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EFFECT OF SALT STRESS AND VITAMIN B1 ON AMYLASE PRODUCTION POTENTIAL IN LOCALLY ISOLATED BACTERIA

SYED SHAHID ALI*, MAHWISH NAZIR AND TANZEELA RIAZ

Biochemistry and toxicology laboratory, Department of Zoology, University of the Punjab, Lahore-54590, Pakistan.

Abstract: Amylases belong to one of the most important enzyme group with wide industrial applications. The amylase activity of 40 bacterial isolates was determined quantitatively. The effect of salt stress and a growth factor vitamin B1 (thiamine HCl) on amylase production potential was evaluated on two *Bacillus* spp., coded as BCTL-X-11 and BCTL-SL-101. In case of isolate BCTL-SL-101, 1% NaCl concentration produced highly significant increase in amylase activity as compared to 2 and 3% but BCTL-X-11 showed increased activity at 3% concentration after 48 hours incubation. Amylase production by BCTL-SL-101 was maximum at 0.1% magnesium sulphate concentration but BCTL-X-11 showed maximum activity at 0.2% concentration after 48 hours incubation. The optimum concentration of dipotassium hydrogen phosphate for BCTL-SL-101 was found to be 0.5% but its optimum concentration for BCTL-X-11 was 1%. Vitamin B1 at 5.0 and 10.0 mg/l concentrations did not produce any rise in amylase activity up to 48 hours incubation, on the other hand highly significant inhibition was noticed at both concentrations.

Keywords: *Bacillus* spp., amylase assay, thiamin HCl, sodium chloride, magnesium sulphate, *di*-Potassium hydrogen phosphate

INTRODUCTION

Enzymes are biological tools which are being widely used in variety of biotechnological processes in industry (Madigan and Marrs, 1997). α-amylase (EC 3.2.1.1) is an important enzyme used in the industry and accounts for approximately 25% of the enzyme market (Pandey, 2000; Oudjeriouat *et al.,* 2003; Peixoto *et al.,* 2003). Thermostable α-amylases have extensive commercial applications in starch

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Corresponding author: shahid.zool@pu.edu.pk

processing, brewing and sugar production, desizing in textile industries and in detergent manufacturing processes (Lévêque *et al*., 2000). α-amylase catalyses the hydrolysis of internal α-glucosidal linkages in starch and other oligo- and polysaccharides, and shows varying action patterns.

The α-amylase (EC 3.2.1.1) is widely distributed in animals, plants and microorganisms. The later are very important for enzyme production, because of their short growth period (Uhling, 1998). The amylases are usually produced by bacteria belonging to the genus *Bacillus* for industrial applications such as *B. amyloliquefaciens*, *B. stearothermophilus*, *B. subtilis* and *B. licheniformis* (Sajedi *et al*., 2005).

Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques (Burhan *et al*., 2003). Optimized growth conditions and nutrients promote the α-amylase production (Pazlarova *et al*., 1984).

Starch or other sugars as a carbohydrate source, and ammonium salts or complex organic compounds as a nitrogen source are needed for bacterial growth and enzyme production. On the other hand, all α-amylases are calcium metallo-enzymes and thus need calcium or other metal ions for their activity (Fogarty, 1983).

In our country these studies are just in infancy and no organized effort is being underway in this direction. The objectives of the present study is to evaluate the effect of some salts *i.e.,* sodium chloride, magnesium sulphate and *di*-potassium hydrogen phosphate and vitamin B1 (thiamine HCl) on the amylase production of some already isolated *Bacillus* spp. from this laboratory.

MATERIALS AND METHODS

Forty bacterial isolates, already stored in the laboratory, were initially used to estimate the amylase activity. Later on two isolates with high amylase production potential (BCTL-SL-101 and BCTL-X-11) were selected to evaluate the effect of salt stress (sodium chloride, magnesium sulphate, *di*-Potassium hydrogen phosphate) and vitamin B1 (thiamine HCl) on amylase production.

Enzyme assay

Amylase production by all forty isolates was carried out quantitatively. Selective medium containing starch was used for the production of amylase enzyme. The ingredients of this medium were peptone 0.5%, yeast extract 0.25%, starch 2.0%, K_2HPO_4 0.5% and magnesium sulphate 0.1%. For preparation of 10ml overnight culture in log phase, the medium was autoclaved for 20 minutes and then it is inoculated with isolate having high amylase production. Inoculum was placed in shaking water bath for 24 hours at 37ºC temperature at 140 rpm.

