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Isolation and Characterization of Thermotolerant Alkaline Serine Protease of *Bacillus* sp. P-02

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ABSTRACT

Twelve bacterial strains isolated from the soils sample were screened for protease production on skimmed milk agar. Among them, strain P-02 was adjudged as the best protease producer and was identified as *Bacillus* sp. The isolate showed maximum protease production (3768 U mL^{-1}) in the presence of wheat bran and peptone at 60°C, pH 9.0 within 48 h of incubation. The enzyme activity was increased in the presence of Ca^{2+}, Mg^{+2} and Mn^{+2} ions (0.1%). Protease activity was strongly inhibited in the presence of Hg and Fe ions. This isolate may be useful in several industrial applications owing to its thermo-tolerant and alkali-tolerant characteristics.

Key words: Protease, *Bacillus* sp., thermotolerant, alkalophilic

INTRODUCTION

In modern times, the products of biological origin mostly enzymes are attracting the attention of researchers. Their role in several biological and commercial processes has been accordingly emphasized. Among all the enzymes, proteases occupy an important position as they were the first to be produced in huge and now constitute 60-65% of total enzymes employed (Amoozegar *et al*., 2007). Proteases are present in all living organisms like plants and animals but microbial proteases are most exploited group of industrial enzymes. Based on their mode of action, they are further classified into four categories viz. alkaline, acid, thiol and metallo proteases (Rao *et al*., 1998). Since alkaline (serine) proteases are active over a broad pH (7-12) and temperature ranges (35-80°C) (Rao *et al*., 1998), they are world wide core of attraction for researchers. Several bacteria, fungi, yeast and actinomycetes are accomplished with the capacity to produce alkaline serine proteases in various environmental and agro-climatic conditions (Prakasham *et al*., 2005; Germano *et al*., 2003; Haki and Rakshit, 2003). Though, bacterial proteases are preferred because they grow rapidly, require less space, can be easily maintained and are accessible for genetic manipulations (North, 1982).

The significant protease producing bacteria are species of *Bacillus*, *Pseudomonas*, *Halomonas*, *Arthrobacter* and *Serratia*. Among all bacterial species, *Bacilli* play a vital role in production of alkaline protease due to their chemo-organotrophic character (Kumar *et al*., 2012; Kennedy and White, 1984). Several species of *Bacillus* are industrially engaged to produce thermostable alkaline protease as they grow easily under extreme pH and temperature conditions (Naidu and Devi, 2005). The improvement of protease production by genetic manipulation has been well studied in

B. cereus, *B. subtilis*, *B. stearothermophilus*, etc., by a number of researchers which further underlines the significance of this enzyme (Gupta *et al*., 2005). Proteases have several applications, mainly in the detergent, food, leather and pharmaceutical industries (Gupta *et al*., 2005). Throughout rising industrial demands for biocatalysts, that can deal with the industrial processes at harsh conditions, the isolation and characterization of efficient strains are feasible ways to enhance the production of such enzymes. The aim of the present study was to isolate alkalophilic thermotolerant extra-cellular protease from the soil and optimize different physico-chemical and nutritional parameters for maximum protease production.

MATERIALS AND METHODS

Isolation, screening and identification of thermoalkaline protease producing bacteria: The soil samples were collected aseptically from different site of University campus to isolate protease producing bacteria. One gram soil was suspended in 9.0 mL sterile distilled water and 0.1 mL suspension was spread over milk agar plates (pH 9.0) containing skimmed milk 2.0% and agar 2.0% and incubated for 24-48 h at 55±1°C. Bacterial colonies showing clear zones were selected, streaked twice on milk agar plates for purification and maintained as pure culture over nutrient agar slants (pH 9.0, 4°C). The isolate having maximum clearance zone was selected for further studies. The selected bacterial isolate was identified by morphological and biochemical characterization as per the Bergey's Manual of Systematic Bacteriology (Holt, 1984).

