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OPTIMIZATION OF Alpha-AMYLASE PRODUCTION BY USING SOLID STATE FERMENTATION TECHNOLOGY FROM *Bacillus subtilis* SSD-I (JQ747516) IN LONAR LAKE [MS] INDIA

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ABSTRACT

Microbial Alpha amylase is highly demanded industrial enzyme in various sectors such as pharmaceuticals, food, textile and detergents, etc. alkaline bacterial stains has better ability to produce such type enzyme. In the present study, alkaline bacterial strains were isolated from water samples collected from saline Lonar Meteorite crater, (MS) India having pH 10.5. Among them one of the bacterial strains was identified by 16S rRNA sequencing showed 95% alignment match with *Bacillus subtilis.* The optimum growth of such organism was 12.5 pH and 16% salt concentrations. Chemical analysis of water shows metal ions and salt concentrations in Lonar crater. The production of α-amylase by producing *Bacillus subtilis* JQ747516 was studied under solid state fermentation (SSF). Different agro-based products as substrates were studied for enzyme production. Production parameters were optimized as incubation time 72 hrs, incubation temperature 37oC, initial moisture 30%, and initial pH 10. Supplementation of the fermentation medium with carbon and metal ion sources decreased α-enzyme production. Among different nitrogen sources supplemented, ammonium nitrate (1%) showed maximum amylase production (1470.3 ± 3.56 U g-1.10-3).

KEY WORDS: 16S rRNA, alpha-enzyme, SSF, Lonar Lake.

INTRODUCTION

Amylases constitute a group of industrial enzymes, which alone covers approximately 30% of the enzyme market. They have opened new frontiers of many commercial biotechnological processes including renewable energy, pharmaceuticals, saccharification or liquefication of starch, detergent industries, warp sizing of textiles, fibers, paper industries, foodstuffs, baking, clarification of haze formed in beer or fruit juices and for pretreatment of animal feed to improve digestibility [1] and[2]. The amylase family of enzymes is of great significance due to its wide area of potential application. The extensive application for food industries, such as brewing, baking, preparation of digestive aids, production of chocolate cakes moist cakes, fruit juices, starch syrups, etc. for their large scale commercial production. The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermostability [3]. Amylases have been obtained from submerged fermentation because of the effortlessness of handling and greater control of environmental factors such as temperature and pH [4] and [5]. However, this process is cost intensive due to low concentrations of products and the consequent handling and disposal of a large volume of water during down-stream processing. The cost of enzyme production in submerged fermentation (SmF) is high, which necessitates reducing the production costs through alternative methods. The use of agricultural wastes makes solid state fermentation (SSF) an attractive alternative method. As the microorganisms in SSF are growing under conditions similar to their natural habitats, they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation [5]. It is now well known that only a small proportion of the microorganisms from an environmental sample can be isolated and cultured in laboratory conditions [6]. PCR amplification followed by cloning of the 16S rRNA genes derived from the total DNA extracted from environmental samples and subsequent phylogenetic analysis of the cloned sequences have enhanced our ability to assess naturally occurring biodiversity in many environments [7]. Contrary to other soda lakes, which are mainly non-polluted due to their inaccessibility, the former alkaline lake of Lonar crater is situated in Maharashtra, suffered many disturbances because of human activities. The Lonar crater is only one meteoritic lake in India, is the only crater in basaltic rock. In the present investigation, isolation of microorganism from Lonar Lake water sample and identified by 16S rRNA cataloging. The isolates were applied to production of biotechnologically and pharmaceutically important enzyme such as amylase.

MATERIALS AND METHODS

Sample collection and chemical analysis: Water samples were directly collected in sterile bottles from different sites of crater. The chemical analysis of water was performed in triplicate by using standard method. The alkalinity of sample was estimated by potentiometric titration in terms of CaCO3, Ca, Mg, Cl as well as Na and K analyzed by Flame Photometer (Elico, India) .[8, 9]

Isolation and identification of bacterial strain

Water sample was used to isolate the different microflora from Lonar crater. Enrichment of water samples was carried out in various growth liquid media, such as Horikoshi I, Horikoshi II [10] and Nutrient broth incubated at 37°C on rotary shaker (120 rpm) for 24 hrs. After enrichment, the samples were streaked on agar plates and incubate at 37°C for 24 hrs. [8]. Identification of bacterial strain was done by 16S rRNA sequencing [11].

