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Effect of Metal Ions on Biphasic Production of Thermostable Amylase by *Bacillus* sp. Isolated from a Local Hot Spring from Odisha, India

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ABSTRACT

The effect of various metal ions on colony growth of five amylase-positive thermo-tolerant *Bacillus* strains was studied. The metal ions used could be grouped into two categories. One group comprised metal ions that have less than 30% inhibition (Ca^{2+} and Mg^{2+}) and the second group consisted of metal ions that impart more than 30% inhibition (Pb^{2+}, Ag^{2+}, Cr^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+}). However, Mn^{2+} had an intermediate effect relative to the different strengths of ions used. All strains showed a biphasic pattern of amylase activity with two peaks at 48 and 96 h of culture. To observe the effect of ions on amylase activity at crucial points in the biphasic amylase production curve, i.e. at 48, 72 and 96 h of incubation, two strains, ARBE LlCrg and ARBE UuSs, were selected on the basis of maximum and minimum amylase activity, respectively. Ca^{2+} and Cu^{2+} were selected from the two groups of metal ions with respect to their effect on colony growth. The general biphasic trend was marked with Ca^{2+} and Cu^{2+} supplementation for both strains, although amylase activity increased with Ca^{2+} supplementation and decreased in the presence of Cu^{2+}. When starch was added the biphasic trend was more pronounced. However, Ca^{2+} supplementation blurred the biphasic trend.

Keywords: *Bacillus*, biphasic amylase activity, metal ions

INTRODUCTION

Amylases are starch hydrolyzing enzymes that produce smaller polymers composed of glucose units. It constitutes a class of industrial enzymes having approximately 25% of the enzyme market (Suman and Ramesh 2010). Thermostable enzymes isolated from thermophilic organisms have a number of commercial applications because of their overall inherent stability (Demirijan *et al.* 2001). The spectrum of application of amylase has widened in clinical, medical, and analytical chemistries. It is used in various industries like paper, textile, detergent, brewing and sugar production, food and fermentation and distilling industries (Pandey *et al.* 2000). It is desirable that α-amylases should be active at the high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize processes, hence, there is a need of more thermophilic and thermostable α-amylases (Sidhu *et al.* 1997). Among various sources, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Grata *et al.* 2008; Rasooli *et al.* 2008). The bacteria belonging to the genus *Bacillus* produces a large variety of extracellular enzymes of which amylases are of significant industrial importance (Reddy *et al.* 2003). Different *Bacillus* species have been widely used for the commercial production of thermostable amylases (Riaz *et al.* 2009).

The composition and concentration of components of medium greatly affect the growth and production of amylase in *Bacillacea* (Otludil *et al.* 2005). Various metal ions are known to affect amylase production and growth of organisms (Otludil *et al.* 2005). The presence of specific metal ions along with nutrient content can inhibit or enhance amylase activity (Dutta *et al.* 2006). To meet the demand of highly thermostable amylases, a series of attempts have been made to propose thermostabilization mechanisms and to find factors that enhance the enzyme thermostability (Sterner and Liebl 2001; Vieille and Zeikus 2001). Among these thermostabilizing factors, calcium ion plays an important role in stabilizing enzymes (Kim *et al.* 2005). It is required to maintain the structural integrity of α-amylase (Vallee *et al.* 1959) and its removal leads to decreased thermostability and/or enzymatic activity (Violet and Meunier 1989), or increased susceptibility to proteolytic degradation (Machius *et al.* 1995). The affinity between divalent metal ions and the α-amylase varies considerably with the source of the enzyme (Saboury *et al.* 2005). The bacterial strains of hot springs are supposed to have adaptable interactions with metal ions, as the water of hot springs are with higher level of metal ions (Marric *et al.* 2010).

So, under the above described scenario, the present investigation aims at finding the effect of metal ions on growth and amylase production of certain *Bacillus* strains isolated from hot spring of Atri, Odisha, India.

MATERIALS AND METHODS

Media

The different media used were prepared in the laboratory. The media used were nutrient agar (5 g/l peptone, 3 g/l beef extract, 15 g/l agar, pH 7.0), nutrient broth (5 g/l peptone, 3 g/l beef extract, pH 7.0) and starch broth (5 g/l peptone, 3 g/l beef extract, 10 g/l soluble starch, pH 7.0). The media were sterilized by autoclaving at 121°C and 15 psi for 45 min.

