Int J Pharm Bio Sci 2015 April; 6(2): (B) 668 - 677

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

HALOALKALIPHILIC *BACILLUS FLEXUS* AW3(2): POTENTIAL FOR BIOTECHNOLOGICAL APPLICATIONS

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ABSTRACT

The traditional cultivation based methods have a great importance in research, providing the chance in investigations of biotechnologically significant bacterial isolates under in vitro. A total of one hundread and fourteen bacteria was isolated from the water and sediment samples collected from the hyperalkalinesaline environment of Lonar crater. Out of them strain AW3(2) was selected for 16S rDNA sequencing, production and, partial characterizations of amylase on the basis of their maximum amylolytic activity. A Gram positive bacterium was identified as *Bacillus flexus* by morphological, physiological and biochemical characterisation 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 flexus* AW3(2). Optimum enzyme activity was found to be at 70°C (1.2 unit/mL), pH 10.0 (5.9 units/mL), and 4% NaCl. The amylase was highly stable over a 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 $BaCl_2$ (7.4 units/mL/min), indicating it was a metalloenzyme. Among the organic nitrogen sources, optimum amylase production was found to be in presence of yeast extract. This is valuable information for enzyme production and optimization of amylase has a 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.

KEY WORDS: **Haloalkaliphiles, Bacillus, Enzyme, Amylase, Biotechnological potential**

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INTRODUCTION

A diverse variety of amylolytic enzymes are produced by bacteria. This interesting catalysts in organic media[1]. With this regards, they have attracted great noticeable resulting in the isolation of a number of novel variants, mainly from microbial species isolated from extremophiles. Extremozymes have been of special interest in this examine appropriate to their extreme properties, access their function in a range of stipulation other than that is commonly the case for enzymes from neutriphilus. Less attention has been given to alkaliphiles, which are organisms with optimal pH for growth at or above pH 9[2]. Group contains enzymes with a wide range of industrial applications and are also considered as Thus far, the main attention has been focused on alkaliphiles, as their enzymes show extreme stability at elevated in alkaline, saline, and also in organic solvents[3,4]. 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[5]. Extremozymes are now-a-day replacing chemical catalyst in manufacturing of various industrial product and agricultural chemicals since the enzymes readiness with suitable properties with the important of new knowledge in biotechnology, the spectrum of enzyme application has widened in many other fields[6]. The prominent has been on alkaline amylase and respective microbes have been looking for their capacity to secrete these enzymes[7].Amylases are enzymes which decompose starch substance to give different things including dextrin and efficient smaller polymers constitute of glucose molecules[8-11]. Amylase is a kind of actual important enzyme and composed a class of industrial enzymes sharing about 25% of the enzyme market[12-13]. Amylases are globally classifying kingdomswise, however, due to their advantages the enzymes from fungal and bacterial sources have dominated applications in industrial sectors[9]. Amylases have many applications in bread and baking industry, starch liquefaction and saccharification, textile desizing, paper industry, detergent industry, analysis in medical and clinical chemistry, food and pharmaceutical industries[10]. Microbial life can be establish in presence of most varied stipulation, including extremes of temperature, pressure, salinity, pH, nutrient concentrations, starvation and, harmful heavy metals and metals ion[14-17]. Since extracellular enzymes by metal ion tolerant microorganisms were possibly stable in the presence of metal ions. Some of these synthesized microbes have been ability to be stream of metal ion stable enzymes, some of which have already been exploit for biotechnological application. Although several extremophilic bacteria have been isolated and their enzymes produced and characterized, there is yet to be need for novel strains competent of producing enhanced levels of enzyme in an economically possible culture system. The appropriate of an enzyme in an industrial diligence depends upon its thermal, alkaline, saline stability and other kinetic properties. A haloalkaliphiles bacterium which produces a great extent of thermohaloalkali-stable amylase is the more suitable microorganism for serve in commercial enterprises[18]. The primary aim of our study was therefore to produce high levels of extremophilic amylase which could be used in the textile and pharmaceutical industry under extreme conditions. The next aim was to elaborate the kinetic properties and various characteristics of amylase for a more excellent understanding of its physicochemical characteristics in specific to make its potential for biotechnological applications to more beneficial.

MATERIALS AND METHODS

Collection and isolation

Enrichment of water samples and sediment samples were carried out in nutrient agar at pH 10.0 with 30 g l−1 sodium chloride [19-20].

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[21-22].