Enzyme production in SSF

In the SSF, the agro-based potential substrates were used for amylase production. In the agro-based like wheat bran, rice husk, coarse meal of corn and cotton stalk (WB, RH, CMC and CS) were used individually. SSF was carried out by taking 3g dry substrate in 100 ml Erlenmeyer flask to which distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121^0C for 15 minutes. Inoculated flasks were shaken 150 rpm at 37^0 C for 168 h. The contents of the flask were harvested and assayed every 24 h [5].

Enzyme Extraction

Crude enzyme was extracted by mixing a known quality of fermented matter twice with tap water. The slurry was squeezed with the muslin-cloth. Extracts were pooled and centrifuged at 4^0C for 15 minutes at 10000 rpm to separate small particles of different substrates. The supernatant was collected and used as crude source of amylase .[5,12]

Enzyme assay

α-Amylase activity were calculated by the method of Bernfeld [13]. The reaction mixture containing 200 µL of 1% substrate, phosphate buffer (pH: 7.0) and enzyme solutions were inoculated for 30 min at 37^0 C. The reaction was stopped by adding DNS followed by heating with water bath and cooling at room temperature, and then added deionized water after that method absorbance were measured at 489 nm with the help of UV-Spectrophotometer (Elico

SL177). One unit of amylase activity was defined as the one number of µ moles of maltose librated by 1 ml of enzyme solution per minute and expressed U g^{-1} of dry state. [2, 5 and 13]

Assay of Protein Concentration

The protein concentration was estimated by the method of Lowry et al. (1951). BSA was used as a standard protein. [13 and 14]

Effect of Different Parameters on α-amylase production in SSF

Combinations of the excellent substrates were used for further optimization of process parameters, like moisture content (20, 30, 40, 50 and 60%), incubation time (24, 48, 72, 96, 120, 144, 168 and 192 h). incubation temperature (30, 37, 40, 45 and 50^0C), initial pH of the medium (pH 7.0 to 10.5), while the nutrient supplementation such as inorganic nitrogen sources (ammonium nitrate, sodium nitrate, ammonium chloride, and ammonium sulphate), organic nitrogen sources (peptone and yeast extract), carbon sources (Lactose, fructose, galactose and glucose) and added some metals salts 0.1% were used for production ($FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$ and $CaCl_2$. [5]

Effect of Temperature on Enzyme activity

Effect of optimum temperature on amylase activity was determined by different temperatures ranging from 37^0C to 70^0C.

RESULTS AND DISCUSSION

Isolation and identification of bacterial strain

The sample was collected from different sites of the Lonar lake having different micro fauna were analyzed. The pH of the collected water sample was 10. The number of colonies was obtained on the surface of Horikoshi I and II agar medium, taking into consideration that 60 colony forming units were observed on the plate as well as the same collected water sample was checked on nutrient agar medium having variable concentrations of salt and pH. The growth of the haloalkaliphilic organisms was directly proportional to the increasing pH and salt concentration at 12.5 and 18% respectively. At lower concentration of pH and salt showed decreasing growth of the isolates. The isolated bacterial strain was morphologically analyzed of its colony character having, gram positive short rods, round colonies, translucent, convex, and white color. The isolated strain was analyzed by 16S rRNA cataloguing and showed 95% resemblance with *Bacillus subtilis* SSD- I (JQ747516). The phylogenetic position of the strain showed in fig: 1 Total genomic DNA was isolated using gene elute genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl2; 1x PCR buffer (Bangalore Genei, Bangalore, India); 200 µM each dCTP, dGTP, dATP, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a final volume of 20 µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene, as described previously. The PCR was performed using the Eppendorf Gradient Master cycler system with a cycle of 94°C for 5 min; 30 cycles of 94°, 60°, and 72°C for 1 min each and final extension at 72°C for 10 min, and the mixture was held at 4°C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice-using 70% ethanol and dissolved in Tris-HCl (10mM, pH 8.0).