Isolation of bacteria

Five amylase positive *Bacillus* strains were isolated from the water sample of the sulfur hot spring at Atri, located 42 km away from Bhubaneswar, Capital city of Odisha, India. This spring is a solitary one with around 1.5 to 2 m in diameter and nearly 4.5-5 m in depth. Continuously water comes out of the spring that has been channelized to different bathing complexes as the water is believed to have some medicinal properties. Though the temperature of the

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hotspring is not much variable, it rises during summer in the month of May-June and falls down during winter during December-January. The temperature of the hot spring measured to be 55°C at the time of collection during December.

Maintenance of bacterial strains

The strains were grown and maintained on nutrient agar slants. The well grown pure cultures of 48 h old were preserved in refrigerator at 4°C. For working culture the strains were maintained in nutrient broth or starch broth up to the required time. The cultures were grown at 37 ± 1°C.

Culture condition for growth

To test the effect of metal ions on growth of the strains, nutrient agar media supplemented with different concentrations (1, 3 and 5 mM) of metal salts including $AgNO_3$, $CaCl_2 \cdot 2H_2O$, $(CH_3.COO)_2Pb \cdot 3H_2O$, $CuSO_4 \cdot 5H_2O$, $HgCl_2$, $K_2Cr_2O_7$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$ were prepared separately. Nutrient agar plates without metal ions served as control. The nutrient agar plates were inoculated with 2 µl of 24 h grown working culture and allowed to grow at 37 ± 1°C to develop colonies. The plates were observed up to 120 h at an interval of 24 h.

Culture condition for amylase production

To find the effect of metal salts on amylase production, both starch broth and nutrient broth were prepared. Selected metal salts in 1 mM were added to the media and pH was adjusted to 7.0. Nutrient broth and starch broth without metal ions served as controls. The fresh media with or without metal ions, as required, were inoculated with 24 h grown culture in starch broth. The volume of the inoculum was 10% in the culture medium. The culture was grown at 37 ± 1°C for different duration.

Enzyme assay

The culture was centrifuged at 4000 rpm for 10 min. The cell free supernatant was used as the crude enzyme extract. The enzyme assay was performed following Bernfeld (1955) with modification. The reaction was carried out in 0.5 ml volume containing 0.25 ml of 1% starch in 0.2 M sodium phosphate buffer (pH 6 for strain ARBE UuSs and pH 8 for all other strains) and 0.25 ml of one-hundredth diluted crude enzyme extract. The reaction was stopped by adding 0.5 ml DNS (3,5-dinitrosalicylic acid) reagent and heating in boiling water bath for 10 min. 0.5 ml of reaction mixture was diluted to 10 ml and optical density was measured at 540 nm. The level of amylase activity was determined by measuring the reducing sugar in terms of maltose released from soluble starch (Nelson 1944). One unit of amylase activity was defined as the amount of enzyme which liberates 1 µmole of reducing sugar as maltose per minute under the conditions of the assay.

RESULTS AND DISCUSSION

Effect of metal ions on growth of the isolates

Nine metal salts (Ca^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Ag^{2+}, Cr^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+}) were used at 1, 3 and 5 mM to find their effect on colony growth of the isolated amylase positive *Bacillus* strains (**Fig. 1**). The metal salts used could be grouped into two categories, one with the salts that have more than 30% inhibitory effect on colony growth and the other with the salts that have less than 30% inhibitory effect. Ca^{2+} and Mg^{2+} fall into the second category and all other metal ions except Mn^{+2} were grouped into the first category. The Mn^{2+} ion could not be placed in either group, as the percentage of inhibition was less than 30% at 1 mM with the exception of ARBE UuSs strain and it exceeded 30% at 3 mM and 5 mM concentrations for all the strains. Considering the effect of Mn^{2+} ion at 1, 3 and 5 mM, it can be said that its effect was intermediate between the effect of first group of metal ions comprising Ca^{2+} and Mg^{2+} and the second group metal ions comprising Pb^{2+}, Ag^{2+}, Cr^{2+}, Zn^{2+},

