International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol5, Issue4, 2014, pp731-742
http://www.bipublication.com

Partial characterization and optimization of alkaline amylase production from *Bacillus lehensis* isolated from alkaline saline Lonar Lake

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[Received 29/10/2014, Accepted-05/12/2014]

ABSTRACT

A Gram positive bacterium isolated from a water sample from the alkaline saline Lonar Lake. It was identified as *Bacillus lehensis* BW3(2) by morphological, biochemical characterization and 16S rDNA sequencing. The bacterium grew in sodium chloride (NaCl w/v) from 0.5 to 7% and at pH 7–12. A substantial level of extracellular alkaline amylase was produced by *Bacillus lehensis* BW3(2). Optimum enzyme activity was found to be at 80°C, pH 10.0, and 2% 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 6.66 mg of starch per mL and a Vmax of 55.55 mg of maltose per mL/ min. The enzyme activity has enhanced by $BaCl_2$ but highly inhibited by KCl, indicating it was a metalloenzyme. Among the organic nitrogen sources, optimum growth and amylase production were supported by the combination of peptone and yeast extract. This is a valuable information for enzyme production and optimization of amylase from *Bacillus lehensis* BW3(2) has bright future towards the improvement and production of novel enzymes for entirely new areas of industrial and biotechnological applications.

Key words: Haloalkaliphiles, Lonar Lake, Bacillus, Enzyme, Amylase

INTRODUCTION:

Alkaline saline environment were occurred naturally in the world, these lakes are characterized by their highly basic pH values ranging from 10-12. These environments generated due to the combination of geological, geographical and climatic condition giving rise to the accumulation of sodium carbonate and sodium chloride which leads to alkaline saline environment [17] Alkaline saline lakes, are characterized by the presence of a high concentration of sodium carbonate formed by evaporative concentration, and are also associated with varying concentration of salinity and low concentration of both Mg2+

and Ca2+ ions [11]. Haloalkaliphiles are vigorously grown in both saline and alkaline environments. Soda lakes are widely distributed [10]; however, as a result of their inaccessibility, few such lakes have been explored from the microbiological point of view.

The microbial population of these lakes, which is considerably diverse phylogenetically, includes Archaea, Cyanobacteria, Proteobacteria and Firmicute *[12,10,15*]. Alkaliphilic microorganisms, in particular Bacillus species, have attracted much interest because of their ability to produce extracellular metabolites that are active and stable at high pH. The unusual properties of these metabolites offer a potential opportunity for their utilization in processes demanding such extreme conditions. Haloalkaliphiles is focused on microbiological classification and genetic characterization, with limited work to discover their industrial application [16]. 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. Extremozymes are now-a-day replacing chemical catalyst in manufacturing of chemicals, textiles, pharmaceuticals, paper, food and agricultural chemicals since the enzymes prepared with suitable properties with the advent of new knowledge in biotechnology, the spectrum of enzyme application has widened in many other fields [29]. The emphasis has been on alkaline amylase and several microbes have been looked for their ability to secrete these enzymes [24]. The enzyme has better resistance to alkali and some other denaturing chemicals in the reaction mixture. It is also thermostable organism growing in naturally alkaline habitats may how analyses with special characteristics [33].

Alkaline amylase producing bacteria are of great importance in detergent and textile industry due to their high thermostability and pH stability and most important industrial enzymes, accounting for about 60% of total enzyme market [5,18]. As there is large demand of amylase, isolation and production of amylase enzyme is most important to fulfill this demand [31]. Lonar Lake an Indian soda lake situated in Lonar, District Buldhana, Maharashtra, India (latitude 19° 8, longitude 76° 36), The alkaline Lonar crater is a unique basaltic rock meteorite impact crater, ranking third in the world and is filled with saline water having an average pH of 9.5-10. During a previous study carried out from 2008 to 2013, 55 alkaliphilic bacterial cultures were isolated from Lonar Lake [33]. These were screened for production of amylase. One of these bacteria, *Bacillus lehensis*, has the ability to produce amylase at alkaline pH. The present work describes the screening and characterization of amylase production from *Bacillus lehensis.*

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 Horikoshi I, Horikoshi II, and nutrient agar at pH 10, nutrient agar at pH 10.0 with 30 g l−1 sodium chloride. Well-isolated and differentiated colonies were transferred to respective medium agar slants [34].

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 5.0, 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 [35]. The isolate BW3(2) was selected for further characterization and detailed studies.

Identification of the bacterial culture: The Gram positive amylase producing Bacterial

culture were examined for their colony, morphological character, motility, capsule staining, spore staining and standard biochemical test such as catalase, oxidase, nitrate reduction, methyl red reaction, voges prauskar test, citrate utilisation by Simmons, urease activity, Indol production and production of acid from glucose, arabinose, mannitol, xylose, lactose, trehalose, sucrose, cellobiose, galactose, maltose, fructose, salicin, sorbitol, raffinose according to Bergey's Manual of systematic bacteriology. The growth of cultures on respective agar plate at incubation temperatures ranging from 37°C to 65°C were examined.

