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EFFECT OF DIFFERENT CONCENTRATIONS OF METAL IONS ON A-AMYLASE PRODUCTION BY *BACILLUS LICHENIFORMIS* DS3

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ABSTRACT

Ten bacterial strains were isolated from banana field soils collected from in the vicinity of Guntur, Andhra Pradesh, India. Isolation of bacteria was done by serial dilution method. Out of 10 isolates, DS3 showed the maximum clearance zone on Starch Agar medium. The identification of the isolate DS3 was confirmed by *Bacillus licheniformis* DS3 after 16S rRNA sequencing analysis. Further, different metal ions were supplemented into Starch agar media for amylase activity. Various metal ions $CaCl_2$, $CuSO_4$, $MgSO_4$, $MnSO_4$ and $FeSO_4$ were supplemented into the medium at different concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 %. Maximum amylase activity was recorded in the presence of Ca^{2+} (154.8 U/ml) at 0.4%. Among the metal ions tested $FeSO_4$ showed the minimum enzyme activity (82.2 U/ml) at 0.2% concentration. Supplementation of metals on certain ions provides best growth of *B. licheniformis* DS3 and also increased the alpha amylase activity. It is thus Ca^{2+} may be considered as best ion for optimum growth and amylase production of *B. licheniformis* DS3. This study reports the addition of metal ions increased the production of amylase.

KEYWORDS: Amylase, *Bacillus licheniformis*, Dinitrosalsylic acid (DNS), Metal ions.

1. INTRODUCTION

Amylases constitute a group of industrial enzymes, which alone covers approximately 25-30% of the global enzyme market. They have opened new frontiers of many commercial

biotechnological processes include renewable energy, pharmaceuticals, saccharification or liquefication of starch, detergent industries, warp sizing of textiles, fibres, paper industries, foodstuffs, baking, clarification of haze formed in beer or fruit juices and for pre-treatment of animal feed to improve digestibility.[1-2] Amylases can be derived from several sources such as plants, animals and microorganisms those obtained from microbial sources generally meet the industrial demands. Among bacteria, *Bacillus* species were widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis B. cereus* and *B. amyloliquefaciens* are known to be good producers of a-amylase and these have been widely used for commercial production of the enzyme for various applications.[3]

Many enzymes require metal ions for increase their production and enzyme activity. A metalloenzyme is one that binds the metal very tightly or requires the metal ion to maintain its stable, native state whereas metal activated enzymes bind to metal ions weakly only during the catalytic cycle. Metals in metal activated and metalloenzymes act as electrophilic catalysts, stabilizing the increased electron density or negative charge that can develop during reactions. Alpha-amylases are glycoprotein and categorized as metalloenzymes. Supplementation of certain metal ions provided best growth of microorganisms and thereby better enzyme production. Ca^{2+} ions are reported to be present in majority of these enzymes. Addition of $CaCl_2$ to the fermentation media increased the enzyme production.[4] Most of amylases were known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, *etc.*[5] Ca^{2+} was reported to increase a-amylase activity of an alkaliphilic *Bacillus* sp. ANT-6 A.[6] The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure.[7] The aim of this study is to investigate, the effect of different metal ions at different concentrations on the activity of amylase from *Bacillus licheniformis* DS3.

2. MATERIALS AND METHODS

2.1 Soil sample collection

Soil samples were collected from various Banana fields in Guntur district, Andhra Pradesh, India. From 3 to 4 cm depth with the help of sterile spatula, soil samples were transferred to sterile plastic bags and maintained in aseptic conditions for further studies.

2.2 Isolation of Bacteria

The Amylolytic *Bacillus licheniformis* DS3 was isolated from banana fields and screened for their maximum α- amylase production. The starch medium used for the isolation of bacteria contained (g/L): Starch, 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 7.0. One gram of each soil sample was suspended in 9.0 ml of sterile water and 0.1 ml of suitably diluted suspension was spread on the agar plates. The plates were incubated at 35^0C, for 24 to 48 hours. The isolated colonies were flooded with iodine solution. Colonies with best colourless halos around them were picked and maintained on starch agar slants at 4^0C. These pure cultures were further assessed for enzyme production in liquid medium. The characterization and identification of the isolate was made following Bergey's Manual of Determinative Bacteriology.[8]

2.3 Amylase production

The medium for enzyme production comprised (gl-1): starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02. Initial pH of the medium was adjusted at 7.0 and 50 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker at 200 rpm at 45^0C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 x g for 10 minutes and cell free culture supernatant fluid used as enzyme source.

