters Km and Vmax of the enzyme produced by Bacillus pseudofirmus DW4(1) was determined from the Lineweaver-Burk plot. This plot indicates that the enzyme has a Km of 5.55 mg of starch per millilitre and a Vmax of 52.63 mg of maltose per millilitre per min.

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 (2.1 Units/mL) at 2 mL of enzyme concentration. The activity of amylase decreases as the enzyme concentration increase with 2.5 mL. The enzyme retained about 76-60% of its activity at enzyme concentration of 2.5 and 3.5 mL respectively. There was very less activity at 4 mL of enzyme concentration revealed 0.6 Units/mL (20%).

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 4.1 Units/mL was shown when the reaction mixture containing enzyme was incubated for 40 minutes. But as the incubation time goes on increased the activity decreased from 45 min to 10 and 60 min. The lowest activity was shown when incubation period was of 60 minutes (2.6 Units/mL).

Influence of Various Organic Solvents on Amylase Activity

The use of organic solvents as reaction media for enzymatic reactions furnish various industrially attractive beneficial features compared to base on response. Despite the advantages, native enzymes almost widely revealed low reactive and stabilities in the presence of organic solvents. The effect of organic solvents on the activity of the amylase was determined. The data elucidate that the enzyme was highly active to Benzene when used as organic solvent as compared to control which was considered as 100%. The loss of enzyme activity was found in presence of all tested organic solvents except Benzene. Thus, all solvents, except Benzene, have an inhibitory effect on the activity of amylase produced by Bacillus pseudofirmus DW4(1). Organic solvent tolerance of an α-Amylase from haloalkaliphilic bacteria as a function of pH, temperature and salt concentrations was studied by Pandey and Singh, (2012). 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 a-amylase from Nesterenkonia sp. These organic solvent-tolerant enzymes are regard to have potential for benefits in industrial chemical processes (Doukyu and ogino, 2010)

Influence of Different Metal Ions on Amylase Activity

The influence of different metal ions on amylase activity of Bacillus pseudofirmus DW4(1) was carried out under the standard assay conditions. Metal ions have different effects on activity of amylase. The enzyme activity without any additives was taken as 100%. The enzyme activity was enhanced by $BaCl_2$ and $CaCl_2$. However, the amylase activity was inhibited by KCl, $ZnSO_4$, $MnSO_4$ and $CuCl_2$. The optimum amylase activity 3.6 Units/mL (102%) was enhanced in presence of $BaCl_2$ and $CaCl_2$.

CONCLUSION

In this work, alkaline-adapted amylase-producing bacterium was isolated and identified as Bacillus pseudofirmus DW4(1) on the basis of its phenotypes, biochemical testing and 16S rDNA gene sequencing. The different characteristics toward the sensitivity to organic solvent, metal ions and enhanced by $Bacl_2$, $CaCl_2$, indicated a novel nature of amylase. The enzyme described in the present report emphasizing several features like to those found in other halophilic enzymes, including salt-dependent activity. Further, the alkaline pH profile and thermal stability closely resembled to features reflected in alkalo-thermophilic organisms. Moreover, the stability of alkaline amylase at multitude of extremities of salt, pH, temperature, metal ions and organic solvents displayed unique novel features for biotechnological applications along with the suitability of this enzyme for industrial status. To the best of our knowledge, this is the first report on a microbial amylase with increased activity in the presence of benzene. It suggests that the mechanism of action of the amylase of Bacillus pseudofirmus DW4(1) is different from reported amylases. In additions, the findings have also highlighted the presence of a novel haloalkaliphilic bacterium from the unexplored saline habitats in Indian Soda lake and these findings also indicated that the Lonar Lake is one of the best resources for screening novel haloalkaliphilic adaptive enzymes. The detection of gene which is responsible for this bacterial strain survive in extremophilic condition and manipulation of the amylase corresponding gene in order to overproduce this enzyme is now in progress simultaneously large scale production of the enzyme with alternate carbon sources and its cost effectiveness are also in progress. In the case of enzyme characterization, the amino acid sequence of an enzyme will be determined and kinetic studies done with a purified enzyme.

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