16S rDNA sequencesing:

DNA was extracted from *Bacilli* culture using standard phenol chloroform protocol [28]. The partial sequence of the 16S rRNA gene was amplified by using polymerase chain reaction and universal primer E. coli specific primers, 16F 27 (5' CCAGAATTGATCMTGGCTCAG-3') and 16R 1525 (5' TTCTGCAGTCTAGAAGGAGGTGWTCCA GCC– 3'). The PCR conditions were used an initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for 1 minute and extension at 72°C for 1 minute and final extension at 72°C for 1 minute. The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl procedure [3] and directly sequenced on the Applied Biosystems Model 3730 DNA sequence (Foster, California USA).

Phylogenetic Analysis:

The 16S rDNA sequences were analyzed using BLAST program [1]. In addition, sequences were analyzed via BLAST to identify the most closely related database sequences. Multiple Sequence Alignment of approximately 900 bp sequence was performed by using Clustal X version 1.8 [39]. The phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of Mega 4 program package [20].

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:

The standard graph of maltose was prepared by adding different concentration of standard maltose (1 mg/mL) into a series of test tubes and added 2mL DNS solution incubate all tubes in boiling water bath for 5 min after incubation add 1mL NaK tartarate to stop the reaction. Estimation of amylase was carried out with 2.5 mL of starch in a test tube; 2.5 ml of PO_4 butter, 1mL of NaCl and 1 mL of enzyme source was added and incubated for 15 min at 37°C. After incubation 1 mL of DNS+NaoH solutions was added and incubates in boiling water bath for 5 min after incubation 1 mL of Nak+ tartarate was added to stop the reaction. The absorbance was measured at 520 nm using double beam UV/VIS spectrophotometer. One enzyme unit (unit/ml) is defined as the amount of enzyme which releases 1µ mole maltose. Characterization of amylase was carried out as described earlier by Tambekar *et al.* [36].

CHARACTERIZATION OF AMYLASE:

Effect of pH on alkaline amylase activity:

The effect of pH on alkaline amylase was determined by assaying the enzyme activity at different pH values ranging from 6.0 to 12 using the PO_4 buffer systems with concentration of buffer was 0.2 M.

Effect of temperature on alkaline amylase activity:

The effect of temperature on alkaline amylase activity was determined by incubating the reaction mixture (pH 10) for 15 min at different temperature ranging from 30°C to 80°C.

Effect of substrate on alkaline amylase activity:

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.

Effect of enzyme on alkaline amylase activity:

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

Morphology and culture characters:

The bacterial strains BW3(2) isolated from Lonar Lake was found to be gram positive, motile rod measuring 3.5 to 4.0 µm in length and approximately 0.5 µm in width, bearing cylindrical endospore which lie at center. Culturally the strain BW3(2) were white pigmented and characteristically very small 1 mm in diameter, shape was circular and elevation was raised with concave belaved with entire edges along with transparent internal structure.

Identification of the Strain:

On the basis of the observed traditional morphological and phenotypic characteristics the BW3(2) 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 lehensis*. The 16S rDNA sequence was submitted to NCBI Genebank Database. The accession number as JX134050. Previously *Bacillus lehensis* was firstly reported by Ghosh *et al.* [9] lehensis pertaining to Leh, in India, where the type strain was isolated. Blanco *et al.* [4] was isolated *Bacillus lehensis* from cassava starch wastewater and studied optimization of parameters for cyclodextrin glycosyltransferase production.

Salt and pH tolerance:

The strain exhibits moderate growth at pH 7 to 12, NaCl concentrations 0.5% to 7% and temperature 37°C to 50°C. *Bacillus lehensis* isolated from Leh was previously isolated and described by Ghosh *et al.* [9], It was examined that the strain MLB2T could grow in the presence of NaCl concentrations up to 12%, pH 7.0–11.0 (optimum, pH 8.0) Growth occurs at temperatures in the range 10–37°C (optimum temperature, 25°C).

The estimation of amylase activity:

It was performed by standard assay conditions. The activity of amylase from *Bacillus lehensis* BW3(2) after 15 min of incubation was found to be 1.53 Units/mL.

PRODUCTION OF AMYLASE

Effect of different Nacl concentration on production of amylase:

The strain *Bacillus lehensis* BW3(2) grew well at various concentration of NaCl ranging from 2-10%. The growth of *Bacillus lehensis* BW3(2) strain was not greatly affected as NaCl concentration was increased from 0 to 10%. The optimum growth was at 2% NaCl and no growth was observed in the absence of NaCl. The enzyme activity without any additives was taken as 100%. The amylase retained 80 and 69% of activity in the presence of 4 and 6% NaCl, respectively. However, more than 49% of the enzyme activity could be detected even at 10% NaCl concentration. As the NaCl concentration goes on increasing, the activity of amylase goes on decreasing. Thus, the salinity was found to be a significant factor in the production of amylase. Same results were obtained by Shanmughpriya *et al.* [29] though their strain was an alkalophilic amylase producer marine isolate *Halobacterium salinarum*, it showed the amylase production in the medium supplemented with 2% NaCl. Similar results also reported by Haifeng *et al.* [13] on the production of amylase from the marine yeast *Aureobasidium pullulans* N13d. They revealed that, the cell growth and production of amylase was not significantly affected as NaCl concentration was increased from 0 to 3.0%. The more production of amylase was observed when the medium prepared with sea water than with distilled water.

