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Full Length Research Paper

Production of extracellular protease in submerged fermentation by *Bacillus licheniformis* ATCC 12759

Nurullah Akcan

Health High School, Siirt University, TR-56100 Siirt – TURKEY. E-mail: nurullah.akcan@gmail.com.
Tel: +90 484 223 10 56. Fax:+90 484 223 51 56.

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The various nutrients belonging to carbon, nitrogen and amino acid sources, were investigated in terms of their effect on the production of extracellular protease by *Bacillus licheniformis* ATCC 12759. Comparison with the control in media containing all the simple sugars resulted in a decrease in proteolytic activity, while there was significant increase in enzyme yield in the case of the supplementation complex carbon source such as wheat flour and rice flour. Urea and sodium nitrate were the best organic and inorganic nitrogen sources, respectively. Among the amino acid sources tested, L-phenylalanine, L-cysteine, glycine and L-valine favored the production, respectively. $FeSO_4$, $ZnSO_4$ and $CuSO_4$ completely repressed protease production. Maximum protease production (10738.2±44.2 U/mg) was obtained in a medium containing 0.1% $MgSO_4$ in 24 h 37°C.

Keywords: *Bacillus licheniformis* ATCC 12759, protease, submerged fermentation, enzyme production

INTRODUCTION

Proteases constitute one of the most important groups of enzymes both industrially and academically (Subba et al., 2009). Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. For economic production of protease, microbes can be the best choice. They can be cultured in large quantities in a relatively short time by established methods of fermentation and they also produce an abundant, regular supply of the desired product (Gupta et al., 2002).Proteases are important from an industrial perspective due to their wide scale applications in the detergents, food, pharmaceuticals, chemicals, leather, paper and pulp and silk industries (Krik et al., 2002; Mukherjee et al., 2008). Owing to the better cleansing properties of protease based detergents and pollution-alleviating capacity over conventional synthetic detergents, alkaline proteases have made their way as key-ingredients in detergent formulations (Krik et al., 2002; Mukherjee et al., 2008; Rai and Mukherjee, 2009). In addition, their functional and thermal stability of protein chemistry and protein engineering are the most important parameters to be investigated to understand their utility in different sectors (Subba Rao et al., 2009). As per the forecast, the global demand for enzymes will rise 7% per annum through 2006 to $6 billion 2011 (McCoy, 2000). Proteases represent one of the major groups industrial enzymes, because of their widespread use in detergents and dairy industry and industrial sales of protease are estimated at more than $350 million annually (Kirk et al., 2002; Kumar et al., 2008). This increasing economic importance has directed the research community to isolate hyperactive strains for the production of novel proteases and subsequent optimisation of the various fermentation parameters for maximising enzyme production (Prakasham et al., 2005, 2006, 2007; Subba Rao et al., 2008). Several microbial strains such as *Bacillus alcalophilus, Bacillus subtilis, Bacillus licheniformis* and *Bacillus lentus* have been isolated from exotic environments to screen their importance at industrial scale with specific catalytic role (Johnvesly and Naik, 2001; Gupta et al., 2002; Prakasham et al., 2005; Subba Rao et al., 2009).The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry (Gupta et al., 2002).

Abbreviations: SmF, Submerged fermentation; **SD,** standard deviation.

Conventionally, commercial production of proteases has been carried out using submerged fermentation (SmF). SmFs are usually carried out with a substrate, which is either dissolved or remains suspended in an aqueous medium (Sumantha et al., 2006). Almost all the large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling (Singhania et al., 2010). To meet the growing demands in the industry, it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production (Gangadharan et al., 2008). Extracellular protease production in microorganisms is also strongly influenced by media components, for example, variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose and metal ions (Varela et al., 1996; Beg et al., 2002). Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium. Besides these, several other physical factors, such as aeration, inoculum density, pH, temperature and incubation, also affect the amount of protease produced (Hameed et al., 1999; Puri et al., 2002; Gupta et al., 2002; Saxena and Singh, 2010).

In commercial practice, the optimization of medium composition is done to balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly toward evaluating the effect of various carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the medium and optimization of environmental and parameters such as pH, temperature, aeration and agitation (Chu, 2007). In addition, no defined medium has been established for the production of proteases from different microbial sources. The present study describes the effects of culture conditions on the production of protease by *B. licheniformis* ATCC 12759.