The same amylase production medium was prepared up to 100 ml in 250ml flasks each and autoclaved for 20 minutes. These 250ml flasks were inoculated with 1% overnight culture from 50ml flasks in triplicate. These flasks were then incubated in shaking water bath at 37ºC temperature and 140 rpm for 48 hours followed by centrifugation at 10,000 rpm for 15 minutes at 4ºC. Supernatants were collected in separate test tubes for estimation of amylase activity.

Dinitro salysilic acid (DNS) method or Bernfeld method (Bernfeld, 1955) was used for amylase assay. Amylase activity *i.e.*, amount of maltose released by the degradation of starch is calculated by using maltose standard factor calculated from the standard curve of maltose according to the following relationship:

$$\text{Enzyme activity}(\mu g/ml/min) = \frac{\text{absorbance of sample} \times \text{standard factor} \times \text{dilution factor}}{\text{incubation time}}$$

Effect of sodium chloride on amylase production

Effect of different concentrations of sodium chloride salt on amylase production by isolates BCTL-SL-101 and BCTL-X-11 was evaluated. Different concentrations of sodium chloride 1, 2 and 3% were added in amylase producing medium for 24 and 48 hours duration, after which the enzyme assay was performed

Effect of magnesium sulphate on amylase production

To study the effect of magnesium sulphate on amylase production, 0.2% magnesium sulphate instead of 0.1% normal conc., was used in the production medium. The activity was calculated by performing enzyme assay.

Effect of di-potassium hydrogen phosphate on amylase production

To estimate the effect of dipotassium hydrogen phosphate on amylase production, its 1% was used in production medium instead of 0.05% in normal medium followed by enzyme assay in order to evaluate the enzyme activity.

Effect of vitamin B1 on amylase production

Effect of vitamin B1 on amylase production was also studied. For this purpose, 0.001 and 0.0005% thiamine HCl was added in the production medium. Enzyme production was followed by enzyme assay to calculate enzyme activity.

Total soluble proteins estimation

For bacterial protein estimation Lowry *et al.* (1951) was followed. A standard curve was prepared using different concentrations of bovine serum albumin (BSA). Total protein content was determined from the BSA standard curve.

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RESULTS

Detection of enzyme activity

The forty available bacterial isolates belonging to *Bacillus spp.* showed variable activity for amylase production using DNS method. Table I showed the amylase activity for all the available *Bacillus spp.* isolates.

Estimation of amylase production by selected isolates

Two isolates BCTL-SL-101 and BCTL-X-11 showing high potential for amylase activities were selected for further study. Effect of different concentrations of salts (sodium chloride, magnesium sulphate and *di*-potassium hydrogen phosphate) and vitamin B1 on amylase activity was evaluated.

Effect of sodium chloride on amylase production

The bacterial isolate BCTL-SL-101 showed 1.33±0.08 and 12.95±0.40 µg/ml/min and BCTL-X-11 showed 0.36±0.14 and 8.92±0.43 µg/ml/min enzyme activity at 24 and 48 hours, respectively under normal

conditions. Following sodium chloride treatment at 1, 2 and 3% concentrations the activity was raised in all cases. The increase in production of amylase by BCTL-SL-101 was 37, 14 and 3% for 1, 2 and 3% sodium chloride concentration, respectively after 48 hours. In case of BCTL-X-11 enzyme production was increased 14.5, 0.94 and 55% for 1, 2 and 3% sodium chloride concentration respectively, after 48 hours (Figures 1-2).

Table I: Amylase activity shown by various bacterial isolates.