Identification of the bacterial culture

Amylase producing bacterial strains were studied 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[23]. 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[24] and directly sequenced on the Applied Biosystems Model 3730 DNA sequence (Foster, California USA). The 16S rDNA sequences were analyzed using BLAST program[25].

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,*[22]. 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 AW3(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 water sample was *Bacillus flexus*. The 16S rDNA sequence was submitted to NCBI Genebank Database and the accession number as JQ319529

The estimation of amylase activity

The estimation of amylase activity was performed by assay conditions. The activity of amylase from *Bacillus flexus* AW3(2) after 15 min of incubation was found to be 1.93 Units/mL.

Effects of Different Nitrogen Sources on Amylase Production

The nitrogen sources have a noticeable influence on the production of amylase of *Bacillus flexus* AW3(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. The result showed that yeast extract was the best nitrogen source having activity of 322% followed by peptone (84%) and beef extract (186%). The enzyme activity without any additives was taken as 100%. On the optimization process, beef extract was found to be the most promising nitrogen source for the production of amylase followed by meat extract. The use of yeast extract was found to be inhibitory for the production of amylase by *Halobacterium* MMD047[6]. Therefore the different nitrogen sources were highly influenced on the production of amylase by different microorganism of genus with different production condition. The extra supplementary of different nitrogen source which were affected on the production of amylase by *Bacillus subtilis*[26]. Different nitrogen 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[27-32]. Suribabu et al.[33]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 *Bacillus flexus* AW3(2) grew well at various concentration of NaCl ranging from 2-10%. The optimum growth was at 4% NaCl and no growth was observed in the absence of NaCl. The enzyme activity without any

additives was taken as 100%. The amylase retained 57 and 77 % of activity in the presence of 2 and 6% NaCl, respectively. However, more than 63 % 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. The high concentration of NaCl affects on the production of amylase by *Bacillus licheniformis*[34] instead in the present studies, the high concentration of NaCl were required for optimum production of amylase.

Effect of pH on activity of enzyme amylase

In the present studies, the effect of pH on amylase activity of *Bacillus flexus* AW3(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 flexus* AW3(2) amylase were found to be 10. The enzyme was active between pH ranging from 9-11. Amylase activity was relatively low at pH 6 (2.4 Units/mL). At pH 6, 7 and 8, the enzyme has relative activities of 40, 66 and 71% respectively. The optimum activity of this enzyme was 5.9 units/mL at pH 10. The activity decreased at pH 11 (5.6 Units/mL) and 12 (4.8 Units/mL). In acidic buffer that is at pH range of 6-8 and increasing the initial pH of buffer above 11 resulted in a decrease in the amylase activity. This showed that the enzyme required a slightly alkaline pH for its activity. Enzyme activity markedly depends upon pH due to the substrate binding and catalysis is often dependent on the charge distribution on both substrate and especially enzyme[35]. In contrast, the optimum activity of amylase enzyme from *Bacillus amyloliquefaciens* ALSHL3 was at pH 6 and activity decreased dramatically at pH 9 and 10[36]. So alkaline amylase from *Bacillus flexus* AW3(2) retain activity at high pH at which detergents function, therefore practically it may be beneficial in the detergents industry and revealed exo-type activity[37-38].

Effect of temperature on activity of enzyme amylase

Influence of temperature on *Bacillus flexus* AW3(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 temperature profile of amylase activity of *Bacillus flexus* AW3(2) showed maximal enzymatic activity of 1.2 Units/mL (100%) at 70°C, which indicated that the enzyme was thermostable at high temperature. The amylase retained more than 91% of the highest activity between 60-70°C. Subsequently, the enzyme activity progressively decreased by 25% at 80°C (0.9 units/mL) and when the temperature was 90°C the enzyme activity was as poor as 0.7 Units/mL). The amylase from *Bacillus amyloliquefaciens* ALSHL3 has activity with temperature to reach an optimum at 60°C. The enzyme maintained 96% of its activity at 70°C[36], similar with those reported for Bacillus sp.PS-7[39] and *Bacillus subtilis* 65[40]. Especially thermostable enzymes have obtained extent to analytical tools and as biocatalysts for large scale application[38]. Applications of these enzymes have despite lot of efforts, frequently minimum by the cost of the enzymes[41].The thermostable amylase has been found advantageous in brewing industries and starch-processing at high temperature for ease and improve the reactant activity[42-44]. Generally Bacillus is enables to production of thermostable amylase which were utilized for commercial purposes due to they have beneficial than fungal amylase because of their less time for production and minimum possibilities of contamination[45].