Enzyme assay: The proteolytic activity was assayed by casein digestion method of Anson (1938). One milliliter of enzyme was incubated with 3.0 mL of casein (1%, w/v) in 100 mM sodium carbonate-bicarbonate buffer; pH 9) at 55±1°C. The reaction was stopped after 10 min by addition of 3.0 mL of 10% (w/v) Trichloro Acetic Acid (TCA). The mixture was centrifuged at 12,000 rpm (4°C) for 10 min and supernatant used to estimate the amount of free tyrosine as per Lowry *et al*. (1951) using tyrosine as standard. One unit of enzyme activity was defined as the amount of enzyme that liberates 1.0 μg of tyrosine (min mL^{-1}).

Biomass determination: Bacterial cells in broth were harvested by centrifugation (10000 rpm for 10 min at 4°C), washed with distilled water and dried in an oven at 80°C until reaching a constant weight. The biomass was reported in the form of dry cell mass (g L^{-1}).

Optimization of physico-chemical and nutritional parameters for protease production: The various process parameters influencing protease production were optimized individually and independently of the others. Therefore, the optimized conditions were subsequently used in all the experiments in sequential order. For optimization, the basal medium (wheat bran 2.0%, peptone 0.3%, yeast extract 0.2%, $MgSO_4$ 0.5% and pH 9.0) was inoculated and incubated at different temperature viz., 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C under the standard assay conditions. The samples were withdrawn at every 8 h interval upto 72 h to study the effect of incubation period. The influence of pH on the enzyme production was determined by measuring the enzyme activity at varying pH values ranging from 7.0-11.0 at 60ºC using different suitable buffers at concentration of 100 mM phosphate buffer (7.0-8.0), Tris-HCl buffer (pH 8.0-9.0) and glycine-NaOH (10-11.0) under standard assay conditions. The growth medium was supplemented with different carbon sources viz., fructose, sucrose, maltose, starch, glucose, lactose, wheat bran, rice bran, rice husk and maize bran (at the level of 2%, w/v). Different organic nitrogen sources

(beef extract, skimmed milk, casein, malt extract, peptone and yeast extract, 0.3% w/v) and inorganic nitrogen sources (ammonium nitrate, ammonium chloride, potassium nitrate and ammonium sulphate 0.3% w/v) were also used for enzyme production. Thereafter, optimized carbon and nitrogen sources were further optimized at different concentrations. The effect of various metal ions (0.1%) on enzyme activity was also investigated using $FeCl_2$, $CaCl_2$, NaCl, $BaCl_2$, $MgCl_2$, KCl, $ZnCl_2$, $AlCl_3$, $CuSO_4$, NH_4Cl, $CoCl_2$ and $HgCl_2$.

Statistical analysis: The experiments were performed thrice, each in triplicate. Standard deviation for each experimental result was calculated using Microsoft Excel.

RESULTS

Isolation, screening and identification of thermoalkaline protease producing bacterial cultures: Twelve bacterial isolates producing variable proteolytic zones on milk agar plates were isolated from the soil samples. The zones of clearance by isolates reflect their extent of proteolytic activity. Those having clearance zone greater than >1.0 cm were considered as significant. Among 12 bacterial isolates, one exhibited good proteolytic activity which was reassessed by loading their culture broth in the wells on milk agar plate (pH 9.0). The culture broth of good protease producers cleared more than >1.0 cm zone within 3-4 h of incubation at 55±1°C, thereby indicating an extra-cellular nature of the proteolytic. The isolate, showing maximum clearance zone diameter was selected for further studies.

The efficient strain P-2 was rod-shaped, gram-positive, aerobe and facultative, motile with positive Voges Proskaver, catalase and oxidase test. It grew over a wide range of pH (5-11), temperature (25-90°C), NaCl concentration (0.0-12%) and was able to hydrolyze gelatin, esculin, starch, tween-20 and 40 and produce acid from glucose. The strain was halotolerant as it grew in the presence of 0.0-12% NaCl (Table 1). On account of morphological and biochemical characteristics, it was identified as *Bacillus* sp.