Effect of Different Carbon Sources on Amylase Production:

It has been reported that microbial amylase is extracellular and inducible enzyme is generally induced by starch (Gupta et al. 2003). Therefore, the influence of different carbon sources on amylase production by the alkaliphilic *Bacillus lehensis* BW3(2) were studied. In the present study, soluble starch was the best carbon source followed by the galactose for production of amylase whether the production of amylase was intensely decrease with fructose. Similar study was done by Sudharhsan *et al.* [32], They have observed repression effect of fructose on amylase production. *Bacillus lehensis* BW3(2) showed comparatively low enzyme production when lactose and mannitol as carbon source. This might due to lactose and mannitol effect that repressed the enzyme production. Contrary to this, Thippeswamy *et al*. [38] were observed the optimum production of amylase with maltose and Sudharhsan *et al*. [32] have studied optimum amount of amylase by a Bacillus sp. with lactose as a carbon source. The maximum activity was reveled in the presence of 1% soluble starch. The production of carbohydrate-degrading enzymes in most species of the genus Bacillus is subject to catabolic suppress by easily metabolizable substrates such monosaccharide [30]. Similar results were also reported by the Marine Yeast *Aureobasidium pullulans* N13d [13]. The hyperthermophilic archaeon *Sulfolobus solfataricus* and *Geobacillus* sp. IIPTN in which glucose repressed production of á-amylase [8,14].

Effects of Different Nitrogen Sources on Amylase Production:

The nitrogen sources have a noticeable influence on the production of amylase of *Bacillus lehensis* BW3(2). Several inorganic and organic nitrogen sources were examined to optimize the source of nitrogen for amylase production generally, organic nitrogen sources have been preferred for production of amylase [13]. The result showed that combination of peptone and yeast extract were the best nitrogen sources followed by individually peptone and yeast extract. 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 [23, 37]. Rajagopalan and Krishnan [27], reported that yeast extract was the best nitrogen source for á-amylase production by *B. subtilis* KCC103. In the present study minimum production was found with beef extract as nitrogen source in this contrast, Lu *et al*. [22] revealed that the optimum production was found with beef extract. The inorganic nitrogen sources such as ammonium sulfate and Ammonium nitrate were also examined for the production of amylase but the production of amylase was found minimum amount while no influence of inorganic nitrogen sources on the production of á-amylase by *Anoxybacillus flavithermus* under solid-state fermentation [26]. In this contrast, ammonium sulfate and ammonium nitrate supported better amylase production by *Aspergillus oryzae* while only ammonium sulfate supported better amylase production by *Aspergillus nidulans* [12]. Therefore the different nitrogen sources were highly influenced on the production of amylase by different microorganism of genus with different production condition.

Effect of pH on activity of enzyme amylase:

The effect of pH on amylase activity of *Bacillus lehensis* BW3(2) 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 lehensis* BW3(2) amylase were found to be 10. The optimum activity of this enzyme was at pH 10 with 1.1 units/mL, which considered as 100%. The activity was decreased dramatically at pH 11 (0.6 Units/mL) and 12 (0.4 Units/mL). At pH 7 and 8, the enzyme has relative activities found to be 68 and 93% respectively. In acidic buffer at pH 6, the activity was found to be 0.2 units/mL. This showed that the enzyme required a slightly alkaline pH for its activity. Earlier studies have shown that amylases were active up to the pH of 5-10. It was comparatively less and the enzyme of *Bacillus*

lehensis BW3 (2) strain has still wider ranges of tolerances than the previously reported for amylases [7,21,]. Annamalai *et al*. [2] reported on amylase production from *Bacillus cereus* and optimum activity was found at pH 8.0 and maintained at pH 11. The Bacillus sp. GM8901 was found extremely alkaliphilic a-amylase produced at pH was 11-12 [19].

Effect of temperature on activity of enzyme amylase:

Influence of temperature on *Bacillus lehensis* BW3(2) 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 optimum activity of enzyme was taken as 100%. The temperature profile of amylase activity of *Bacillus lehensis* BW3(2) were showed maximal enzymatic activity of 0.9 Units/mL (100%) at 80°C, which indicated that the enzyme was thermostable at high temperature. The amylase retained more than 22% of the highest activity between 60-70°C. Subsequently, the enzyme activity progressively decreased at 90°C (0.5 units/mL) and when the temperature was 100°C the enzyme activity was obtained 0.4 Units/mL. Previously *Geobacillus* sp. IIPTN was able to grow and produce amylase in the temperature ranges from 50°C to 80°C and showed maximum production of amylase at its optimum growth temperature 60°C [8]. Many of the amylases were revealed thermostable but not alkaline active whereas some of them were alkaline active but not thermostable. But the amylase from present study was thermostable (80ºC) and also alkaline active (pH 10.0) which is important for biotechnological potential.