2.4 Enzyme Assay

One ml of crude enzyme supernatant was taken in test tube and 1.0 ml of substrate (starch solution) was added in test tube. The test tubes were covered and incubate at 35°C for 15 minutes in water broth. Then 2.0 ml of DNS reagent was added in each tube and the reaction was stopped by boiling the reaction mixture in water bath for 10 minutes. After cooling at room temperature, the absorbance (O.D) was measured at 540 nm by spectrophotometer and the released sugar was determined from maltose standard curve [9]. One unit of amylase activity was defined as the amount of enzyme that released 1µmol reducing sugar equivalent maltose per minute under the assay condition.

Amount of reducing sugar = Absorbance at 540 nm/ Slope of maltose standard

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Amount of reducing sugar} \times 1000}{\text{Molecular weight of maltose} \times \text{time}}$$

2.5 Effect of metal ions on amylase activity

The effect of metal salts on α- amylase production was studied by adding different metal salts like $CaCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$ and $CuSO_4$ and in the medium at 0, 0.1, 0.2, 0.3, 0.4 and 0.5 % concentration. The estimation of enzyme was determined in the presence of 1% soluble starch as substrate. The relative enzyme activity was measured under standard assay conditions.

3. RESULTS

Out of 10 bacterial isolates, the strain DS3 which showed maximum zone of clearance was further identified up to species level through 16 S rRNA sequencing analysis. The sequences were deposited in Gen bank (NCBI). The strain name with allotted accession number used in the study are *B. licheniformis* DS3 (Accession No. MG870112) isolated from banana field soil tadepalli, Guntur district of Andhra Pradesh. Various metal ions at 0, 0.1, 0.2, 0.3, 0.4 and 0.5% concentrations were supplied in the production medium to determine their influence on enzyme production. Here in the presence of Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+} ions, there was maximum enzyme production (154.8 U/ml) was observed in Ca^{2+} at 0.4% concentration (Figure- 1).

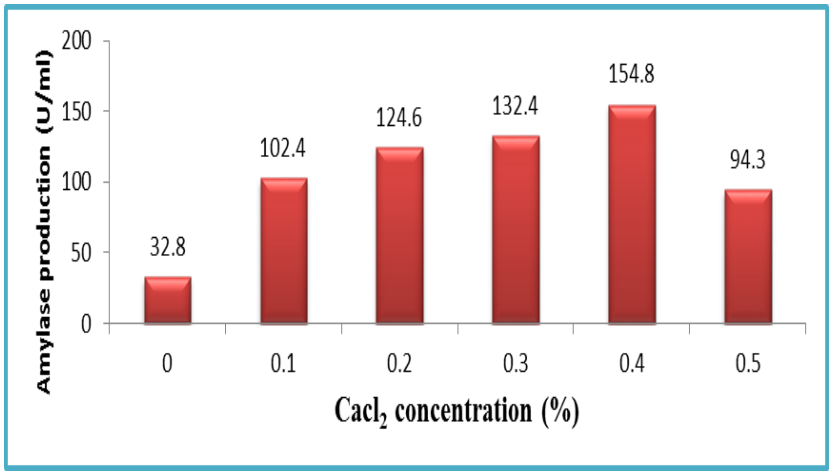

Figure 1: Effect of $CaCl_2$ on amylase production by *Bacillus licheniformis* DS3.

*The overall model is significant with $p<0.05$

Among the metal ions the calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions were best to enhance the enzyme production as they induced amylase production in all 5 concentrations tested. At 0.4% concentrations while the Ca^{2+} ion enhanced the enzyme production when compared to control. Further increase the metal ion concentration the α-amylase production was decreased.

The other metal ion Mg2+ also showed the (128.4 U/ml) maximum enzyme activity at 0.4% concentration. Except Fe^{2+}, rest of the metal ions showed the maximum enzyme activity at 0.4% concentration (Figure 2).