Table 1: Morphological, physiological and biochemical characteristics of the selected P-02 isolate

Characteristics	Results	Characteristics	Results
Morphological tests		Gelatin hydrolysis	+
Gram's reaction	+	Esculin hydrolysis	+
Shape	Cylindrical	Starch hydrolysis	+
Motility	+	Urea hydrolysis	_
Physiological tests		Nitrate reduction	_
Growth on NaCl (%) 0-12.0	+	Ornithine decarboxylase	_
Growth at pH 5.0-11.0	+	Lysin decarboxylase	_
Growth at temperature 25-90°C	+	Catalase test	+
Growth under anaerobic condition	+	Oxidase test	+
Biochemical tests		Arginin decarboxylas	_
Growth on MacConkey agar	_	Tween 20 hydrolysis	+
Indole test	_	Tween 40 hydrolysis	+
Methyl red test	_	Gas production from glucose	_
Voges Proskauer test	+	Acid production from	
Citrate utilization	_	Dextrose	+
H_2S production	_	Lactose	_

+: Positive, -: Negative

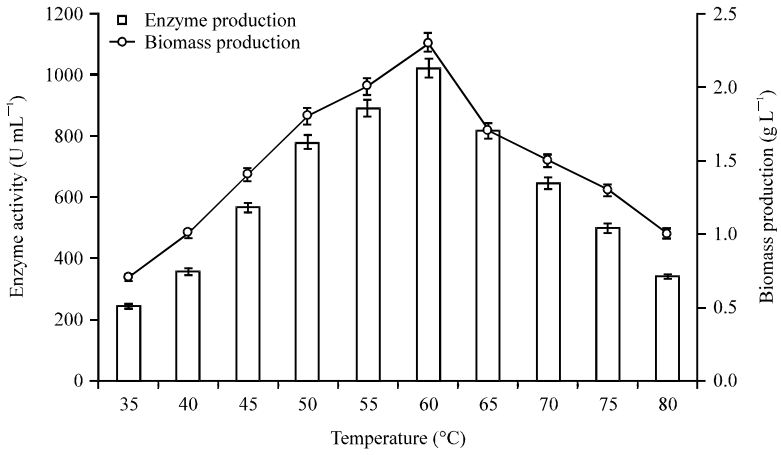

Fig. 1: Effect of temperature on protease production. The flasks inoculated with culture in the medium were incubated at different temperature (35-80°C) for 48 h at pH 9.0. For enzyme activity, reaction mixture was incubated at 55°C for 15 min and reaction was conducted as standard assay method. Error bars presented Mean±Standard deviation of triplicates of three independent experiments

Effect of temperature on protease production: The influence of temperature on protease production and biomass production were studied by varying the incubation temperature from 35-80°C. Protease producing *Bacillus* sp. was active at all temperatures employed with maximum protease production at 45-75°C. At 60°C, it exhibited maximum protease production (1020 U mL^{-1}) with biomass production (2.3 g L^{-1}) (Fig. 1). The remarkable protease production in the temperature range of 45-75ºC revealed thermotolerant as well as thermophilic nature of the strain as well as enzyme. Further, increase in temperature enzyme production and biomass production was slightly decreased.

Effect of pH on protease production: The influence of pH on protease production and biomass production were studied by varying the pH from 7-11 while other parameters were maintained constant. From Fig. 2, it was observed that maximum protease production (1234.5 U mL^{-1}) was achieved at pH 9.0 with 2.3 g L^{-1} of biomass development. Beyond pH 9.0, decrease in protease production and biomass production were observed. It appeared that more acidic pH (below 9.0) has a detrimental effect on protease production and minimum activity was found at pH 4.0.

Effect of incubation time on protease production: Time course of protease production was studied along with the growth of the bacterium. With increase in time, there was increase in cell mass, increased protease production and biomass production. Maximum protease production (1156 U mL^{-1}) was attained in 24 h with 2.2 g L^{-1} biomass production (Fig. 3). Further increase in the incubation period did not increase the protease production, however, increase in biomass development was recorded.