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 during the period of 10 minutes incubation at 37°C, 95% of substrate was utilized (4.5 Units/mL) but maximum substrate utilization (4.7 Units/mL) occurred at 3 mL of substrate concentration. The amylase produced by *Bacillus flexus* AW3(2) showed Michaelis-Menten type kinetics with soluble starch. A Lineweaver-Burk plot indicates that this enzyme has a Km of 2.94 mg of starch per millilitre and a Vmax of 90.90 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 the assay conditions. The enzyme amylase

from *Bacillus flexus* AW3(2) shows very less activity (0.9 Units/mL) at 0.5 mL of enzyme concentration. The enzyme shows maximum enzymatic activity (5.3 Units/mL) at 3 mL of enzyme concentration. The activity of amylase decreases as the enzyme concentration increase above 3 mL. The enzyme retained about 88% and 62% of its activity (4.7 and 3.3 Units/mL) of enzyme concentration at 3.5 and 4 mL respectively.

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 4.9 Units/mL was shown at 37°C when the reaction mixture containing enzyme was incubated for 35 minutes. The enzyme retained about 61% and 51% of activity at 30 and 40 minutes respectively. But as the incubation time goes on increasing the activity was decreasing from 40 min to 60 min. The lowest activity was shown when incubation period was about 60 minutes (1.6 Units/mL).

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. Thus, all the solvents had an inhibitory effect on the activity of amylase produced by *Bacillus flexus* AW3(2).

Table 1
Effect of different metal ions on activity of enzyme amylase

Metal ions	Relative activity (%)
Control	100
$BaCl_2$	274
$CuCl_2$	274
$CaCl_2$	214
$MnSO_4$	203
$ZnSO_4$	203
KCl	51

Influence of different metal ions on activity of enzyme amylase

The influence of different metal ions on activity of *Bacillus flexus* AW3(2) amylase was carried out under the assay conditions. Metal ions have different effects on activity of amylase. The enzyme activity was enhanced by $BaCl_2$, $CuCl_2$, $CaCl_2$, $MnSO_4$ and $ZnSO_4$. However, the amylase activity was inhibited by KCl (49%). The optimum amylase activity 7.4 Units/mL (274%) was enhanced in presence of $BaCl_2$ and $CuCl_2$. The activity decreased (5.8 and 5.5 Units/mL) about 60 and 71% in presence of $CaCl_2$ and $ZnSO_4$ respectively. In previous reports, most amylase activity were inhibited in presence of Cu^{2+},Zn^{2+}[46] and activity of amylase from *Nesterenkonia* sp.strain F was completely inhibited by Fe^{3+}, Cu^{2+}, Al^{3+} and Zn^{2+},Ca^{2+} and Mg^{2+} has no significant effect on the enzyme activity[47].

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 (1.6 Units/mL) was found at 2 and 2.5M of NaCl concentration. The lowest activity (0.8 Units/mL) was observed at 0 and 0.5 M concentration of NaCl concentration.

CONCLUSION

In the present studies, isolated *Bacillus flexus* AW3(2) from haloalkaline Lonar Crater and tested its ability to produce amylase under different culture conditions. Considering the characteristics of AW3(2) amylolytic activity in high alkaline and saline conditions. The finding that the investigated amylase were active at elevated temperatures (70ºC) and high pH (pH 10). The discovering of the amylolytic enzymes, they were further characterized to determine their tolerance with salt alterations and their kinetic parameters were calculated. A Lineweaver-Burk plot indicates that enzyme has a Km of 2.94 mg/mL and a Vmax of 90.90 mg/mL/ min. In addition to their resistance against metal ion

makes these enzymes very attractive for biotransformation Processes. It is now known that enzymes display striking novel properties in the presence of metal ions. The role of metal ion stable enzymes needs to be explored and could result in novel applications. These amylase from *Bacillus flexus* AW3(2) may be make up industrial enzyme and are used predominantly in the food industry for operations such as brewing, baking and potential for biotechnological application.

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.