Effect of substrate concentration on activity of enzyme amylase:

The influence of different concentrations of substrate was assayed ranging from 0.5 to 4 mL under constant assay conditions. The activity at 4 mL of substrate concentration was 1.4 Units/mL. However, the activity was 79% with 1.3 Units/mL of substrate concentration from 2.5-3.5 mL. There was very less activity at 0.5 mL of substrate concentration (0.1 Units/mL). The amylase produced by *Bacillus lehensis* BW3(2) showed, a Lineweaver-Burk plot indicates that enzyme has a Km of 6.66 mg of starch per mL and a Vmax of 55.55 mg of maltose per mL/ min. Similar result also reported by *Bacillus circulans* [36], the Michalies Menten constant (KM) and Maximum velocity (VMax) was found to be 1250 µg/ml and 400 µg/ml by Line weaver-Burk plot.

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 was 3.5 Units/mL at 1.5 mL of enzyme concentration. The activity of amylase decreases as the enzyme concentration increase with and above 2 mL. The enzyme retained about 85% of its activity 2.9 Units/mL of enzyme concentration at 2 and 2.5 mL. There was very less activity of enzyme concentration at 4 mL revealed 2.4 Units/mL.

Time interval for hydrolysis of starch:

The activity of enzyme amylase from *Bacillus lehensis* BW3(2) was examined at different time intervals ranging from 15-60 min. The highest activity with 3.2 Units/mL was shown at 37□C when the reaction mixture containing enzyme was incubated for 40 min. But as the incubation time goes on increasing the activity was decreasing from 45 min to 60 min. The lowest activity was shown when incubation period was of 60 min (0.1 Units/mL).

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 (2 Units/mL) was found at 3M of NaCl concentration Which was considered as 100%. The lowest activity (0.8 Units/mL) was observed at 0 M concentration of NaCl concentration (40%).

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 inactive to all organic solvents tested as compared to control

which was considered as 100%. The loss of enzyme activity was found in presence of all tested organic solvents. Thus, all the solvents have an inhibitory effect on the activity of amylase produced by *Bacillus lehensis* BW3(2).

Influence of different metal ions on activity of enzyme amylase:

The influence of different metal ions on activity of *Bacillus lehensis* BW3(2) amylase was carried out under the 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 $BaCl_2$, $CuCl_2$, $CaCl_2$, $MnSO_4$ and $ZnSO_4$. The optimum amylase activity 6.9 Units/mL (268%) was enhanced in presence of $BaCl_2$. The activity decreased (3.3 Units/mL) about 88% in presence of $ZnSO_4$. while in which $MnSO_4$and $CaCl_2$ catalyzed the enzyme activity as well as stability. Mn^{2+} and Ca^{2+} ions catalysed the enzyme activity and also these metals may act as co-factor which is required to increase the enzyme activity [2]. However, the amylase activity was inhibited by KCl (35%). In previous reports, most amylase activity were inhibited in presence of Cu^{2+}, Zn^{2+} [6] and amylase from *Bacillus subtilis* was strongly inhibited by Zn^{2+} and Cu^{2+}. Such ions on amylase activity may be due to competition between the exogenous cations and the protein-associated cations, consequence in decreased metalloenzyme activity [21] Thus similar to other amylases, BW3(2) amylase was metalloenzyme [25].

CONCLUSION

The haloalkaliphilic *Bacillus lehensis* BW3(2) isolated from the alkaline saline Lonar Lake, India and exhibited amylase activity at extremophilic stipulation. However, novel features of the enzyme such as stability over the wide range of pH 6-12, temperature 40-100ºC and salt 0.5-10% make it an attractive candidate for future studies and development process. The production of the enzyme with these sources would be economically attractive preposition. It was found that, amylase production was affected by the nitrogen and carbon sources. This is a valuable information for enzyme production and optimization by extremophilic *Bacillus lehensis* BW3(2). Amylase from *Bacillus lehensis* BW3(2) has bright future towards the improvement and production of novel enzymes for entirely new areas of industrial and biotechnological applications. Further research on structural characterization, gene regulation of amylase and cost of production are in progress.

REFERENCES

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman OJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.
2. Annamalai N, Thavasi R, Vijayalakshmi S, Balasubramanian T (2011) Extraction, purification and characterization of thermostable, alkaline tolerant a-Amylase from *Bacillus cereus*. Ind J Microbiol 51(4):424–429. DOI 10.1007/s12088-011-0160-z
3. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (2002) The Polymerase chain reaction. In: Short Protocols in Molecular Biology, 5th Edn., Vol. II,Wiley, New York
4. Blanco KC, Cristian JB, de Lima, Rubens M, Joaquim M, Bernardi NS, Contiero J (2012) *Bacillus lehensis*—an alkali-tolerant bacterium isolated from cassava starch wastewater: optimization of parameters for cyclodextrin glycosyltransferase production. Ann Microbiol 62:329–337. DOI 10.1007/s13213-011-0266-x.
5. Borsodi AK, Micsinai A, Rusznyak A, Vladar P, Kovacs G, Toth EM, Marialigeti K (2005) Diversity of alkaliphilic and alkalitolerant bacteria cultivated from decomposing reed rhizomes in a Hungarian soda lake. Microb Ecol 50:9–18.
6. Cordeiro CAM, Martins MLL, Luciano AB (2002) Production and properties of α-amylase from thermophilic Bacillus sp. Braz J Microbiol, 33: 57- 61.
7. Das K, Doley R, Mukherjee AK (2004). Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular a-amylase from Bacillus subtilis DM-03, a strain isolated