Sr. No.	Bacterial isolates	Amylase activity (µg/ml/min)	Sr. No.	Bacterial isolates	Amylase activity (µg/ml/min)
1	BCTL-AL01	7.07±0.01	21	BCTL-WF57	7.94±0.02
2	BCTL-AL02	8.83±0.01	22	BCTL-X01	5.74±0.01
3	BCTL-AL03	6.55±0.03	23	BCTL-X02	7.29±0.12
4	BCTL-WL51	8.32±0.01	24	BCTL-X03	8.69±0.01
5	BCTL-WL52	8.42±0.03	25	BCTL-X04	6.57±0.05
6	BCTL-WL53	8.43±0.02	26	BCTL-X05	8.38±0.30
7	BCTL-WL54	8.20±0.06	27	BCTL-X06	8.23±0.01
8	BCTL-SL101	12.95±0.40	28	BCTL-X07	8.90±0.56
9	BCTL-SL102	7.95±0.40	29	BCTL-X08	8.76±0.01
10	BCTL-SL103	7.40±0.07	30	BCTL-X09	8.54±0.04
11	BCTL-SL104	7.49±0.04	31	BCTL-X10	8.21±0.04
12	BCTL-SL105	8.83±0.07	32	BCTL-X11	8.92±0.43
13	BCTL-SL106	8.12±0.01	33	BCTL-X12	6.67±0.15
14	BCTL-SL107	7.97±0.01	34	BCTL-X13	7.09±0.03
15	BCTL-SL108	7.21±0.06	35	BCTL-X14	8.26±0.09
16	BCTL-SK109	7.89±0.01	36	BCTL-X15	7.64±0.01
17	BCTL-SK110	8.01±0.07	37	BCTL-X16	8.03±0.12
18	BCTL-SK111	7.92±0.02	38	BCTL-X17	7.98±0.01
19	BCTL-WF55	6.67±0.04	39	BCTL-X18	8.13±0.21
20	BCTL-WF56	8.81±0.03	40	BCTL-X19	7.94±0.07

Values are given in terms of mean± SEM. Each value is average of 3 replicates.

Effect of magnesium sulphate on amylase production

The bacterial isolate BCTL-SL-101 showed 1.33±0.08 and 12.95±0.37 µg/ml/min and BCTL-X-11 showed 0.36±0.14 and 8.92±0.43 µg/ml/min amylase activity at 24 and 48 hours respectively, under normal conditions. Following magnesium sulphate treatment at 0.2% concentration, the amylase activity in case of BCTL-SL-101 showed 25% increase after 24 hours treatment while 48% decrease was found after 48 hours incubation. In case of BCTL-X-11 at 24 hours treatment 25% elevation was observed while at 48 hours treatment in 0.2% concentration, 2.8 fold increase was found in amylase activity (Figures 3-4).

Effect of di-potassium hydrogen phosphate on amylase production

The bacterial strain BCTL-SL-101 showed 1.35±0.08 and 12.29±0.55 µg/ml/min amylase activity while BCTL-X-11 showed 0.47±0.08 and 8.81±0.37 µg/ml/min enzyme activity at 24 and 48 hours respectively, under normal conditions. Following 1% *di*-potassium hydrogen phosphate treatment, there was an increase of 69% for BCTL-SL-101 and 3.4 fold increase for BCTL-X-11 after 24 hours incubation. However a decrease of 42% was observed for BCTL-SL-101 during the same period (Figures 5-6).

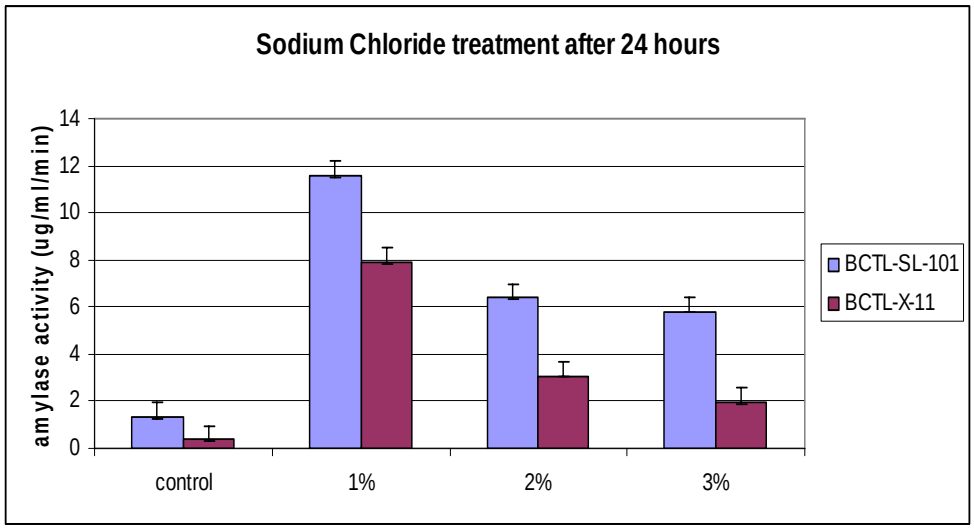

Figure 1: Effect of 1, 2 and 3% sodium chloride on amylase production by BCTL-SL-101 and BCTL-X-11 after 24 hours incubation.