Effect of carbon sources on protease production: Protease production was monitored by using different carbon sources such as glucose, maltose, fructose, sucrose, lactose, starch, wheat bran, rice bran, maize bran and rice husk at a concentration of 2.0% (w/v). Wheat bran was found to be the

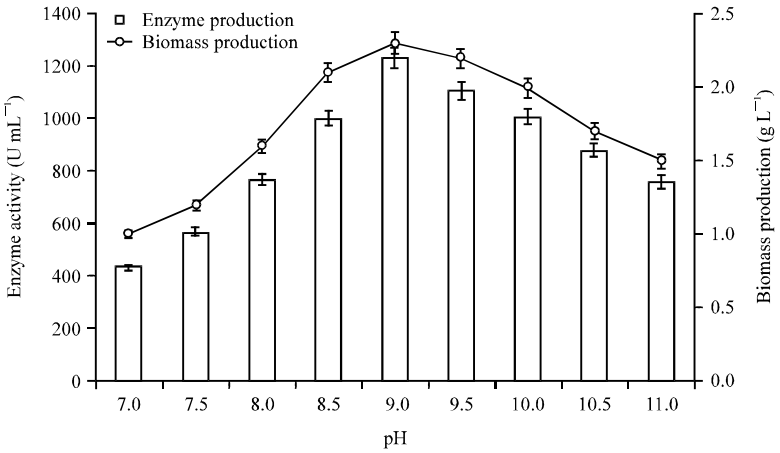

Fig. 2: Effect of pH on protease production. The flasks inoculated with culture were incubated at different pH (7-11) for 48 h at 60°C. For enzyme activity the reaction was assayed at respective pH with buffers (100 mM) at 60°C for 15 min. Error bars presented Mean±Standard deviation of triplicates of three independent experiments

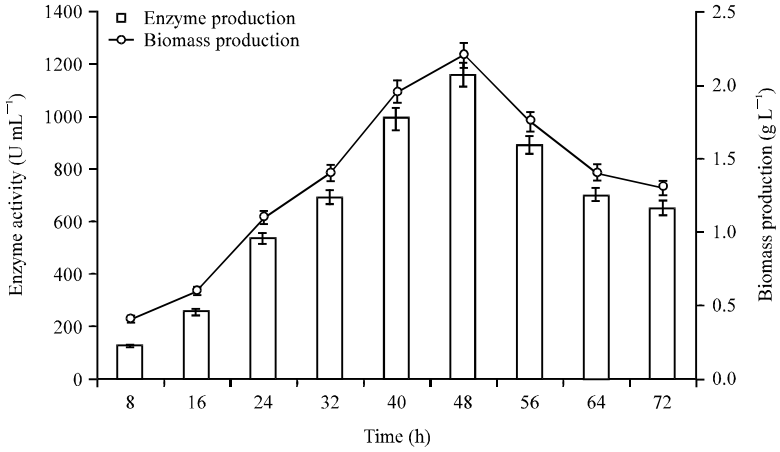

Fig. 3: Effect of incubation periods on protease production. The flasks inoculated with culture were incubated at different incubation periods (8-72 h) at initial pH 9.0, 60°C. For enzyme activity, the reaction was assayed at respective incubation periods at 60°C for 15 min. Error bars presented Mean±Standard deviation of triplicates of three independent experiments

best carbon source, allowing maximum protease production (1489.5 U mL^{-1}) with 2.5 g L^{-1} biomass production (Fig. 4). Rice bran, maize bran, rice husk, lactose and sucrose were found to be fairly good substrates allowing average protease production. Whereas, maltose, fructose, starch and glucose were relatively poor substrates showed inhibitory effects on protease production.