The chemical analysis of water sample was analyzed according to American Public Health organization (APHA). The five parameters were analyzed by potentiometric titration method and showed concentration of Ca, Mg and Cl was 174.2, 150.9, 3219.9 mg/l. The sodium and potassium was analyzed by flame photometer (Elico India) having concentration 3619.8 and 17.2 mg/l respectively.

Screening of substrates for SSF

The agro-based solid substrates were effects on the production of enzyme. As it is shown in Table 1, all selected substrates were supported amylase formation by the *Bacillus subtilis*. The cotton stem has superior to the other substrates. The CS was optimum production of α-amylase production (1022.3 ± 5.79 U $g^{-1} \cdot 10^{-3}$). CS was potent for enzyme production and these results were slightly matches with [5].

Optimization of SSF

Effect of temperature on α-amylase production

The effect of temperature on amylase activity was analyzed at different temperatures. Among the used temperature the optimum activity was found at 37^0C for CS with 826.6 ± 5.03 U $g^{-1} \cdot 10^{-3}$ in (Fig. 1). Previous studies revealed that the 37^0C were reported as optimum temperature for amylase production by several authors [13] and [15]. Temperature plays a significant role in biological process as it protein denaturation, enzyme inhibition. [5]

Effect of moisture level on α-amylase production

Initial moisture content of the substrate was known to critically influence bacterial growth and enzyme production in SSF [5]. The optimal moisture content of substrate for enzyme production was found to be 30% with 962.6 ± 5.50 U $g^{-1}\cdot 10^{-3}$ when compared to 20, 40, 50 and 60% CS (Fig. 2). Low and high moisture levels of the substrate affect the growth of the microorganism resulting lower enzyme production. High moisture content leads to reduction in substrate porosity, changes in the structure of substrate particles and reduction of gas volume [12].

Table 1: ***Effect of different substrates on the production of Bacillus subtilis JQ747516 α-amylase by SSF***

Substrates				
Hours	**WB**	**RH**	**CMC**	**CS**
Enzyme production (U $g^{-1}\cdot 10^{-3}$)				
24	195.6 ± 3.51	656.6 ± 4.50	400.0 ± 4.58	723.0 ± 3.60
48	129.0 ±5.00	679.6 ±3.05	424.6 ± 3.51	922.0 ± 4.00
72	944.0 ± 4.00	948.6 ± 4.02	802.0 ± 4.58	1022.3 ± 5.79
96	126.0 ±5.09	751.6 ± 3.05	726.6 ± 3.51	980.6 ± 3.51
120	102.6 ± 4.16	693.3 ± 4.04	341.6 ± 4.16	841.3 ± 4.04
144	97.6 ± 5.03	619.3 ± 4.53	314.3 ± 3.56	673.3 ± 4.04
168	91.3 ± 4.55	608.0 ± 4.58	302.3 ± 4.72	636.6 ± 3.51

Effect of pH on α-amylase production

The pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion [5]. The enzyme production was maximum when initial medium pH was 10, which give in 948.6 ± 4.04 U $g^{-1}\cdot 10^{-3}$, depicts (Fig.3). this might be attributed to the requirement of alkaline pH by bacteria for production of α-amylase. Our findings are in accordance with the earlier reports [15,16 and17].

Effect of Incubation Time on α-amylase production

The *B. subtilis* strain was produced higher 1442.3 ± 4.04 U $g^{-1}\cdot 10^{-3}$, at 72 h of incubation (Fig. 4). The production of enzyme decline after 72 h for CS. Lily (2012) revealed that the α-amylase synthesis started within 6 h of growth achieving maxima at 72 h. Incubation from 96 h to 168 h resulted in sharp decline in total α-amylase production. Similar results were found previously by Qader et al. in *Bacillus* sp. [16].

Effect of Inorganic and Organic Nitrogen Sources on α-amylase production

Addition of inorganic nitrogen sources such as ammonium nitrate, sodium nitrate, ammonium chloride, and ammonium sulphate, and organic nitrogen sources such as peptone and yeast extract to the medium were also analysed. In our investigation, as illustrate in Table 2, in comparison with control (1192 ± 3.0 U $g^{-1} \cdot 10^{-3}$), there was enzyme production were increases due to supplementation of ammonium nitrate (1470.3 ± 3.56 U $g^{-1} \cdot 10^{-3}$) which proved that the ammonium nitrate were better to among all nitrogenous sources. Ammonium nitrate was found to be suitable with CS in SSF [5].