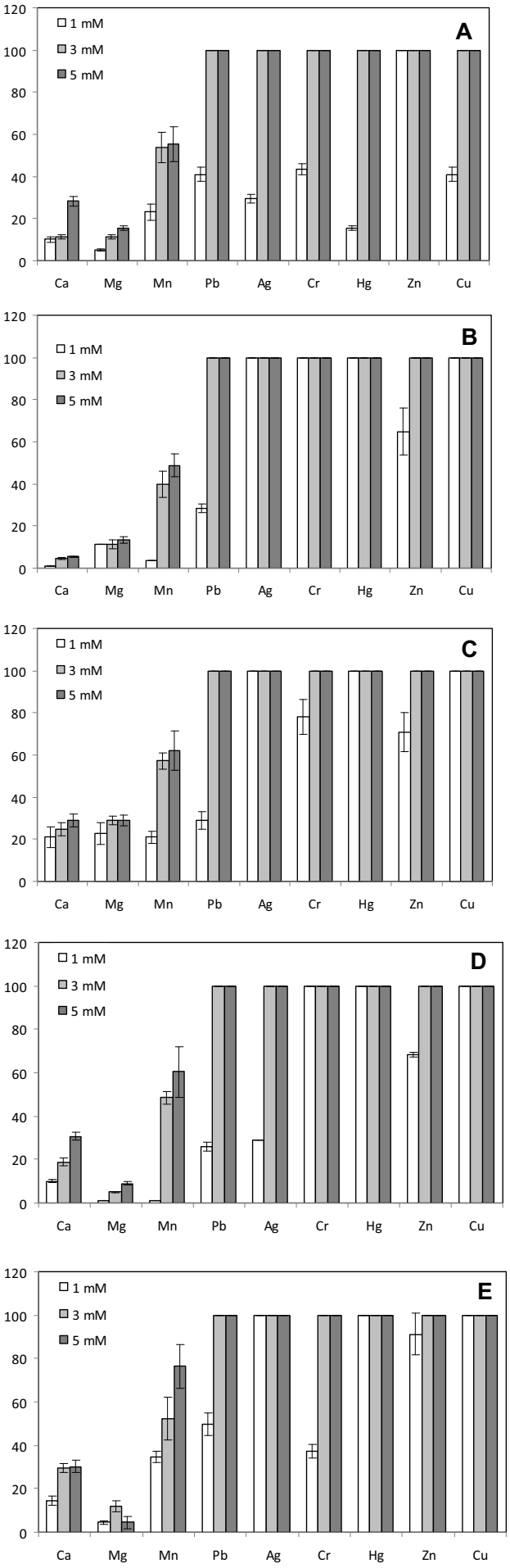

Fig. 1 Effect of metal ions in terms of percentage of inhibition of colony growth of the isolates at 96 h of culture. (**A**) ARBE LlCrg. (**B**) ARBE LlCra. (**C**) ARBE UlC2c2. (**D**) ARBE LlC2c2. (**E**) ARBE UuSs.

Hg^{2+} and Cu^{2+}.

Nies (1999) observed the influence of metal ions on nutrient uptake mechanism, thereby influencing the colony growth. Sterner and Liebl (2001) have shown that the ion Ca^{2+}, Mg^{2+} and Mn^{2+} used to participate in the process of metabolism and thereby essential metal for the growth. The inhibition of growth by metal salts was also reported by Nies and Silver (1995). Kalantari (2008) reported the inhibitory effect of metal ions on the growth of *Bacillus cereus* strain, whereas Shafee *et al.* (2005) observed the increase in growth of *Bacillus cereus* strain 146 by supplementation of metal ions. Pavani *et al.* (2011) also reported the inhibitory effect of metal ions on the colony growth of the *Bacillus circulans* strain. Some of the metal ions used in this investigation were also found to have inhibitory effect on colony growth of *Bacillus* sp. (Marric *et al.* 2010).

Amylase activity

The amylase activities of the five strains were studied in the reaction mixture with buffer at their respective pH optima incubated at 100°C for 3 min. For all experiments the above conditions were treated as the standard condition. The amylase activities in terms of enzyme unit at different durations for different strains are shown in **Fig. 2**. All the strains were found to show a biphasic pattern of amylase activity. The amylase activity increased up to 48 h, where after there was a decline of the amylase activity at 72 h. From 72 to 96 h, there was an increase in amylase activity which was then declined at 120 h. This trend was observed for all the strains.