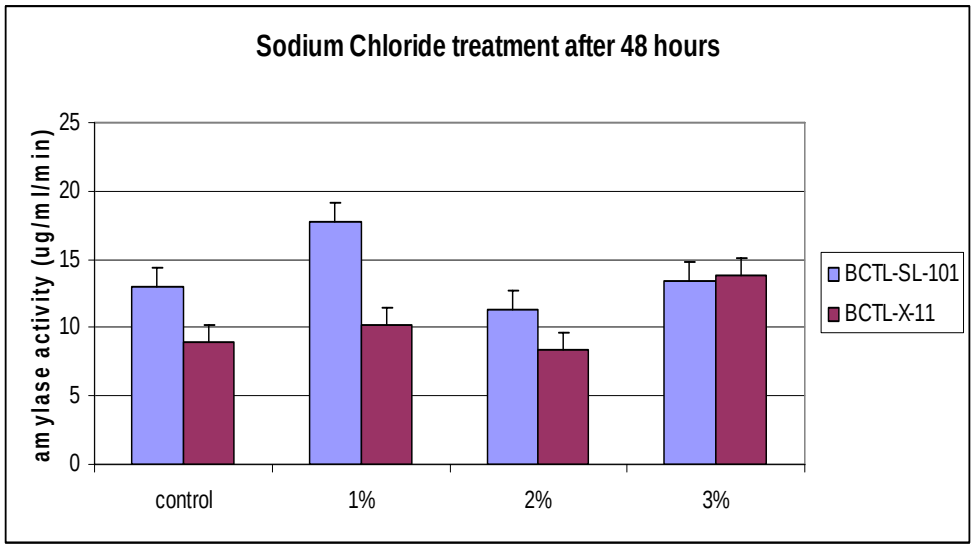

Figure 2: Effect of 1, 2 and 3% sodium chloride on amylase production by BCTL-SL-101 and BCTL-X-11 after 48 hours incubation.

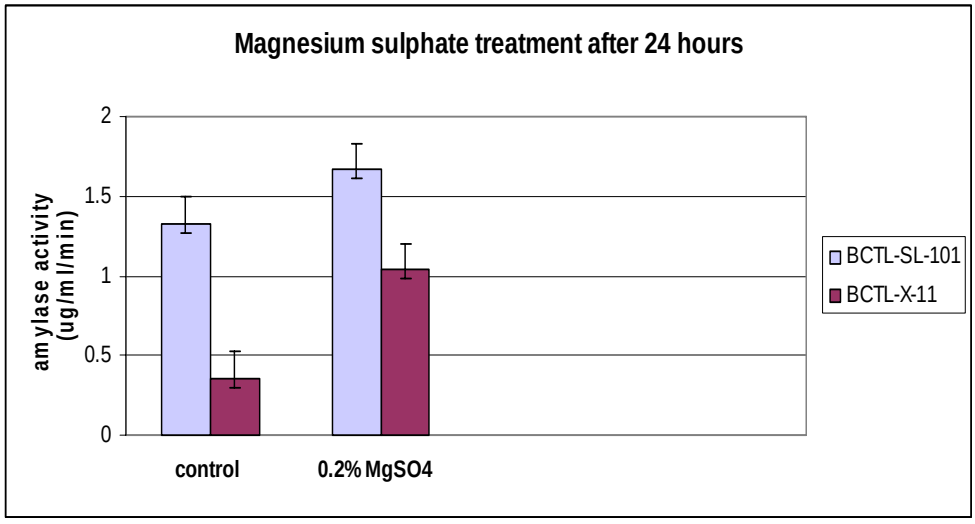

Figure 3: Effect of 0.2% magnesium sulphate on amylase production by BCTL-SL-101 and BCTL-X-11 after 24 hours incubation.