Effect of nitrogen sources on protease production: The influence of various organic and inorganic nitrogen sources (0.5%, w/v) on protease production and biomass production were also investigated for 48 h of incubation. It was observed that except ammonium nitrate, potassium

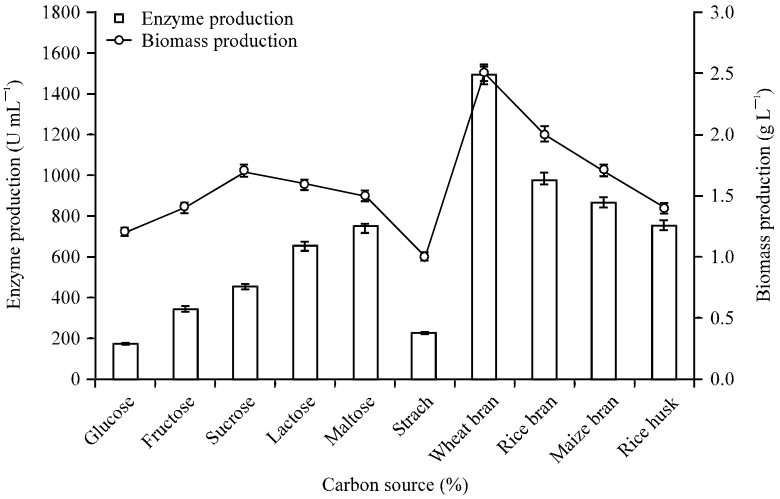

Fig. 4: Effect of different carbon sources on protease production. Test flasks contained different carbon sources in the medium at a level of 2% (w/v). The flasks were inoculated with culture and incubated at 60°C for 48 h at pH 9.0. Error bars presented are Mean±Standard deviation of triplicates of three independent experiments

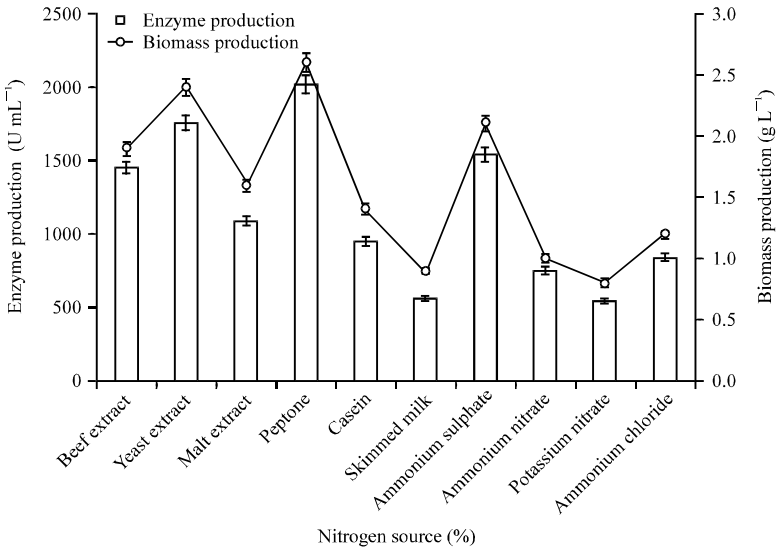

Fig. 5: Effect of different nitrogen sources on protease production. Test flasks contained different nitrogen sources in the medium at a level of 0.5% (w/v). The flasks were inoculated with culture and incubated at 60°C for 48 h at pH 9.0. Error bars presented are Mean±Standard deviation of triplicates of three independent experiments

nitrate and skimmed milk in presence of other nitrogen sources enhanced the protease production alongwith biomass production. Peptone was found to be the best nitrogen source and showed higher protease production (2020 U mL^{-1}) with 2.6 g L^{-1} biomass production (Fig. 5).

