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Research Article

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Effect of metal ions on amylase production using *Bacillus subtilis* isolated from soil of Almora District, Uttarakhand, India

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ABSTRACT

Amylase producing bacteria were isolated from soil samples collected from Almora district of Uttarakhand, India thereafter screened for amylase activity on starch agar medium. The isolate shown maximum clearance zone was selected for strain identification morphologically and biochemically. 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+}. Almost similar activities was recorded with Zn^{2+}, Fe^{2+} and Na^{2+} and were observed to be comparatively better enzyme enhancer than Cu^{2+}. It is thus Mg may be considered a better ion for optimum growth of the bacteria and amylase production.

Key words: *Amylase, metal ions, Bacillus subtilis*

INTRODUCTION

Microorganisms are preferred over plant and animal for amylase production due to the relative ease of handling, availability, favourable growth conditions, and cheap nutrient requirements. Amylase occupies around 25% of total world enzyme market owing to its high demand eliminating chemical hydrolysis of starch in the starch liquefaction process[1]. It has been utilized in textile, food, brewing and paper pulp industries etc.[2]

Alpha-Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal 1, 4-*O*-glycosidic bonds in polysaccharides with the retention of alpha-anomeric configuration in the products. Many enzymes require metal ions for increase their 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. They belong to family GH-13 of the glycoside hydrolase group of enzymes[3]**.** Its single polypeptide chain of about 475 residues has SH group and 4 disulphide bridges and contains a tightly bound Ca^{2+}. Calcium stabilizes the interface between the central A domain (291 residues) with $(\beta/\alpha)_8$ barrel structure and the more variable B domain (104 to 206 residues)[4,5] **.** Since the inception of its discovery, the research continues relating microorganisms as a potential source of amylase.

The present study is mainly focused on the isolation of potent, indigenous amylase producing bacteria from soil and to perceive the effect of various metal ions on the activity of the enzyme.

MATERIALS AND METHODS

Collection of soil samples

The soil samples were collected randomly during October 2011 from the sites of Almora District, Uttarakhand, India using standard procedure. Samples were dried under shade, mixed and representative samples were collected in a clean zip lock bag. These samples were preserved for microbiological analysis at 4°C temperature.

Isolation of bacterial strains

Isolation of soil bacteria was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to starch agar plates. The sample was spread uniformly. The plates were incubated at 37°C for 24 hr. The bacterial isolates were further sub cultured to obtain pure culture. Pure isolates on starch agar slants were maintained at 4ºC.

Screening of potent amylase producing bacteria

Bacterial isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The microbial isolates were streaked on the starch agar plate and incubated at 37°C for 72 hours. After incubation, iodine solution was flooded with dropper for 30 seconds on the starch agar plate. Presence of blue colour around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The isolates produced clear zones of hydrolysis were considered as amylase producers[6].

Identification of amylase producing bacteria

The culture showing maximum zone of hydrolysis was selected for identification of bacteria by performing and comparing various staining and biochemical tests according to the Bergey's manual.

Fermentation medium

The inoculum was prepared by inoculating the loopful of bacterial strain into nutrient broth media and it was incubated in shaker for 24 hrs. 100µl of 24 hr old inoculum was transferred aseptically to 100 ml nutrient broth production medium and incubated in shaker for 72 hrs at 150 rpm.

Effect of metal ions on amylase

1% (w/v) of various metal ions: Mg^{2+} ,Ca^{2+}, Cu^{2+} , Na^{2+} , Zn^{2+} and Fe^{2+} were added to the production broth medium and activity of the enzyme was measured under standard assay conditions.

Extraction of crude enzyme

Three ml of production media culture was transferred into centrifuge tubes and spinned for 20 minutes at 5000 rpm. After 20 minutes, the supernatant portion was decanted i.e. crude enzyme extract.

Assay of Amylase activity

The DNS (dinitrosalicylic reagent) method[7] used involved estimating the amount of reducing sugar produced, using 1% soluble starch as substrate. The reaction mixtures consisted 0.5 ml of substrate solution (1% soluble starch in 0.05 M phosphate buffer, pH (6.9) and 0.5 ml of the cell free extract. The reaction mixture was incubated for 10 min at 37°C. The reaction was terminated by the addition of 1 ml of DNSA. The mixture was heated at 100 °C for 5 min and cooled. The optical density was read at 540 nm in spectrophotometer. Maltose was used as standard substrate. One unit of enzyme activity was defined as the amount of enzyme that formed 1 mg of reducing sugar in 1 min.