The peak amylase production time is variable for different *Bacillus* species. Swain *et al.* (2006) observed maximum amylase production during 36 h of growth for *Bacillus subtilis* strain CM3, whereas Asgher *et al.* (2007) obtained the peak production during 48 h for *B. subtilis* JS2004. Maximum amylase production was marked at 24 h for some *Bacillus* sp. (Rasooli *et al.* 2008; Riaz *et al.* 2009; Ramesh and Suman 2010; Anupama and Jayaraman 2011), whereas it ranged from 36 to 60 h for some other *Bacillus* spp. (Kiran *et al.* 2005; Devi *et al.* 2010; Ahamadi *et al.* 2010; Joshi 2011). Though biphasic trend of amylase production is not frequently reported in *Bacillus* sp., Kelly *et al.* (1997) observed biphasic trend of amylase production by *Bacillus flavothermus* with two peaks at 24 and 52 h. The result of the present investigation corroborates the findings of Kelly *et al.* (1997).

Effect of metal ions on biphasic trend of amylase production

Out of five strains, two strains, ARBE LlCrg and ARBE UuSs were selected on the basis of maximum and minimum amylase activity respectively for further studies. From among each of two groups of metals considered in this investigation with respect to colony growth and also our earlier study (Marric *et al.* 2010), Ca^{2+} and Cu^{2+} were chosen to observe their effect on amylase activity at crucial points in the biphasic amylase producing curve, i.e. at 48, 72 and 96 h of incubation.

The effect of calcium and copper salts on amylase activities of ARBE LlCrg during 48-96 is shown in **Fig. 3**. In general, application of Ca^{+2} enhanced the amylase activity at all the durations tested as compared to control. The reverse was observed in case of Cu^{2+}. The result indicated the maintenance of biphasic trend of amylase production in all the treatments except in case of calcium treatment without starch where the biphasic trend was not so distinct.

Effect of calcium and copper salts on amylase activity of ARBE UuSs during 48-96 h of culture is depicted in **Fig. 4**. Similar to the ARBE LlCrg strain, this strain had also shown increase in amylase activity with the addition of calcium salt at all the durations considered in this experiment. Starch addition found to supplement the enhancing effect of calcium on amylase activity. Copper salt inhibited amylase activity as relative to control both at the presence and absence of starch. The biphasic trend of amylase activity was exhibited by all types of supplementation like the ARBE LlCrg strain. However, in this strain also calcium supplementation resulted in blurring of biphasic trend.

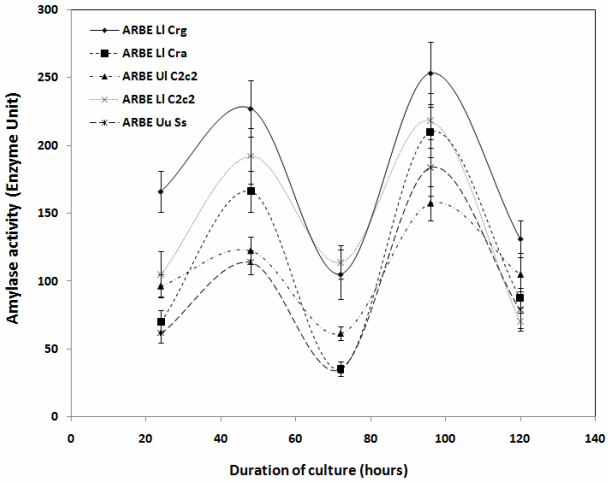

Fig. 2 Amylase activity of different strains at different time intervals.

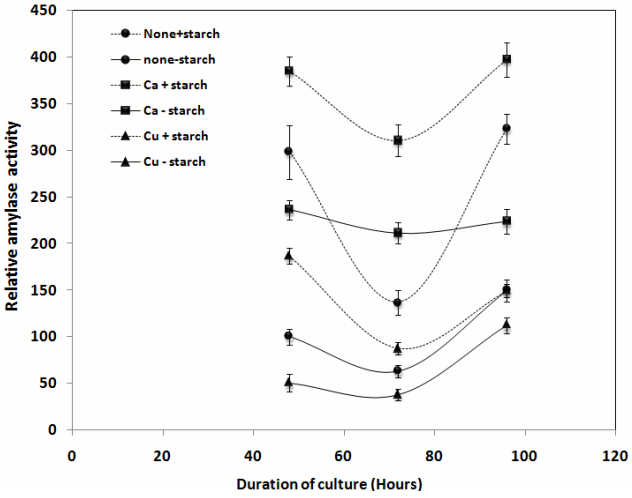

Fig. 3 Effect of Ca^{2+} and Cu^{2+} on amylase activity of "ARBE Ll Crg".