Effect of Carbon Sources and Metal ion sources on α-amylase production

Addition of carbon sources such as lactose, fructose, galactose and glucose at 1% concentration in the production of α-amylase by Bacillus subtilis (JQ747516) to the medium and obtain for better production as shown in Table 3, as per the comparison of standard control (1142.3 ± 2.59 U $g^{-1} \cdot 10^{-3}$), there was no significant growth in the case of additional carbon sources. Comparison between carbon sources galactose had no effect on production but lactose, fructose and glucose has exhibited repressive effect, similar results were found [5]. Additional supplement of metal ion sources to the medium were investigated. Among these metal salts $FeSO_4 \cdot 7H_2O$ and $CaCl_2$ were found to increase amylase production (Table: 4). The addition of Fe^{2+} and Ca^{2+} further increases whereas Zn^{2+} and Cu^{2+} repressed amylase production by B. subtilis JQ747516. Positive effect of metal cations on amylase production have also been confirmed other workers [15, 16, 17].

Effect of Temperature on Enzyme activity

The effects of temperature on the amylase activity in the ranges from lower to higher temperature (Fig. 5). At 100°C, 78% activity was observed compared to the optimum enzyme activity was observed at 55°C. [5,12]

Table 2: ***Effect of nitrogen sources on the production of Bacillus subtilis JQ747516 α-amylase.***

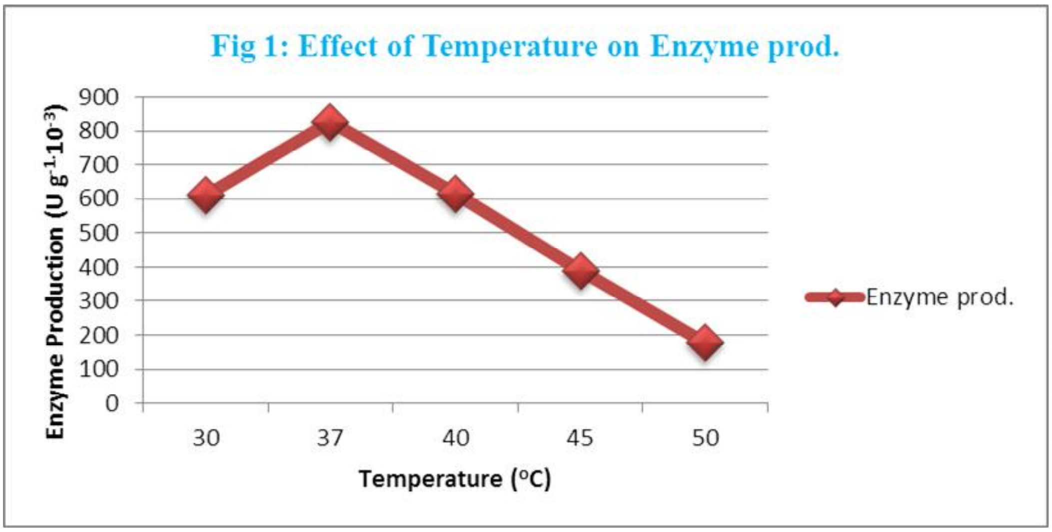
Nitrogen Source (1%)	Enzyme Production (U $g^{-1} \cdot 10^{-3}$)
Ammonium nitrate	1470.3 ± 3.56
Sodium nitrate	851.6 ± 4.50
Ammonium chloride	1062.3 ± 5.03
Ammonium sulphate	859.3 ± 3.56
peptone	1027.3 ± 3.56
yeast extract	691.3 ± 4.04