Effect of metal ions on protease production: The influence of various metal ions (0.1%, w/v) on protease production and biomass production was also investigated for 48 h of incubation. It was

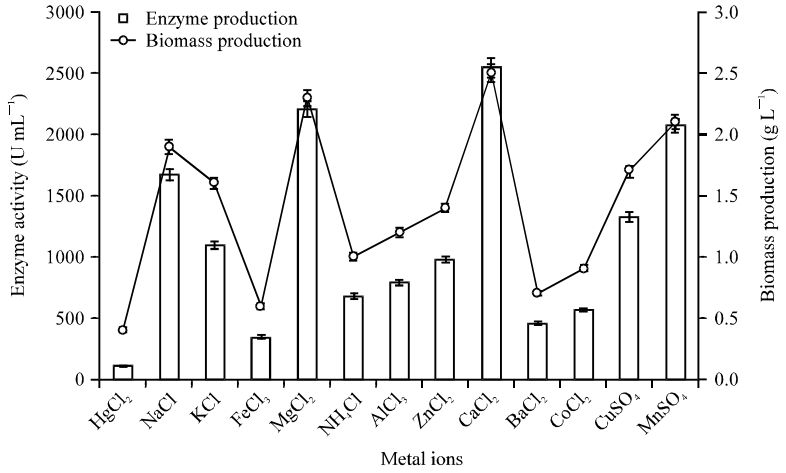

Fig. 6: Effective of metal ion on protease production. The flasks contained different metal ions is the medium at a level of 0.1% (w/v). The flasks were inoculated with culture and incubated at 60°C for 48 h at pH 9.0. Error bars presented Mean±Standard deviation of triplicates of three independent experiments

observed that $CaCl_2$ enhanced the protease production (2547 U mL^{-1}) alongwith biomass production (2.6 g L^{-1}) followed by $MnCl_2$ and $MgCl_2$. $HgCl_2$ and $FeCl_2$ inhibitory effect on protease production when compared with others (Fig. 6).

DISCUSSION

To study the effect of various temperatures on the growth and alkaline protease production, different temperatures (35-80°C) were used in the study. Protease production at different temperatures was examined keeping other conditions constant, the results were depicted in Fig. 1. It was noted that high protease production (1020 U mL^{-1}) with 2.3 g L^{-1} biomass production was achieved at 60°C from *Bacillus* sp. that indicating the thermophilic nature of the enzyme (Fig. 1). It was capable of producing protease in the range of 35-75°C. However, increase in temperature beyond 75°C led to decline in enzyme production. Nadeem *et al.* (2007) also reported that *Bacillus lichniformis* N-2 showed maximum protease production at 60°C.

The initial pH of the culture strongly affects enzymatic processes and transport of compounds across the cell membrane. Maximum protease production (1234.5 U mL^{-1}) with 2.3 g L^{-1} biomass production was achieved at pH-9.0 by *Bacillus* sp. (Fig. 2). The enzyme production was increased as pH of the medium was increased reaching maximum at pH-9.0 and above this pH enzyme production was strongly decreased. Results suggest that there is a stimulation of enzyme production at alkaline pH. The obtained results coincide with several earlier reports, that protease production is high at pH-9 for *Bacillus* sp. (Adinarayana *et al.*, 2003; Joo *et al.*, 2004; Swamy *et al.*, 2012).

Incubation time plays a substantial role in the enzyme production. Enzyme synthesis is related to cell growth and therefore, there is a co-relation between incubation period and enzyme production. In this experiment, maximum protease production (1156 U mL^{-1}) with 2.2 g L^{-1} biomass production from the *Bacillus* sp. was observed at 48 h (Fig. 3). Similar results also reported by Qadar *et al.* (2009) from *Bacillus* sp. showed maximum protease production in 48 h, whereas, Naidue and Devi (2005) and Gibb and Strohl (1988) reported that *B. subtilis* 3411 and *B. subtilis* K-30 gave maximum production in 72 h and 96 h, respectively. Under most growth conditions,

Bacillus sp. produce extra-cellular protease during the post exponential growth phase (Dawson and Kurz, 1969). Further incubation resulted in a sharp decline in the enzyme production. This decline might be due to cessation of enzyme synthesis together with auto proteolysis. Similar finding was also reported in which maximum enzyme production was observed at 48 h of growth.