MATERIALS AND METHODS

Microorganisms and production of protease

Protease producing *B. licheniformis* ATCC 12759 which was procured from MicroBioLogics, Inc. was used as biological material. *B. licheniformis* ATCC 12759 was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to to Laura broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCl, (w/v), pH 7.0). One hundred milliliters of LB was inoculated with 1mL of the inoculum ($3x10^6$ cells=mL) and was incubated at 37°C for 24 h. After incubation, the crude enzyme was obtained by centrifugation of the culture broth at 10,000xg for 10 min at 4°C. The cell-free supernatant containing the enzyme was assayed for protease activity.

Enzyme assay

Protease activity was determined using sulphanilamide azocasein according to the method of Leighton et al. (1973). The reaction mixture containing 250 µl 1% (w/v) substrate in 0.1M Tris/HCl buffer (pH 9.0) and 150 µl of enzyme solution was incubated for 30 min at 37°C. After incubation, the enzyme was inactivated by addition of 1.2 ml trichloroacetic acid solution (10%, v/v) and then the solution was neutralized using 800 µl of 1.8 N NaOH solution. The absorbance was read at 420 nm. One unit of proteolytic enzyme activity was defined as the amount of azocasein that hydrolyzed during 30 min incubation at 37°C for millilitre of solution of extract. All experiments were conducted in triplicate and the mean at three with standard deviation (SD) was represented.

Assay of protein concentration

The protein concentration was determined by the Lowry method by using bovine serum albumin as standard (Lowry et al., 1951).

Effect of incubation period

The effect of incubation period was determined by incubating production medium for different incubation periods (12, 24, 48, 72, 96, and 120 h) at 37°C taking other conditions into consideration.

Effect of carbon sources

Different carbon sources such as soluble starch, wheat starch, potato starch, corn starch, wheat flour, rice flour, corn flour, soy flour, mannose, xylose, lactose, galactose, arabinose, glucose, sucrose, and fructose were employed to find the suitable carbon source for protease production by *B. licheniformis* ATCC 12759. All these sources were studied at 1% (by mass per volume) initial concentrations.

Effect of nitrogen sources

Two categories, viz. organic nitrogen sources and inorganic nitrogen sources were employed. The growth medium was initially supplemented with different organic nitrogen sources, that is, yeast extract, tryptone, beef extract, peptone, casein, urea each at 1% (by mass per volume). Among the inorganic nitrogen sources, ammonium nitrate, ammonium chloride, ammonium sulphate and sodium nitrate again at 1% (by mass per volume) were tested.

Effect of amino acid sources

This experiments was carried out in order to investigate the effect of different amino acids on protease production. Different amino acids (glycine, L-lysine, L-tyrosine, L-cysteine, glutamic acid, L-alanine, L-phenylalanine, L-isoleusine, L-valine, L-tryptophane and L-metionine) were added into production medium at 0.01% (by mass per volume).

Effect of metal salts

The effect of metal salts on protease production is determined by adding different metal salts in the fermentation medium. The metal salts selected for the present study are $FeSO_4$, $MgSO_4$, $CaCl_2$, $CuSO_4$, and $ZnSO_4$, at 0.1% concentration.