from the traditional fermented food of India. Biotechnol Appl Biochem 40:291–298.
8. Dheeran P, Kumar S, Jaiswal YK, Adhikari DK (2010) Characterization of hyperthermostable *α*-amylase from Geobacillus sp. IIPTN. Appl Microbiol Biotechnol 86:1857–1866. DOI 10.1007/s00253-009-2430-9.
9. Ghosh A, Bhardvaj M, Satyanarayana T, Khurana M, Mayilraj S, Jain RK (2007). *Bacillus lehensis* sp. nov., an alkalitolerant bacterium isolated from soil. Int J Syst Evol Microbiol 57(2):238-242.
10. Grant WD, Jones BE, Mwatha WE (1990) Alkaliphiles: ecology, diversity and applications. FEMS Microbiol Rev 75:255–270
11. Grant WD, Tindall BJ (1980) The isolation of alkalophilic bacteria. In: Gould GW, Corry ICL (Eds.) Microbial Growth and Survival in Extremes of Environment. Academic Press, London, 27–36.
12. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial amylase: a biotechnological perspective. Process Biochem 38:1599-1616.
13. Haifeng LI, Zhenming C, Xiaohong W, Chunling M A (2007) Amylase production by the marine Yeast *Aureobasidium pullulans* N13d. J Ocean University China 6 (1):60-65.
14. Haseltine C, Rolfsmeier M, Blum P (1996). The glucose effect and regulation of á-amylase synthesis in the hyperthermophilic archaeon Sulfolobus solfataricus. J Bacteriol 178:945–950.
15. Jones BE, Grant WD, Duckworth AW, Owenson GG (1998) Microbial diversity of soda lakes. Extremophiles 2:191–200.
16. Joshi A, Kanekar PP (2011) Production of exopolysaccharide by Vagococcus carniphilus MCM B-1018 isolated from alkaline Lonar Lake, India. Ann Microbiol 61:733–740. DOI 10.1007/s13213-010-0189-y.
17. Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Wani AA, Borgave SB, Sarnaik (2007). Cultivable bacterial diversity of alkaline Lonar Lake, India. Microb Ecol, 55: 163-172.
18. Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS (2000) Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresource Technol 85(1): 87- 93.
19. Kim TU, Gu BG, Jeong JY, Byun SM, Shin YC (1995) Purification and characterization of a maltotetraose forming alkaline amylase from an alkalophilic *Bacillus sp.* GM8901. Appl Environ Microbiol 61:3105-3112.
20. Kumar S, Tamura K, Nei M (2004) MEGA 3: Integrated software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Brief Bioinfo 5:150–163.
21. Lin LL, Chyau C, Hsu WH (1998) Production and properties of a tawstarch-degrading amylase from the thermophilic and alkaliphilic *Bacillus sp. TS-23*. Nature.6: 68-74.
22. Lu MS, Fang Y, Li H, Liu H, Wang S (2010) Isolation of a novel cold-adapted amylase-producing bacterium and study of its enzyme production conditions. Annals Microbiol 60: 557-563.
23. Malhotra R, Noorwes SM, Satyanarayan T (2000) Production and partial characterization of thermostable and calcium-independent á-amylase of an extreme thermophilic *Bacillus thermooleovorans* NP54. Lett Appl Microbiol 31:378–384.
24. Mehta VJ, Thumar JT, Singh SP (2006) Production of alkaline protease from an alkaliphilic actinomycete. Bioresource Technol 97:1650–1654.
25. Nielsen JE, Borchert TV (2000) Protein engineering of bacterial α-amylases. Biochim Biophys Acta 1543(2): 253-274.
26. Ozdemir S, Matpan F, Okumus V, Dundar A, Ulutas MS, Kumru M (2012) Isolation of a thermophilic *Anoxybacillus flavithermus* sp. nov. and production of thermostable α-amylase under solid-state fermentation (SSF). Ann Microbiol DOI 10.1007/s13213-011-0385-4
27. Rajagopalan G, Krishnan C (2008) Optimization of medium and process parameters for constitutive α-amylase production from a catabolite derepressed *Bacillus subtilis* KCC103. J Chem Technol Biotechnol 8:654–661.
28. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor, New York.
29. Shanmughapriya S, Kiran GS, Selvin J, Gandhimathi R, Baskar TB, Manilal A, Sujith S (2009) Optimization, production, and partial characterization of an