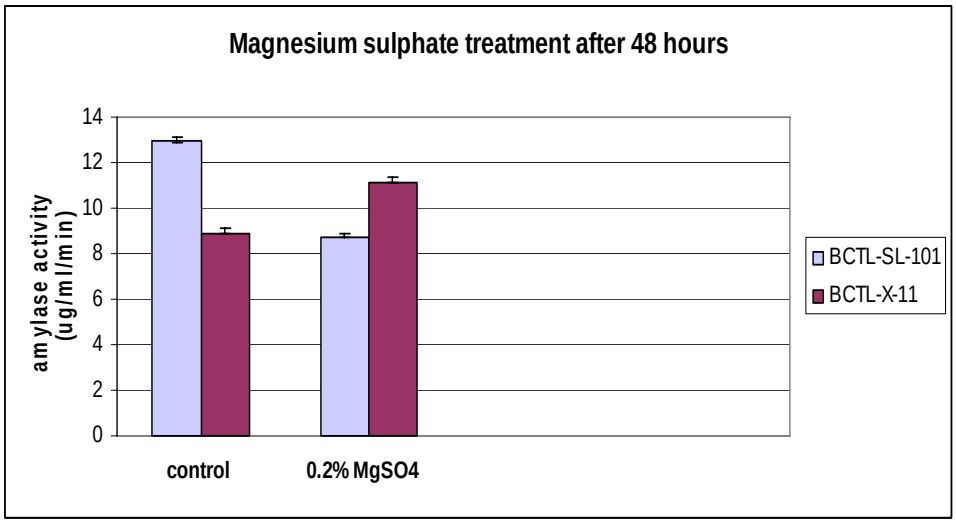

Figure 4: Effect of 0.2% magnesium sulphate on amylase production by BCTL-SL-101 and BCTL-X-11 after 48 hours incubation.

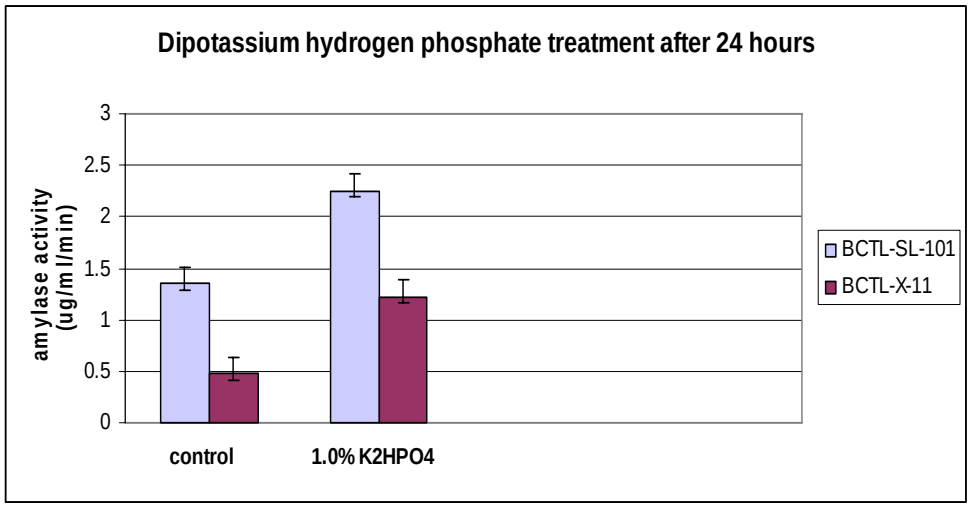

Figure 5: Effect of dipotassium hydrogen phosphate (1%) on amylase production potential by BCTL-SL-101 and BCTL-X-11 after 24 hours incubation.