RESULTS AND DISCUSSION

In world market, fully half of the enzymes produced are

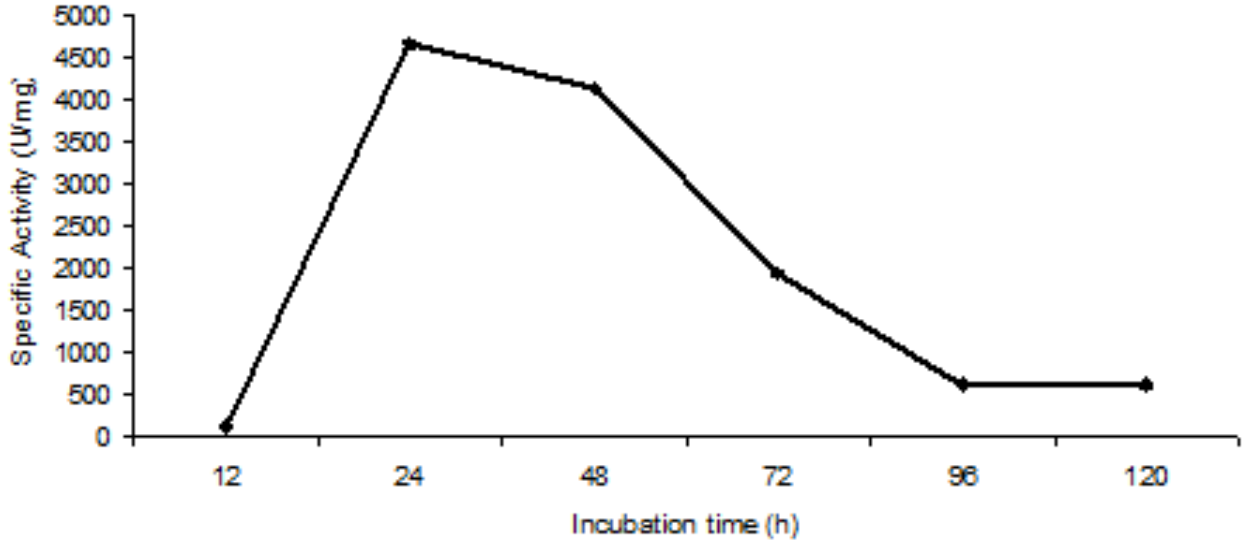

Figure 1. Effect of incubation time on the production of protease from *Bacillus licheniformis* ATCC 12759.

generated by *Bacillus* species, and approximately 60% of these are proteases (Reddy et al., 2008). *Bacillus* genus gained importance because of extracellular enzyme production under submerged fermentation conditions. There is no general medium for protease production by different microbial strains (Pandey et al., 2000). Every microorganism evidences its own idiosyncratic physico-chemical and nutritional requirements for growth and enzyme secretion (Reddy et al., 2008).

Most proteases produced by bacterial cells are extracellular in nature and help in the processing of complex proteinaceous compounds present in the fermentation medium in order to provide the amino compounds required by growing cells. These growth-promoting metabolites are produced during the exponential phase of the growth curve (Subba et al., 2009). The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme production. At different time courses, the production of protease is shown in Figure 1. The results indict that *B. licheniformis* ATCC 12759 exhibited its maximum ability to biosynthesize protease was 24 h (4641.2±43.8 U/mg). This result is in complete accordance with the finding of many investigators (Abdul-Raouf, 1990; Johnvesly et al., 2002; El-Hadj-Ali et al., 2007). However, Olajuyigbe and Ajele (2008) found that a high level of extracellular protease from *B. licheniformis* Lbbl-11 was observed after 48 h incubation. A prolonged incubation time beyond this period did not increase the enzyme yield. The reduction in protease yield after the optimum period was probably due to depletion of nutrients available to microorganism (Sandhya et al., 2005).

The nature and amount of carbon source in culture media is important for the growth and production of extracellular protease in bacteria. Protease production was tested in fermentation medium containing different carbon source at a 1% concentration. As shown in Figure 2, comparison with the control in media containing the all simple sugars resulted in a decrease in proteolytic activity. This may be attributed to the repressive effect of readily metabolizable sugars on the mechanism of protease production (Elibol and Moreira, 2005). In addition, glucose completely repressed protease production. Similar to our results, several studies have reported that proteins and peptides are necessary for protease production, while glucose repressed protease formation (El-Hadj-Ali et al., 2007; Chen et al., 2004; Akcan and Uyar, 2010). However, other works have reported better protease synthesis in the presence of glucose as carbon source (Prakasham et al., 2006; Nadeem et al., 2008; Mukherjee et al., 2008). These results show that the addition of readily matabolizable sugars in the medium was no sufficient to stimulate the proteases production from *B. licheniformis* ATCC 12759.

Cheaper sources of both carbon and nitrogen sources are the key attraction for commercialization of the production processes and thus, ability of the microbial agent to grow and produce enzymes using these sources has been arguably a point of interest (Patel et al., 2005). In Table 1, comparison with the control (4695.7±15.1 U/mg), there was significant increase in enzyme yield in the case of the supplementation wheat flour (6102.2±65.5 U/mg) and rice flour (5874.3±20.3 U/mg) such as complex carbon sources. Similar effect of wheat flour on protease production by *Bacillus* sp. and *Bacillus licheniformis* N-2 have been observed in earlier investigations (Patel et al., 2005; Nadeem et al., 2008).

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components.