alkalophilic amylase produced by sponge associated marine bacterium *Halobacterium salina rum*.MMD047. Biotechnology and Bioprocess Engineering 14: 67-75, DOI/10.1007/s12257-008-0060-1.
30. Snatos EO, Martins ML (2003). Effect of the medium composition on formation of amylase by Bacillus sp. J Braz Arch Biol Technol 46: 129-134.
31. Srinivasan TR, Das S, Bal Krishnan V, Philip R, Kannan N (2009) Isolation and characterization of thermostable protease producing bacteria from tannery industry effluent. Rec Res Sci Technol 1(2): 63-66.
32. Sudharhsan S, Senthilkumar S, Ranjith K (2007) Physical and nutritional factors affecting the production of amylase from species of Bacillus isolated from spoiled food waste. Afr J Biotechnol 6:430–435.
33. Tambekar DH, Tambekar SD (2012) Partial characterization and optimization of alkaline protease production of *Bacillus pseudofirmus* from Lonar Lake. Int J Adv Pharm Biol Sci 2(1): 130-138.
34. Tambekar DH, Dhundale VR (2012) Studies on the physiological and cultural diversity of *bacilli* characterized from Lonar lake (MS) India. Biosci Discovery 3(1): 34-39.
35. Tambekar DH, Dhundale VR (2013) Phylogenetic analysis of amylase producing Bacilli from extremophilic environment. Int J LifeS. Bt Pharm Res, 2(1): 103-113.
36. Tambekar DH, Raut NS, Dhundale VR (2013) Partial characterization and optimization of alkaline amaylase production by *Bacillus circulans* from Lonar Lake Biosci Discovery 4(2):202-207.
37. Tanyildizi MS, Ozer D, Elibol M (2007) Production of bacterial α- amylase by *Bacillus amyloliquefaciens* under solid substrate fermentation. Biochem Eng J 37:294–297.
38. Thippeswamy S, Girigowda K, Mulimani VH (2006) Isolation and identification of α-amylase producing *Bacillus sp*. from dhal industry waste. Ind J Biochem Biophys 43:295–298.
39. Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving sensitivity of progressive multiple sequence alignments through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–7680.

Tables and Figures:

Table 1:- Effect of various organic solvents on activity of amylase

Organic Solvent	Relative activity (%)
Control	100
Methanol	68
Acetone	62
1-Butanol	62
Chloroform	25
Benzene	56
Toluene	93

Table 2:- Effect of different metal ions on activity of enzyme amylase

Metal ions	Relative activity (%)
Control	100
$BaCl_2$	268
$CuCl_2$	236
$CaCl_2$	185
$MnSO_4$	200
$ZnSO_4$	180
KCl	65

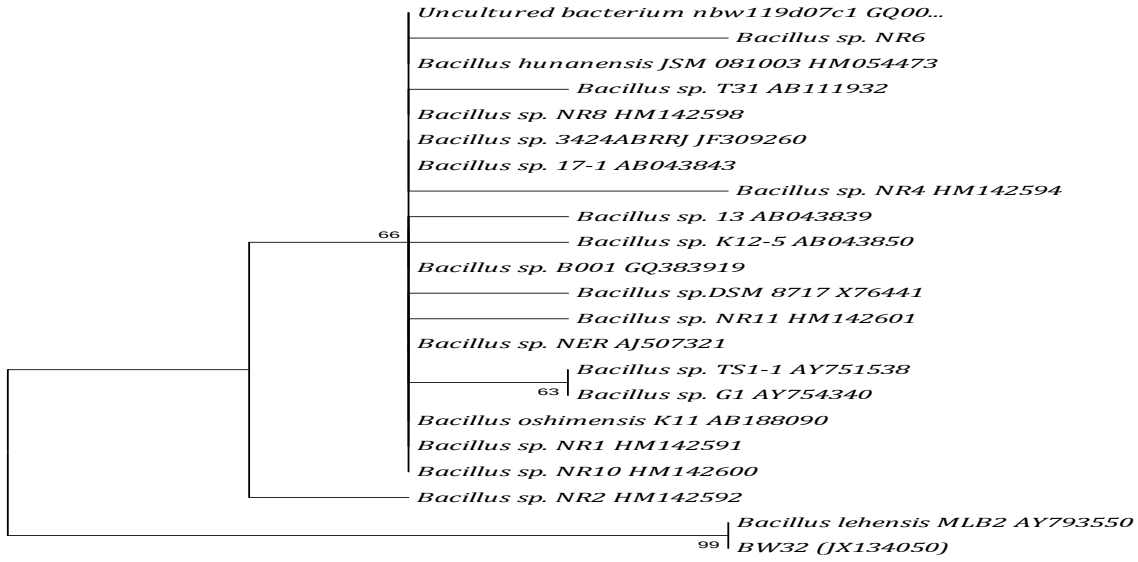

Fig. 1: Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates and some of their closest phylogenetic relatives. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1,000 replications.