The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzyme (Saxena *et al.*, 2007). Among the carbon sources, wheat bran was found to support maximum protease production (1489.5 U mL^{-1}) with 2.5 g L^{-1} biomass production by *Bacillus* sp. (Fig. 4). It was reported earlier that addition of starch to the culture medium induced protease synthesis and that production was dependent on the starch type (Feng *et al.*, 2001). In contrast, other sugars such as glucose, fructose, maltose and lactose were reduced enzyme production although growth was observed optimal. This negative effect of glucose on protease production is attributed to catabolite repression (Kaur *et al.*, 2001; Mehta *et al.*, 2006) and was similar to that observed for *Bacillus horikoshii* as reported previously (Joo *et al.*, 2003). In some *Bacillus* strains such as *Bacillus subtilis* NS (Nisha and Divakaran, 2014), *Bacillus* sp. AR-009 (Gessesse and Gashe, 1997) and *B. licheniformis* ATCC 21415 (Mabrouk *et al.*, 1999), however, enhanced protease yields were reported on supplementation of glucose and lactose.

The effect of various nitrogen sources in the form of organic or inorganic on alkaline protease production was also studied to discriminate the suitable nitrogen source as the requirement for particular nitrogen source differs from organism to organism (Kumar and Takagi, 1999). In this experiment maximum protease production (2020 U mL^{-1}) with 2.6 g L^{-1} biomass production was observed in the presence of peptone followed by yeast extract (Fig. 5). Similarly, Qadar *et al.* (2009) also reported that maximum enzyme production was observed in the presence of peptone followed by yeast extract from *Bacillus* sp. Whereas, Nadeem *et al.* (2007) reported maximum enzyme production in the presence of yeast extract followed by peptone. No significant outcome was observed on protease production when combinations of various organic nitrogen sources were used. From results, it was cleared that single organic nitrogen source was most appropriate than using them in combinations. Hossain *et al.* (2007) also reported that mixture of nitrogen sources were not valuable to enhance the production of protease by *B. licheniformis* MZK-3. In contrast to our results, Nejad *et al.* (2009) reported that a mixture of peptone and yeast extract were the best nitrogen sources for protease production by *B. licheniformis* BBRC 100053. In case of inorganic nitrogen sources, maximum enzyme production was attained in the presence of ammonium sulphate while minimum production was observed in case of ammonium nitrate and potassium nitrate, respectively (Fig. 5). Similar results were also reported by Sarker *et al.* (2013) from *Bacillus licheniformis* P003. However, Bhunia *et al.* (2010) found maximum production in case of sodium nitrate at a concentration of 0.5% followed by potassium nitrate, ammonium chloride, ammonium nitrate and ammonium sulfate respectively. Several researchers have also reported that organic nitrogen sources were found better for enzyme production than inorganic (Feng *et al.*, 2001; Joo *et al.*, 2003).

The effect of various metal ions on protease production was evaluated. Among these ions, Ca^{+2} increases protease production (2547 U mL^{-1}) with 2.6 g L^{-1} biomass production followed by Mn^{+2} and Mg^{+2} and rest metal ions showed inhibitory effect on the protease production (Fig. 6). Qadar *et al.* (2009) and Nadeem *et al.* (2007) also reported that protease produced by *Bacillus* spp. was enhanced by Ca^{+2}, Mn^{+2} and Mg^{+2}. These metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature (Johnvesly and Naik, 2001). In addition, protease required a divalent cation like Ca^{+2} and Mn^{+2} or combination of these

cations for its maximum activity (Beg and Gupta, 2003). Selection of effective ions and their appropriate concentrations for balanced thermo-stability to the enzymes are most essential for their applications at industrial levels.

CONCLUSION

A novel thermotolerant strain of *Bacillus* sp. was isolated to enhance production of alkaline protease from soil sample. The isolate was able to produce higher protease in broad temperature and pH range. Maximum protease production (2547 U mL^{-1}) with 2.6 g L^{-1} biomass production was obtained within 48 h in the presence of wheat bran, peptone and $CaCl_2$. The potential of this strain to produce higher protease production in the presence of cheap carbon source and short period promises its candidature for the industrial applications.

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