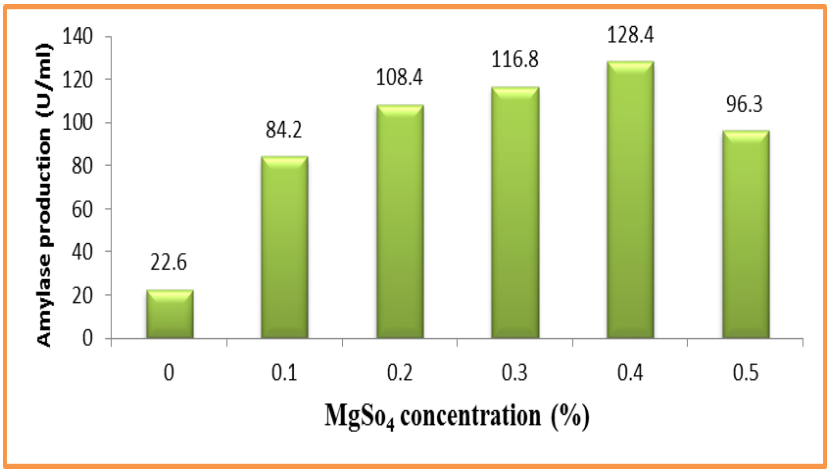

Figure 2: Effect of $MgSo_4$ on amylase production by *Bacillus licheniformis* DS3.

*The overall model is significant with $p<0.05$

Therefore, Mg ions were considered to be the best ion for optimum growth of the bacteria as well as best inducer for amylase production. Although, inhibitory effects of some of the metals may be related to the pH changes associated with their use in the medium.

The metal ion MnSo4 showed the enzyme activity of (100.2 U/ml) at 0.4% concentration. The enzyme activity increased with increase in metal ion concentration up to 0.4% (Figure 3). Further increase in the concentration the enzyme activity decreased. CuSo4 also showed the enzyme activity of (87.9U/ml) at 0.4% concentration (Figure 4). Interestingly the metal ion Fe^{2+} showed the maximum enzyme activity (104.2 U/ml) at 0.2% concentration (Figure 5). Further increase the concentration of metal ions decrease the enzyme production.

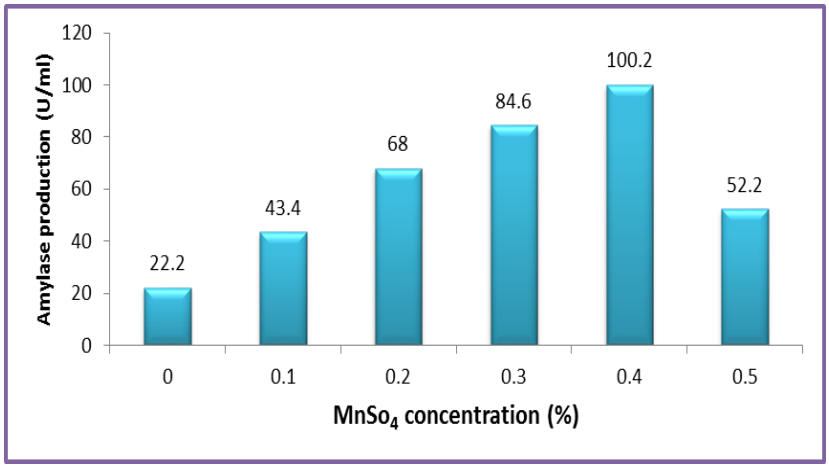

Figure: 3 Effect of $MnSo_4$ on amylase production by *Bacillus licheniformis* DS3.

*The overall model is significant with $p<0.05$

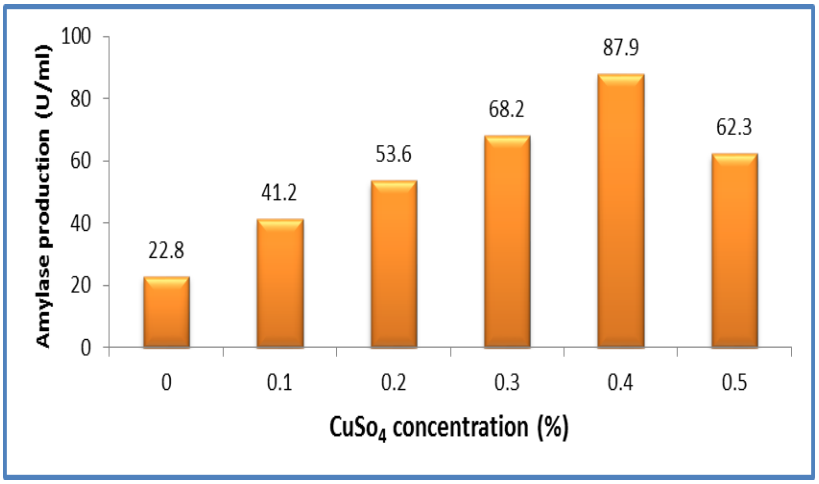

Figure: 4 Effect of $CuSo_4$ on amylase production by *Bacillus licheniformis* DS3