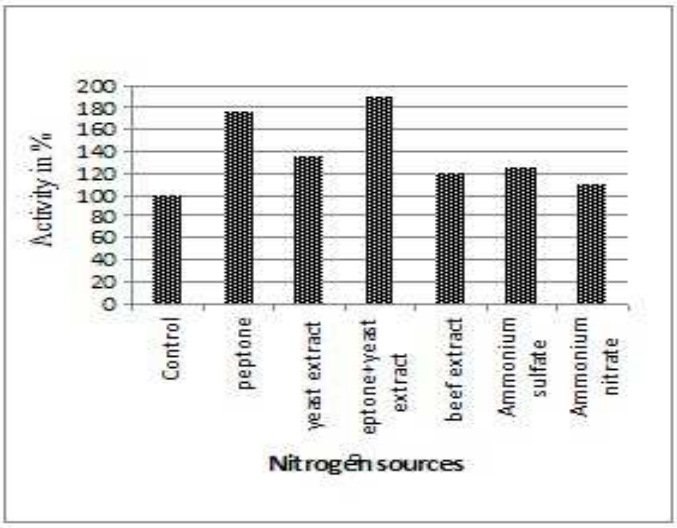

Fig 2: Effect of different nitrogen sources on amylase production

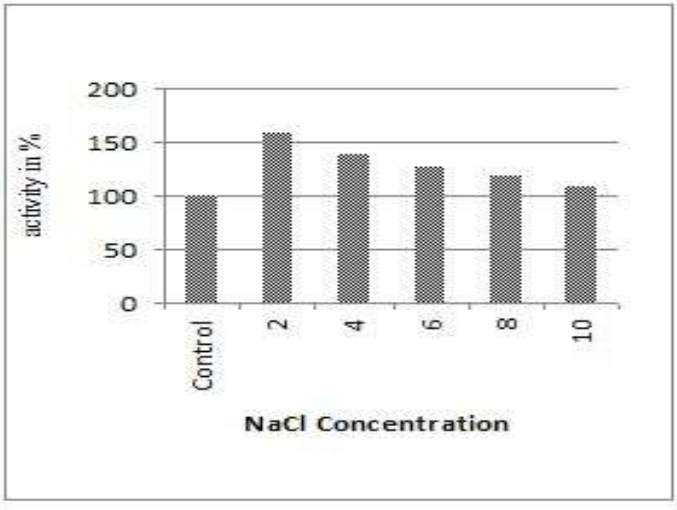

Fig 3: Effect of NaCl concentration on production of amylase

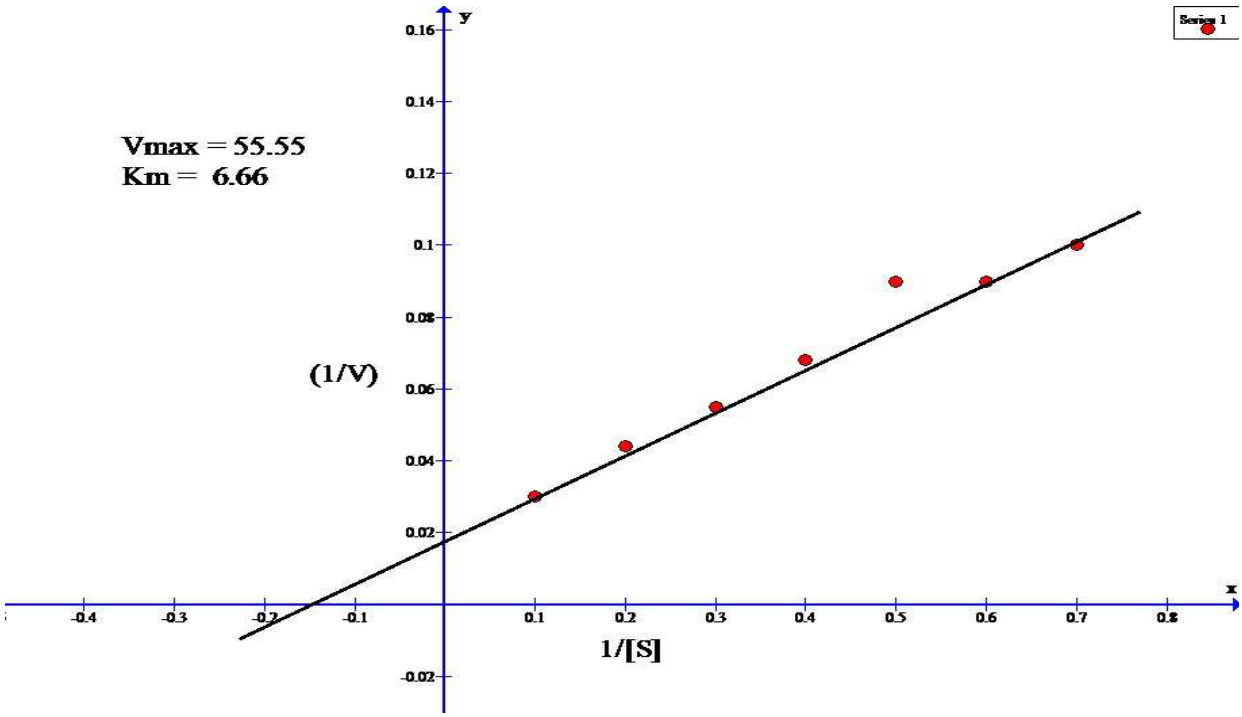

Fig 4- Lineweaver and Burk Plot For *Bacillus lehensis* BW3(2)