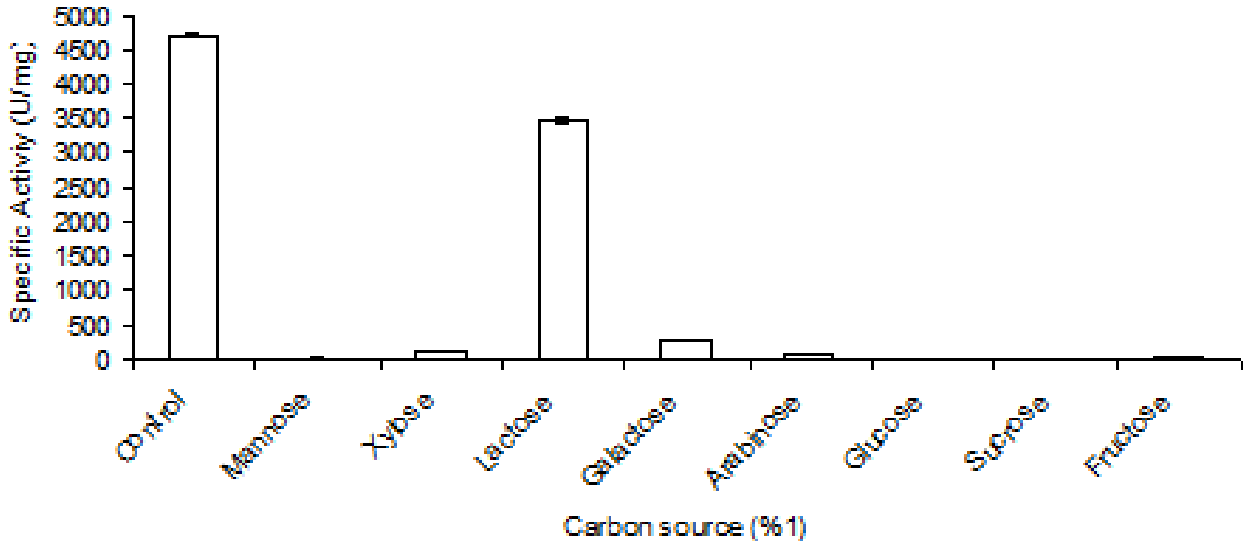

Figure 2. Effect of simple sugars on the production of protease from *Bacillus licheniformis* ATCC 12759.

Table 1. Effect of complex carbon sources on the production of protease from *Bacillus licheniformis* ATCC 12759.

Complex carbon source (1%, w/v)	Specific Activity (U/mg)
Control	4695.7±15.1
Soluble starch	804.1±56.9
Wheat starch	640.3±39.6
Patato starch	1426.5±60.9
Corn starch	805.6±71.2
Wheat flour	6102.2±65.5
Rice flour	5874.3±20.3
Corn flour	943.6±84.9
Soy flour	4827.0±65.6

Although, complex nitrogen sources are usually used for alkaline protease production, the requirement for a specific nitrogen supplement differs from organism to organism (Kumar and Takagi, 1999). Considering these facts, the effect of different nitrogen sources in the form of organic and inorganic on protease production and growth of *Bacillus licheniformis* ATCC 12759 was studied to investigate the suitable nitrogen source. Organic nitrogen sources at initial concentration of 1% (by mass per volume) showed significant effect on protease yield (Figure 3). Comparison with the control (4695.7±15.1) in media containing urea was found the most suitable source for protease production. Many other researchers have also reported that organic nitrogen sources are suitable for protease production (El-Hadj-Ali et al., 2007; Nadeem et al., 2008; Mukherjee et al., 2008). However, Nadeem et al. (2008) found that the addition of urea repressed the enzyme biosynthesis as well as the growth of *B. licheniformis* N-2. On the other hand, the protease production decreased dramatically when tryptone, yeast extract and peptone was used. Similar to our results, several studies have reported that some organic nitrogen sources are repressed protease production (Nilegaonkar et al., 2002; Nadeem et al., 2008; Lazim et al., 2009). Among the different inorganic nitrogen sources tested, sodium nitrate supported maximum protease synthesis (8307.0±84.8 U/mg). Yang et al. (2000) also found that protease production by *B. subtilis* Y-108 was repressed by most of the nitrogen sources used with the exception of sodium nitrate which enhanced protease production. Medium containing ammonium nitrate repressed completely protease production. Conversely, both ammonium chloride and ammonium sulphate decreased protease production. A similar result was reported by other authors (Joo et al., 2002; Nadeem et al., 2008; Lazim et al., 2009). All these findings suggest that the level of protease production by *B. licheniformis* ATCC 12759 is highly responsive to the nature of nitrogen sources.