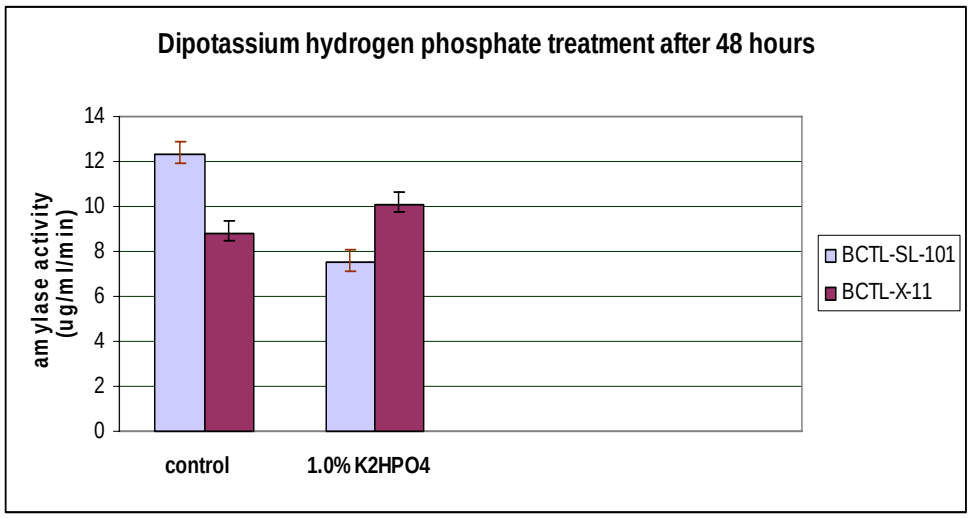

Figure 6: Effect of dipotassium hydrogen phosphate (1%) on amylase production by BCTL-SL-101 and BCTL-X-11 after 48 hours incubation.

Effect of vitamin B1 on amylase production

BCTL-SL-101 and BCTL-X-11 were treated with two concentrations (5 and 10 mg/l) of vitamin B1 (Thiamine HCl). The bacterial isolate BCTL-SL-101 showed 1.35±0.03 and 13.78±0.15 µg/ml/min enzyme activity at 24 and 48 hours respectively, under normal conditions. Following thiamine HCl treatment at 5mg/l and 10mg/l concentrations, the amylase activity decreased in both treatments by both isolates (Figures 7-8). The decrease in amylase activity by BCTL-SL-101 at 5 and 10mg/l concentration was 72 and 18% respectively, after 24 hours while 82 and 75% decrease was observed in 48 hours treatments respectively (Figures 7-8). The bacterial isolate BCTL-X-11 showed 0.45±0.01 and 9.07±0.12 µg/ml/min enzyme activity at 24 and 48 hours, respectively, under normal conditions. Following vitamin B1 treatment at 5mg/l and 10mg/l concentration, the activity decreased in all the cases. The decrease in amylase activity at 5 and 10mg/l was 53% and 33.3% respectively, after 24 hours while 79 and 65% decrease was observed in amylase activity following 48 hours treatment respectively (Figures 7-8). The data shows that vitamin B1 has no enhancing effect on amylase production.

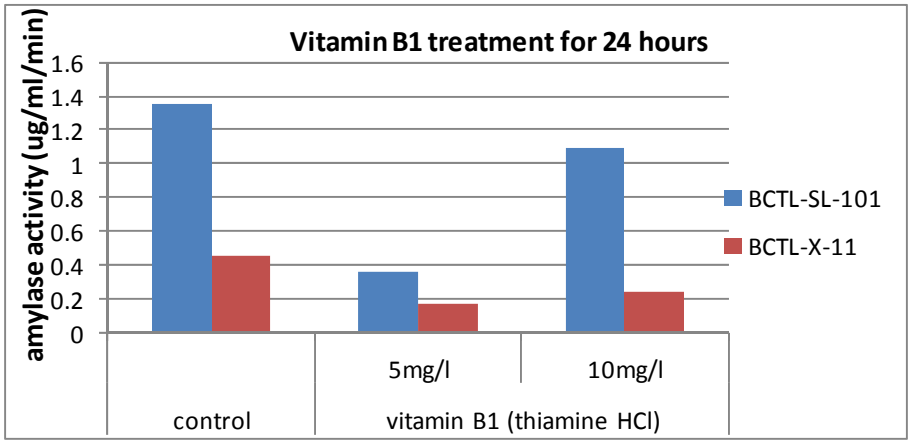

Figure 7: Effect of vitamin B1 (5 and 10mg/l) on amylase production by BCTL-SL-101 and BCTL-X-11 after 24 hours incubation.

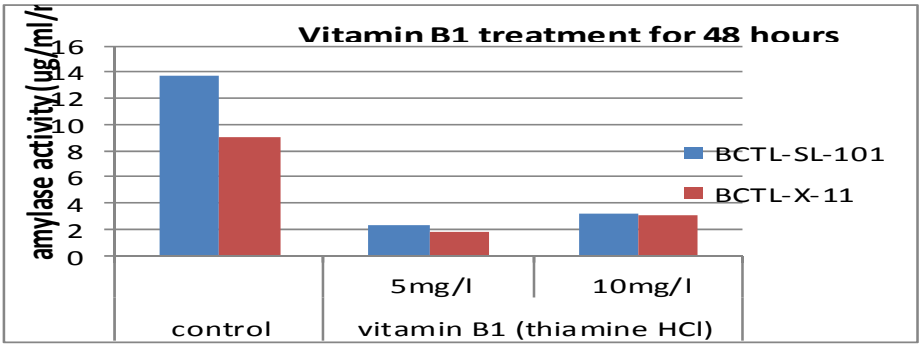

Figure 8: Effect of vitamin B1 (5 and 10mg/l) on amylase production by BCTL-SL-101 and BCTL-X-11 after 48 hours incubation.