The amylase activity was determined in IU/ mL/min by applying the following formula[8].

$$\text{Amount of reducing sugar} = \text{Absorbance at 540} / \text{Slope of maltose standard}$$

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

RESULTS AND DISCUSSION

The selected screened amylolytic bacterial strain shown maximum zone of hydrolysis (**Plate 1)**, was taken for its morphological and biochemical identification. The strain was seen to be short rods with single arrangement having a discrete, off white non pigmented colony and was found to be positive for starch hydrolysis, Gram reaction, Voges-Proskauer, catalase and glucose fermentation, while gave negative results for nitrate reduction and methyl red. The bacterial isolate was thus identified as *Bacillus subtilis* (**Table 1).**

Table 1: Morphological and Biochemical characteristics of *Bacillus subtilis*

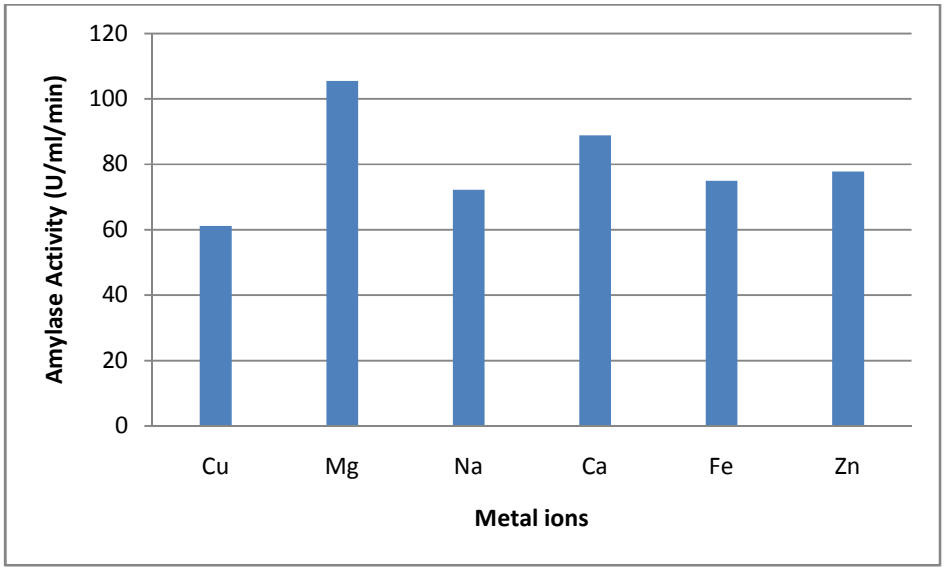
S.NO.	TEST	RESULT
Morphological characteristics		
1	Colony configuration	Discrete
2	Pigmentation	nil
3	Gram reaction	+
4	Shape	Short rod
5	Arrangement	Single/chain
6	Opacity	opaque
7	Colour	Off white
Biochemical characteristics		
1	Starch hydrolysis	+
2	Methyl red	_
3	Voges-Proskauer	+
4	catalase	+
5	Glucose fermentation	+
6	Nitrate reduction	-

Plate 1: Selected bacterial isolate showing zone of clearance for amylolytic activity by starch hydrolysis test

Different metals exhibit different behaviours in their ability to act as effectors[9]. Metallic cofactors are important in the enzymatic reaction, because their presence or absence regulate enzyme activity. The presence of specific metallic ion along with essential nutrient source can inhibit or enhance amylase activity **[10].** Therefore the supplementation of metal ions in production broth media was evaluated in the present study **(Figure** 1). It was observed that confluent bacterial growth was seen in presence of Mg^{2+} and Ca^{2+}. However maximum activity of 105.55 IU/ml/min was obtained with Mg^{2+} followed by Ca^{2+}

showing an activity of 88.88 IU/ml/min. Zn^{2+}, Fe^{2+} and $Na^{2+}$are also equally good metal ions having positive effect on bacterial growth as their supplementation yielded almost similar activity i.e. 77.55, 75.00 and 72.22 IU /ml/min respectively, which was higher than the production of amylase using Cu^{2+} (61.11 IU/ml/min) in the culture media. 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. These results are in confirmation with the previously done research where maximum activity of amylase and bacterial growth with Mg^{2+} was reported[11,12]. Addition of Ca^{2+} to the broth culture amplified the enzyme production and also had significant effects on physiology and metabolism of bacteria[13]**.** An enhanced amylase activity with the addition of Cu^{2+} and Na^{2+} to the media was also observed[14]. Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} was reported to increase α-amylase activity of an alkaliphilic *Bacillus* sp. ANT-6. [15, 2].

Fig. 1: The effect of metal ions on amylase activity



The effects of metal ions have been well studied on several amylases from fungi and bacteria. Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Mn^{2+} Mg^{2+}, Zn^{2+} , Fe^{2+}, *etc.*[16] .Enhancement of amylase activity in presence of ions such as $Mn^{2+,}$ $Ca^{2+,}$ $Co^{2+,}$ Fe^{2+} and Ba^{2+} could be based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid [17]. The stabilizing effect of Ca^{2+} on thermo stability 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[18]. It was found that metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme[19].

CONCLUSION

The present study concluded that soil being a rich source of many hydrolytic enzymes, can be exploited to isolate many potent indigenous microorganisms. The genus *Bacillus* no doubt produces a wide range of economically important enzymes including amylases. It appears that metal ions play a very important role in the growth and production of amylases and the action of metallic ions on amylase vary from one species to other.

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