*The overall model is significant with $p<0.05$

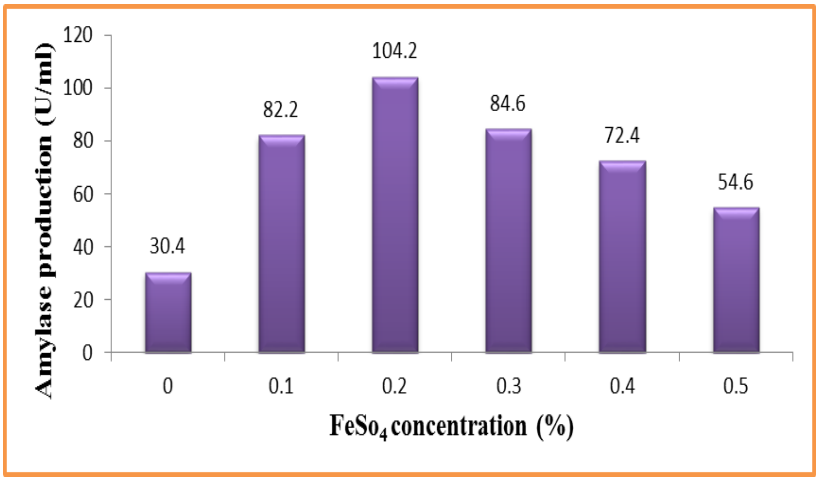

Figure: 5 Effect of $FeSo_4$ on amylase production by *Bacillus licheniformis* DS3.

*The overall model is significant with $p<0.05$

4. DISCUSSION

The present study mainly focuses the metal ions enhanced the amylase enzyme activity. Addition of Ca^{2+} to the broth culture amplified the enzyme production and also had significant effects on physiology and metabolism of bacteria.[10] Enzyme production was increased with increasing concentration upto 0.4%. Further increase the conc. the enzyme production decreased. The alpha-amylase production on *Bacillus amyloliquefaciens by* Ca^{2+} and Mg^{2+} exhibit positive influence. The amylase production is higher in the presence of Ca^{2+} (0.439) IU/ml/min at 7g/l concentration in comparison of other metal ions. The enzyme activity of Mg^{2+} (0.321) IU/ml/min at 2g/l concentration.[11] Similarly results reveal the production of α- amylase by *B. subtilis* KC3 was increased in the presence of 0.1% $CaCl_2$ (28.83 U/ml) Ca^{2+} had significant effects on the metabolism.[12] And physiology of bacteria and that was also found to be effective on enzyme activity.[13]

The strain was identified as *Bacillus subtilis* which was confirmed later on through genomic sequencing. Further, different metal ions were supplemented in production media of *B. subtilis* for amylase production and found that Mg ion be the best inducer that exhibited 105.55 IU/ml/min activity followed by Ca^{2+} showing an activity of 88.88 IU/ml/min whereas less activity was observed with Cu^{2+} in the production medium.[14] Our results are in confirmation with the previously done research where maximum activity of amylase and bacterial growth with Mg^{2+} was reported earlier.[15-16] The purification and characterization of alpha amylase from *Bacillus licheniformis* CUMC 305 metal ions Cu2' and Fe2+ could partially revive enzyme activity on the medium.[17] Effect of heavy metal ions on Amylase production from *Bacillus amyloliquefaciens* was strongly inhibited at 0.4 g/L by Cu^{2+} and less affected by Mg^{2+}, Fe^{2+}, and Mn^{2+} at higher concentrations.[18] The increased activity in the presence of metal ions could be attributed to their role in stabilizing the enzyme structure and thus increasing its stability.[19]

5. CONCLUSION

It can be concluded that soil being a rich source of many hydrolytic enzymes can be exploited to isolate many potent indigenous microorganisms. The genus *Bacillus* species produces a wide range of economically important enzymes including amylases. The present strain *B. licheniformis* DS3 isolated from agriculture field soil and it showed maximum enzyme activity in presence of metal ion Ca^{2+}. Metal ions play a very important role in the production of amylase.

6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

7. ACKNOWLEDGEMENTS

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