DISCUSSION

Though amylases originate from different sources (plants, animals and microorganisms), the microbial amylases are mostly used in industry, due to their productivity, thermostability, suitability over wide pH range and biocompatibility (Shah *et al.*, 1991). As per present study, the effect of vitamin B1, sodium chloride, magnesium sulphate and *di*-potassium hydrogen phosphate was studied on amylase production from BCTL-SL-

101 and BCTL-X-11 bacterial isolates. Sodium chloride showed considerable effect on amylase production. In case of both (BCTL-SL-101 and BCTL-X-11) isolates amylase production increased maximum up to 37.4% and 55% when sodium chloride was added. The results showed that by increasing sodium chloride concentration (within the range used in this study), the activity of amylase was increased significantly.

It has already been reported that salt stress causes series of biological and physiological changes in plants (Greenway and Munns, 1980; Boyer, 1982). Increase in salt concentration affects the biomass and soluble proteins in plants (Lutts *et al.*, 1999). Effect of sodium chloride stress on ammonia assimilation enzymes and the related parameters were determined in the roots of three rice (*Oryza sativa*) varieties differing in salt tolerance (Wei *et al.,* 2004). The enzymes from extremely halophilic bacteria represent a fascinating example of adaptation. These enzymes perform their functions *in vivo* at 4-5M sodium chloride concentration, losing activity rapidly when exposed to low concentration (Lanyi, 1974). It is remarkable that a considerable amylase activity was detected even at 30% salts concentration (Coronado *et al.,* 2000). Present results precisely explain that whenever sodium chloride concentration is increased up to particular threshold level the enzyme activity also increased. After that threshold level the activity started decreasing. In the present study, the amylase activity for BCTL-SL-101 and BCTL-X-11 increased when provided 1% and 2% sodium chloride concentration, but it started getting lower when concentration increased to 3%, but still it is more than that of control which was deficient of sodium chloride. Our findings are in contrast to Karlekar *et al*. (1985) who reported that amylase activity decreased in the presence of NaCl by *Cladosporium sphaerospermum* (mold) and in thermophilic *Bacillus sp.* The amylase production was decreasing continuously by increasing salt concentration (Carvalho *et al*., 2008). The effect of magnesium sulphate stress on amylase production was also observed in this study. The amylase activity decreased (48%) in BCTL-SL-101 isolate at 0.2% concentration while it was increased (25%) in case of BCTL-X-11 as compared with 0.1% concentration in the normal medium which indicated that magnesium sulphate showed prominent effect on amylase activity. Bajpai *et al*. (1992) reported 0.1% magnesium sulphate gives maximum α-amylase production by *Bacillus subtilis.* Vishwanathan (2001) showed that 0.02% magnesium sulphate was best for amylase production by *Aspergillus flavus* in case of banana peel medium.

During this study, the effect of dipotassium hydrogen phosphate was also worked out. The results showed inverse relationship of salt with enzyme activity. The amylase activity decreased by 42% in BCTL-SC-101 while BCTL-X-11 exhibit 13% rise in enzyme activity.

Vitamins play critical roles in increasing enzyme activity by serving mostly as co-enzymes (Klat and Goldman, 1996). The role of vitamin B1 was also tested on amylase production by BCTL-SL-101 and BCTL-X-11 bacterial isolates. Vitamin B1 is a water soluble vitamin and is a part of the B-complex group which is essential for normal metabolism, cardiovascular and nervous system health. The results obtained during this study showed sufficient inhibition (up to 80%) of amylase activity following administration of two doses (5 and 10 mg/l) of vitamin B1. This might be due to higher vitamin doses which are responsible for inhibition of bacterial growth. Further experimentation and studies are required to evaluate the effect of different concentrations of vitamin B1 to detect the range of this vitamin which is required for maximum growth with no inhibitory effect. It has also been shown in various studies that vitamins at lower amounts may cause deficiency diseases in living systems while higher concentration may be toxic and play inhibitory role in metabolic activities.

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