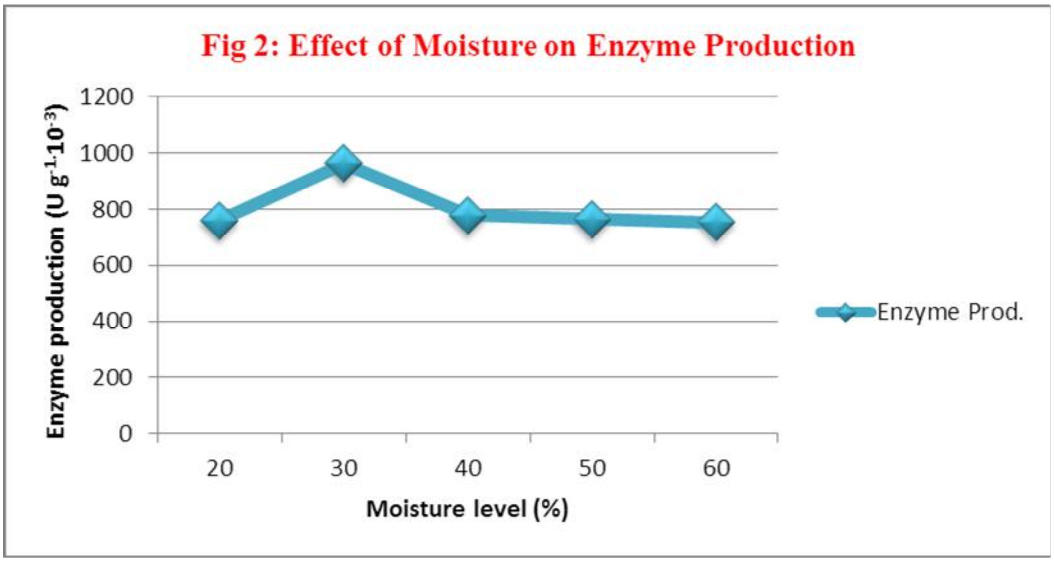
Table 3: ***Effect of Carbon sources on the production of Bacillus subtilis JQ747516 α-amylase***

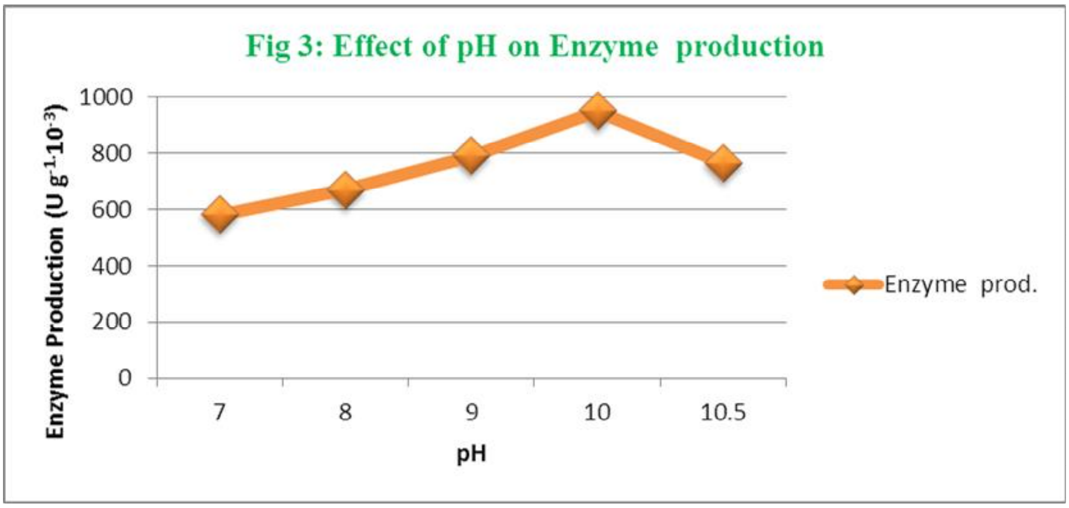
Carbon Source (1%)	Enzyme Production (U $g^{-1}\cdot 10^{-3}$)
Lactose	885.3 ± 3.56
Fructose	813.6 ± 2.51
Galactose	1136.6 ± 3.51
Glucose	606.0 ± 3.60