investigation gave stimulatory effects concerning Four amino acids (L-phenylalanine, L-cysteine, glycine

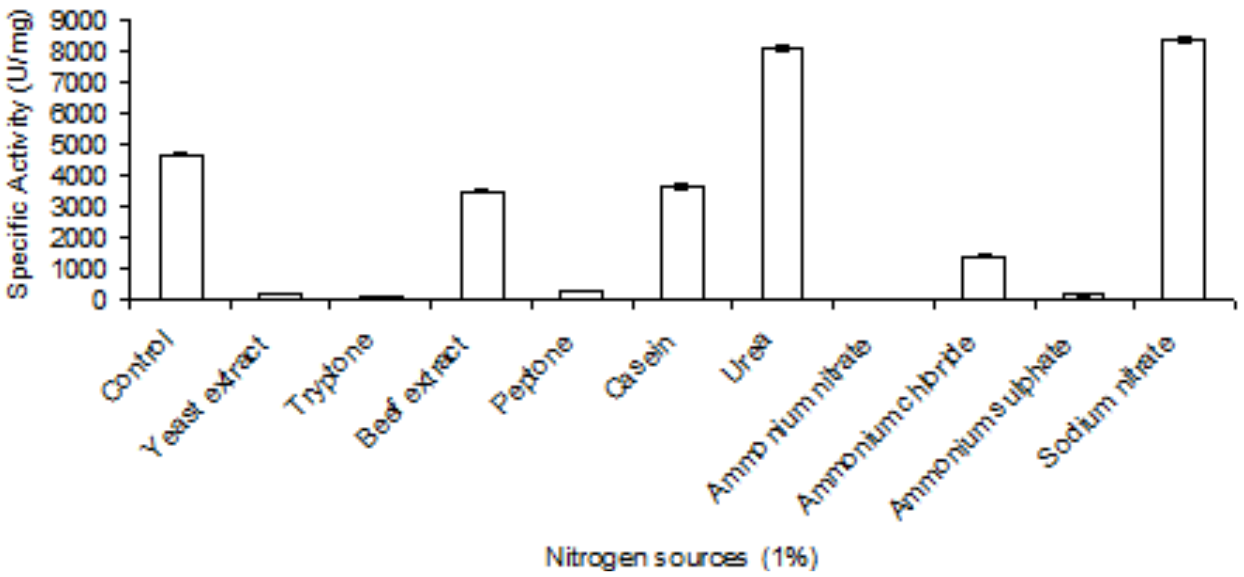

Figure 3. Effect of nitrogen sources on the production of protease from *Bacillus licheniformis* ATCC 12759.

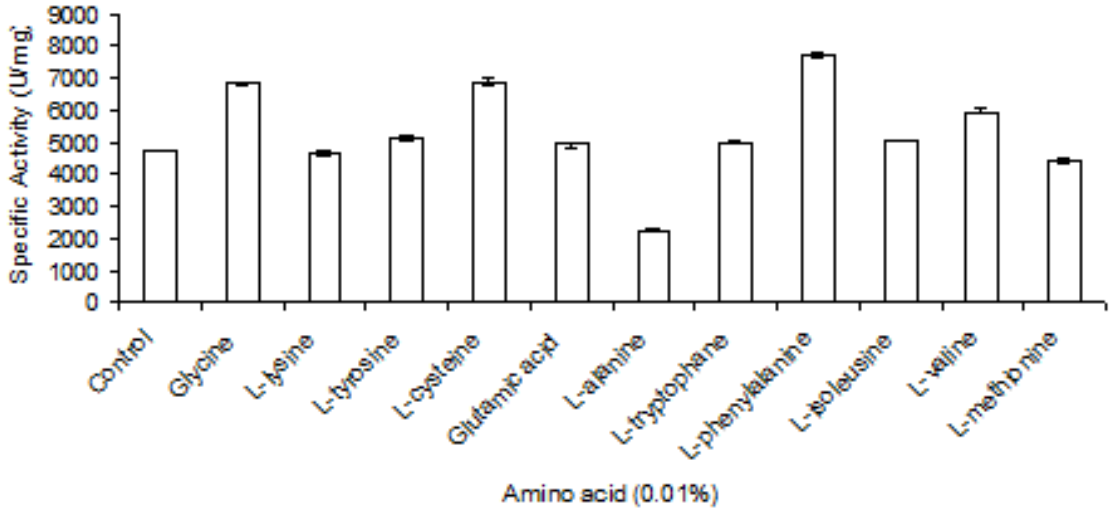

Figure 4. Effect of amino acid sources on the production of protease from *Bacillus licheniformis* ATCC 12759.