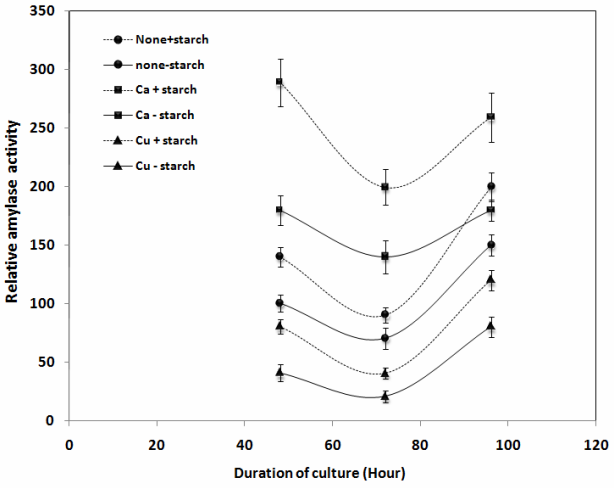

Fig. 4 Effect of Ca^{2+} and Cu^{2+} on amylase activity of "ARBE UuSs".

The general biphasic trend was marked with supplementation of Ca^{2+} and Cu^{2+} for both the strains, though the amylase activity increased with supplementation of Ca^{2+} and decreased in the presence of Cu^{2+} at all the three durations considered.

The extent of increase or decrease in amylase activity

Table 1 Effect of metal ions on amylase activity (%) of the isolates.

Metal ions	% of increase (+) and decrease (-) in amylase activity					
	ARBE LlCrg			ARBE UuSs		
	48 h	72 h	96 h	48 h	72 h	96 h
Ca + Starch	+29.0	+126.46	+23.0	+106.61	+121.27	+29.89
Ca - Starch	+136.3	+236.68	+49.7	+79.44	+99.0	+19.9
Cu + Starch	-37.39	-36.13	-53.7	-42.64	-55.127	-39.86
Cu - Starch	-49.56	-39.44	-24.85	-59.58	-70.72	-46.44

with respect to control is depicted in **Table 1** for the strains ARBE LlCrg and ARBE UuSs. The increase in amylase activity was found to be high in ARBE LlCrg strain (236.68%) at 72 h of culture whereas, that of ARBE UuSs strain was 121.27%. The interesting part of the result was the extent of increase of amylase activity with or without starch for the strain ARBE LlCrg. Calcium salt with starch supplementation decreased the extent of increase marked in calcium salt without starch supplementation. Starch was found to modify the amylase activity antagonistically in the presence of Ca^{2+}. The maximum inhibition of amylase activity was marked with copper salt in ARBE LlCrg strain at 96 h of culture (53.7%). In ARBE UuSs strain, the inhibition was more pronounced at 72 h of culture (70.72%) under Cu^{2+} treatment without starch.

In general the calcium salt increased and copper salt decreased the amylase activity in both the strains. There are sufficient literatures in favour of positive effect of calcium on amylase activity (Obi and Odibo 1984; Shih and Labbe 1995; Sarikaya and Gurgun 2000; Asgher *et al.* 2007). The results of this investigation are in accordance with others reported in literature. However, the starch supplementation distinguished these strains with respect to amylase activity. Both the strains were observed to behave in an opposite way. The ARBE UuSs strain had shown an increase in the amylase activity with supplementation of starch to calcium salt treatment but interestingly such an effect could not be marked in ARBE LlCrg strain. Rather, the ARBE LlCrg strain pointed that the starch supplementation may have certain role in inhibition of the amylase production. There are many reports suggesting enhancement of amylase production by supplementation of starch (Shah *et al.* 2006; Asgher *et al.* 2007). As the expression of amylase gene is reported to be constitutive in nature, starch is not an essential factor for production of amylase. Babu and Satyanarayan (1995) also reported inhibitory effect of starch on amylase production by *Bacillus coagulans* in solid state fermentation.

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