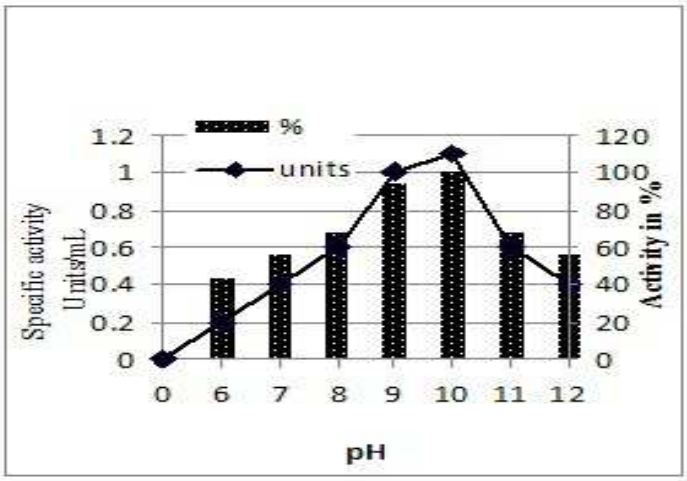

Fig 5: Effect of pH on activity of amylase

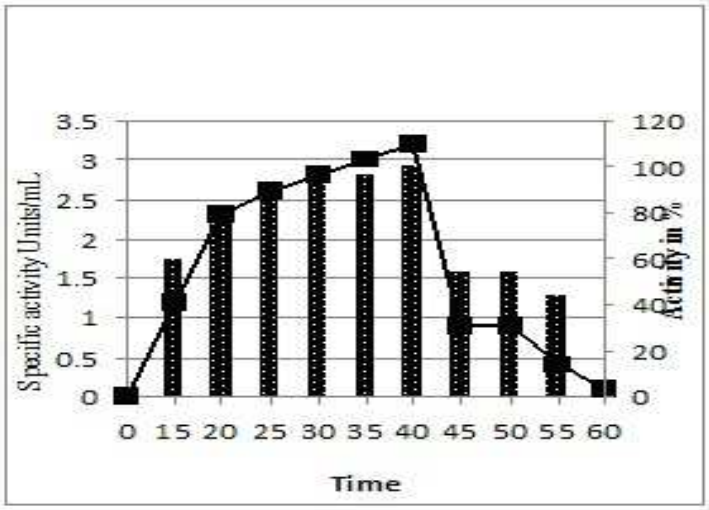

Fig 6: Effect of time on activity of amylase

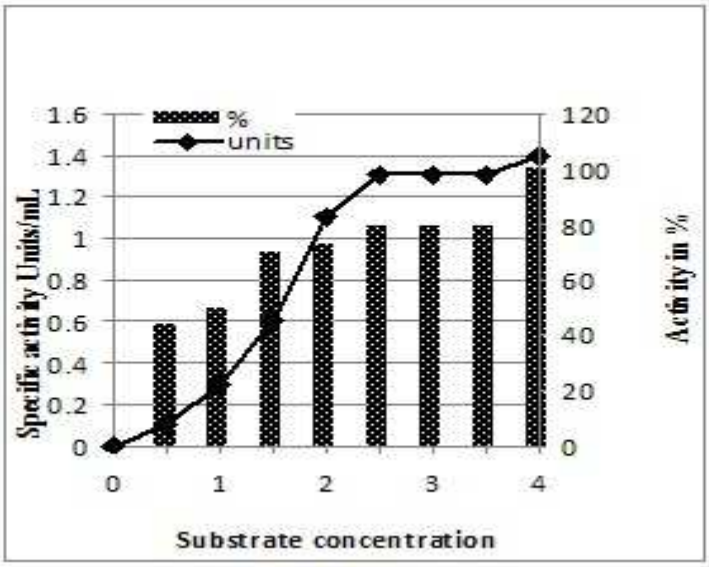

Fig 7: Effect of substrate concentration on activity.

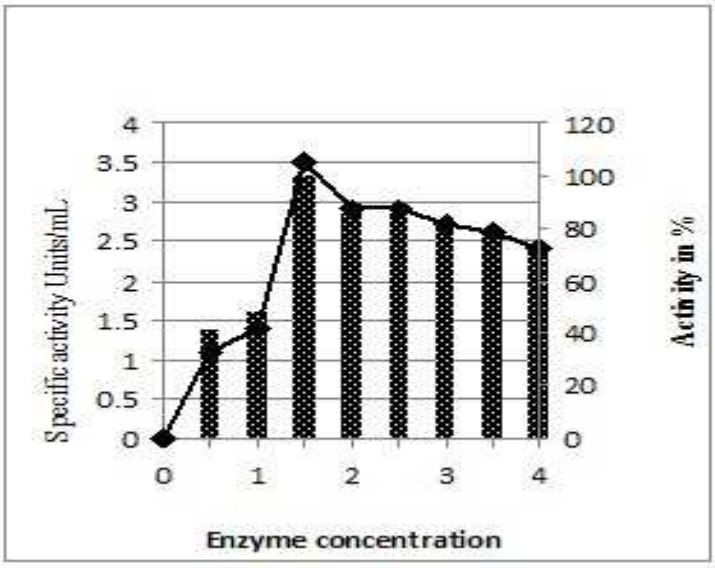

Fig 8: Effect of enzyme concentration on activity.