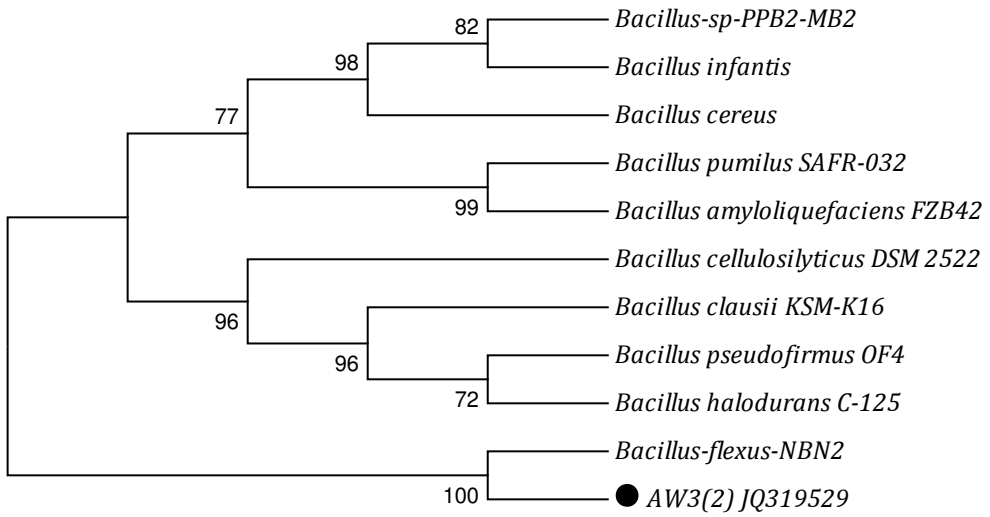


Fig. 2- Effect of different Nitrogen sources on production of amylase

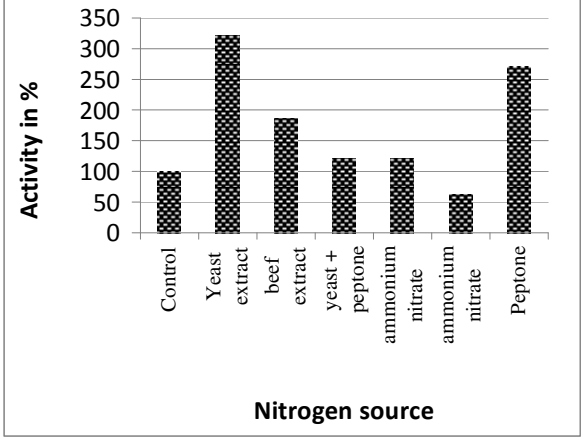
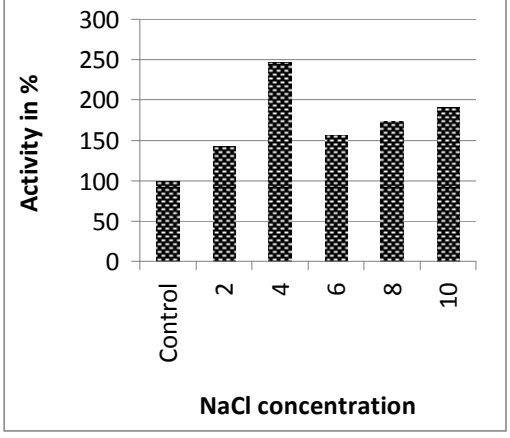
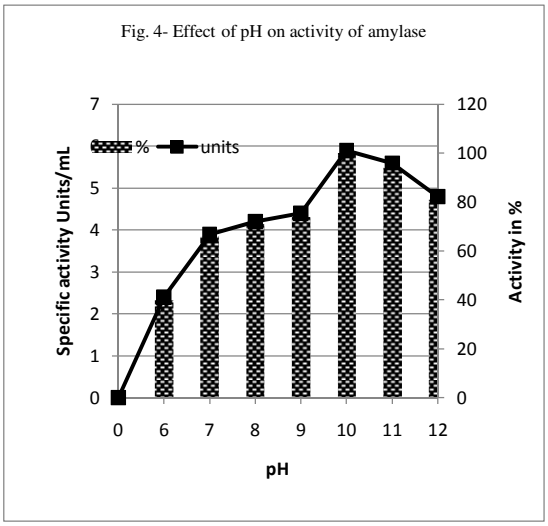