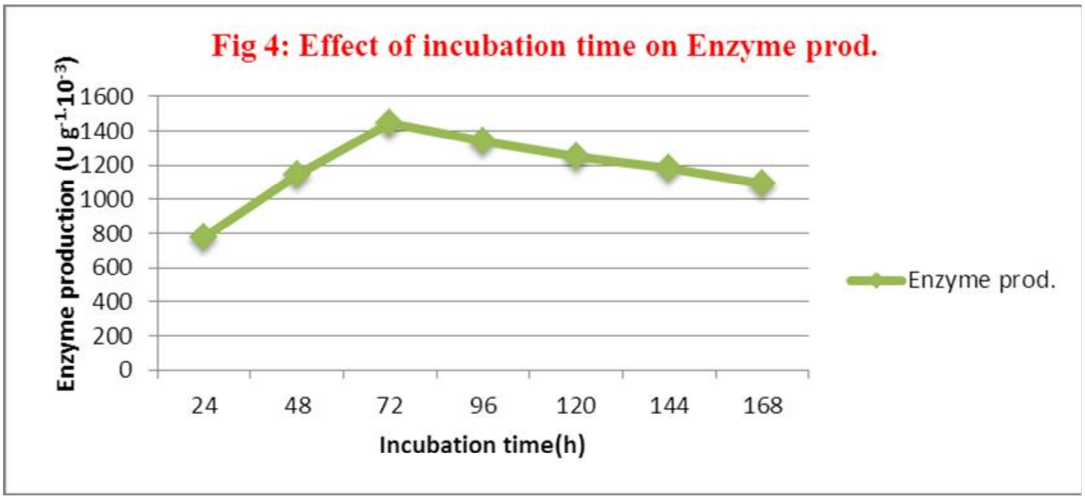
Table 4: ***Effect of metal ions sources on the production of Bacillus subtilis JQ747516*** **α-amylase**

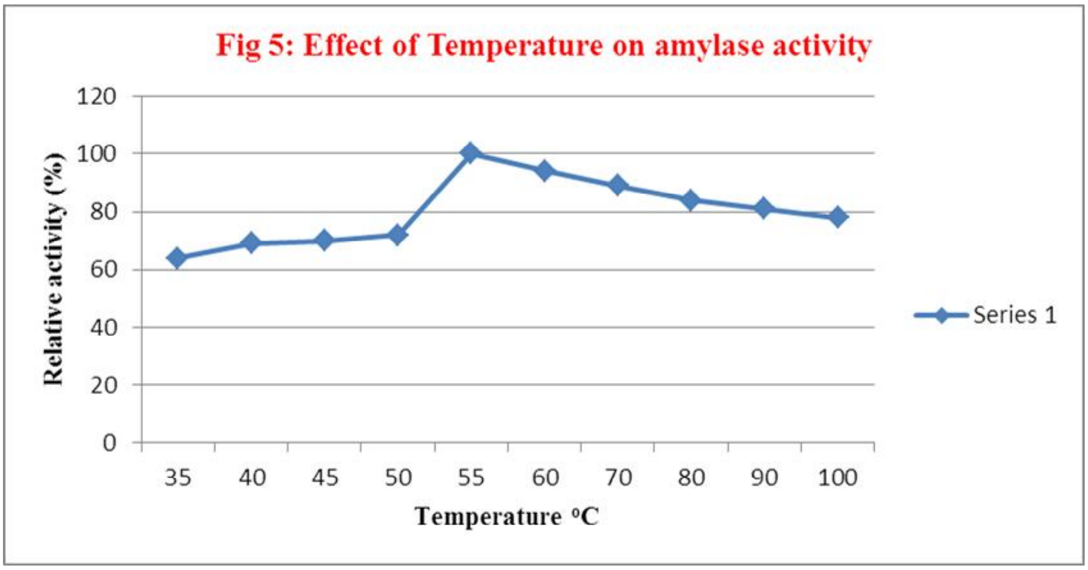
Metal ions	Enzyme Production (U $g^{-1}\cdot 10^{-3}$)
$FeSO_4$	1094.0 ± 4.24
$CuSO_4$	1055.6 ± 3.05
$ZnSO_4$	786.3 ± 3.05
$CaCl_2$	1060.6 ± 3.51











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