and L-valine) out of the twelve amino acids under protease production in comparison to the control and other amino acids under investigation (Figure 4). El-Safey and Abdul-Raouf (2004) reported that valine was found to enhance the protease production from *B. subtilis*. Addition of certain amino compounds were shown to be effective in the production of extracellular enzymes by alkalophilic *Bacillus* sp. (Ikura and Horikoshi, 1987). However, some amino acids appeared inhibitory effects on protease production (Ong and Gaucher, 1976; Chudsama et al., 2010).

In order to investigate the effect of metal salts on proteases production, these salts were individually added to a main medium at 0.1%. The result of the impact of metal salts on enzyme production is shown in Figure 5. The proteases activities were increased when production medium was supplemented with $CaCl_2$ and significant improvement in proteases yield was observed with supplementation of $MgSO_4$. This indicated that Mg^{2+} and Ca^{2+} were necessary for enzyme induction and/or enzyme stabilisation (Ghorbel-Frikha et al., 2005; Nilegaonkar et al., 2007; Chudsama et al., 2010). Positive effects of metal cations including Mg^{2+} and Ca^{2+} on protease stability have also been demonstrated by other authors (Johnvesly and Naik, 2001; Kumar, 2002). However, $FeSO_4$, $CuSO_4$, and $ZnSO_4$ completely inhibited protease

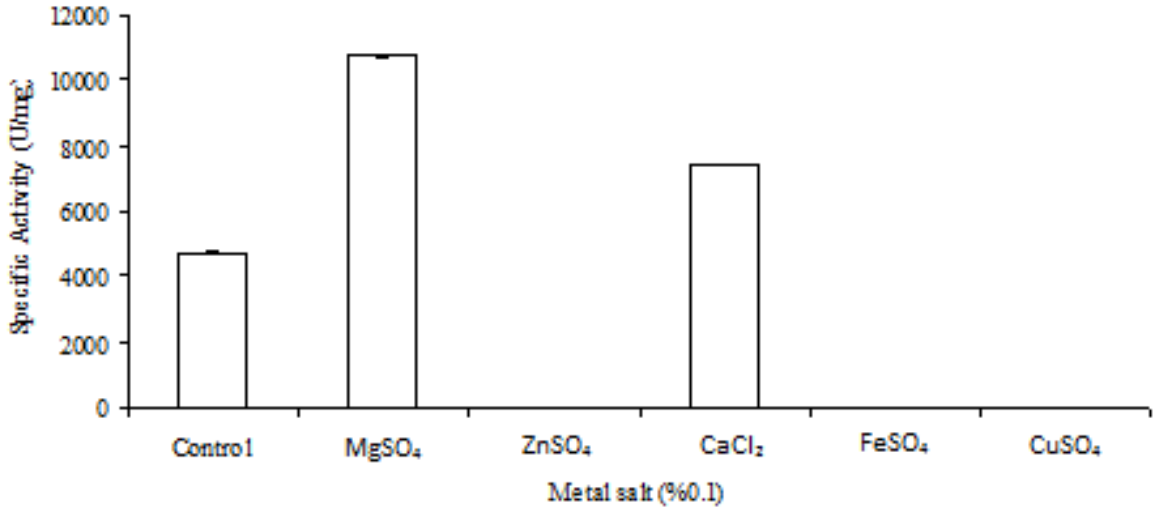

Figure 5. Effect of metal salts sources on the production of protease from *Bacillus licheniformis* ATCC 12759.

production.

The results obtained in this study showed that there is appreciable high production. This suggests that *B. lichenisformis* ATCC 12759 can be a potential producer of extracellular protease which could find applications in the industry and biotechnology. The enzyme thus produced is presently under optimization. Due to the importance of these findings, further studies will be carried on in order to commercialize the production process after necessary optimization for enhanced enzyme production.

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