Fig. 3- Effect of different NaCl concentration on production of amylase





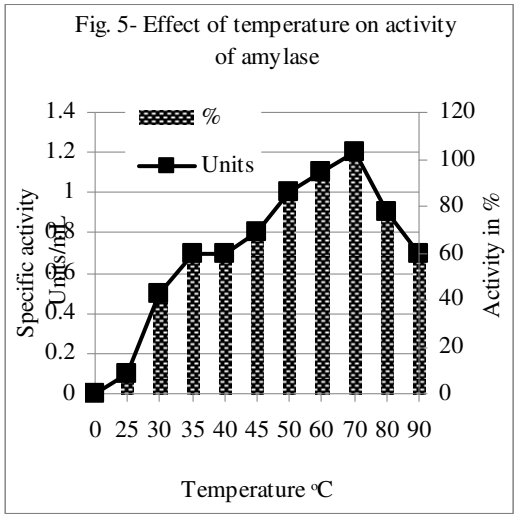
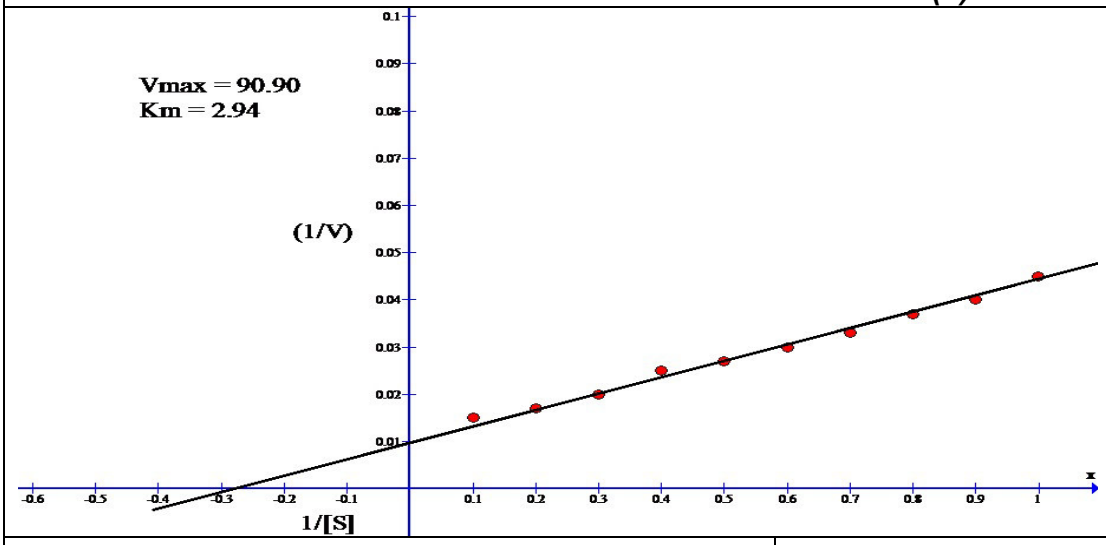
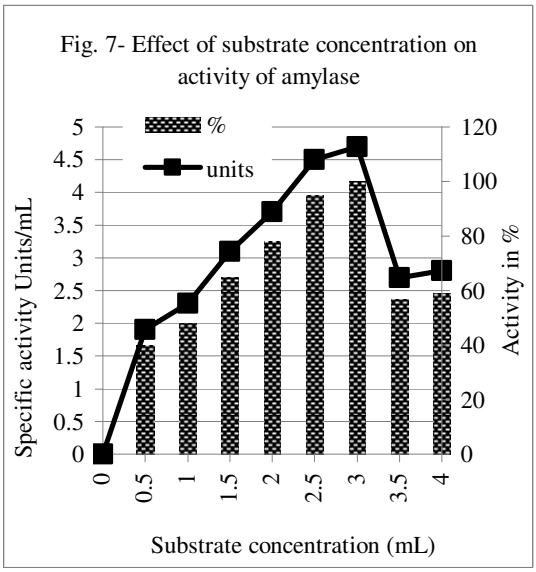
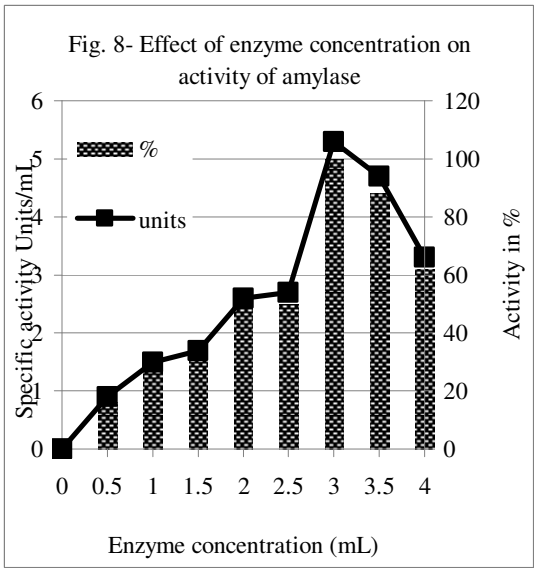
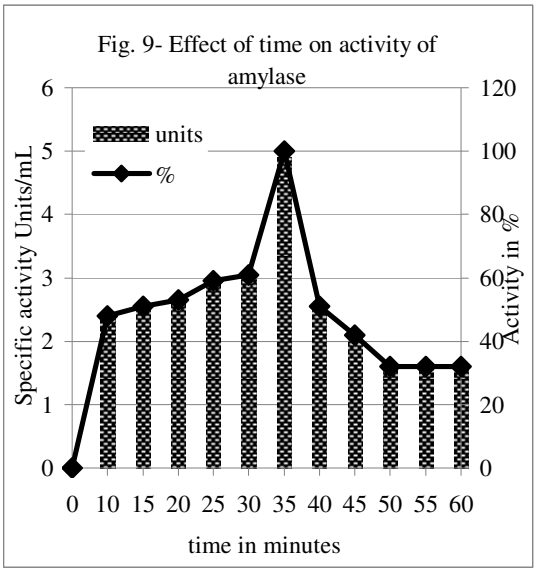


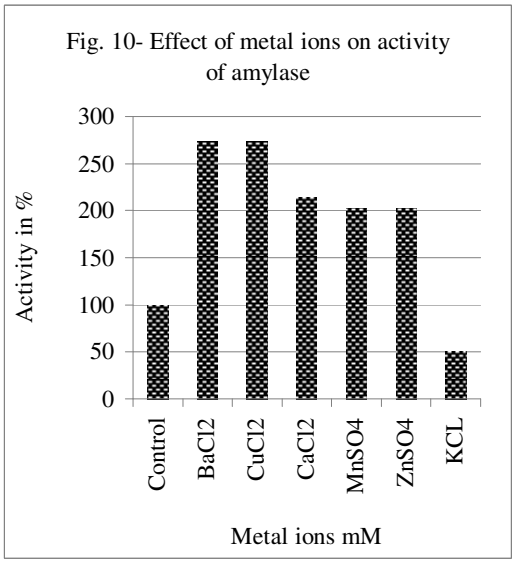
Figure *6*
Lineweaver and Burk Plot for AW3(2)

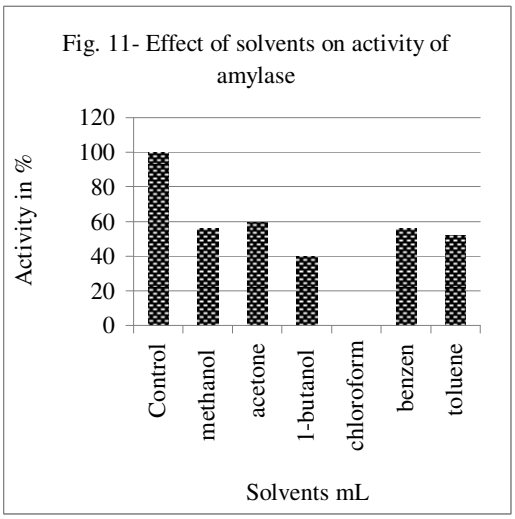


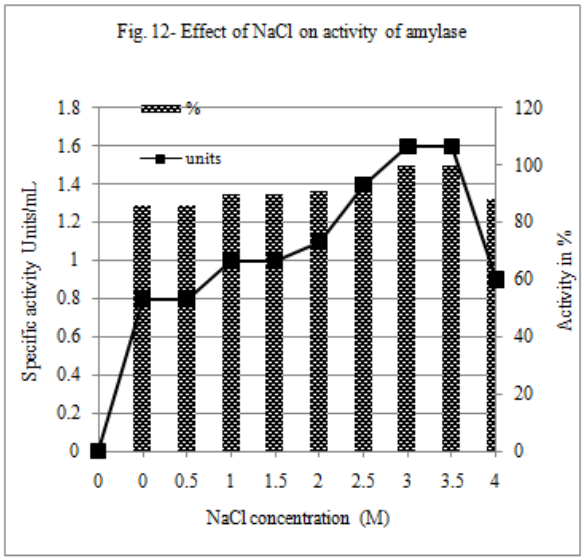












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