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Full Length Research Paper

Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil

K. Kalaiarasi* and P. U. Sunitha

Department of Textiles and Clothing, Avinashilingam University for Women, Coimbatore 641 043, Tamil Nadu, India.

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A protease producing bacteria was isolated from meat waste contaminated soil and identified as *Pseudomonas fluorescens.* Optimization of the fermentation medium for maximum protease production was carried out. The culture conditions like inoculum concentration, incubation time, pH, temperature, carbon sources, nitrogen sources and metal ions were optimized. The optimum conditions found for protease production was 37°C at pH 9, with 2% inoculum in the medium for 24 h. Wheat bran and peptone stimulates the production of protease. Magnesium sulphate has less inhibitory effect among the metal ions tested.

Key words: Alkaline protease, casein agar, meat waste contaminated soil, *Pseudomonas fluorescens.*

INTRODUCTION

Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications (Mohen et al., 2005). They constitute two thirds of the total enzymes used in various industries and it account for at least a quarter of the total global enzyme production (Kumar et al., 2002). These enzymes occupy a pivotal position due to their wide application in food processing (Pastor et al., 2001), pharmaceutical industries (Anwar and Saleemuddin, 1998; Gupta et al., 2002), meat tenderization process (Takagi et al., 1992; Wilson et al., 1992), peptide synthesis (Kumar and Hiroshi, 1999), infant formula preparation (American Academy of Pediatrics Committee on Nutrition, 1989), leather processing (George et al., 1995) and in weaving processing (Helmann, 1995). Proteases are complex multienzyme system which catalyses the hydrolysis of amide bond in a protein molecule hence it has been used in the field of textile processing for degumming of silk and processing of wool (Ravel and Banerjee, 2003; Adinarayana et al., 2005). With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry. To meet the current largely increased demand, studies on the cost-effective production of industrially important enzymes have become the need of today.

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Habitats that contain protein are the best sources to isolate the proteolytic microorganism. Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products (Dalev, 1994; Gaustevora et al., 2005). Hence the present study aims to isolate protease producing bacteria from meat shop waste discharged soil. The yield of extracellular enzymes is significantly influenced by physicochemical conditions. Hence physical parameters are optimized for the maximum production of protease.

This study presents effect of different cultural conditions on production of protease from *Pseudomonas fluorescens* isolated from meat waste contaminated soil.

MATERIALS AND METHODS

Screening and isolation of proteolytic bacteria

One gram of the meat waste contaminated soil sample was

*Corresponding author. E-mail: kalaiarasik@rediffmail.com.

weighed aseptically into 100 ml of sterile distilled water, agitated for 45 min on a shaker and 0.2 ml was spread on casein agar plates (Nutrient agar with 1% casein) and incubated at 37°C for 2 days. A total of 58 bacterial isolates from enriched sample was plated over nutrient agar medium containing 0.4% gelatin (Harrigan and Mc Cance, 1966). After 24 h of incubation, plates were flooded with 1% tannic acid. Isolates having a higher ratio of clearing zone to colony size were grown in liquid broth and the amount of protease production was determined from culture filtrate. The isolate which showed higher protease activity was selected and maintained on nutrient agar slants and subcultured after every fifteenth day. Selected isolate was identified based on morphological and biochemical characteristics as per the method described by Kannan (2002).

Enzyme production medium

Yeast extract casein medium was used as production medium (Naidu and Devi, 2005) contained (g/l) glucose 10, casein 5, yeast extract 5, KH_2PO_4 2, Na_2CO_3 10. Hundred milliliter of yeast extract casein medium was taken in a 250 ml conical flask. The flasks were sterilized in autoclave at 121ºC (15 lb) for 15 min and after cooling the flask was inoculated with 2% over night grown bacterial culture. The inoculated medium was incubated at 37°C in shaker incubator for 24 h. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm at 4°C for 15 min to obtain the crude extract, which served as enzyme source.

Protease assay

Protease was determined by the method of Tsuchida et al. (1986). One unit of protease activity is defined as the amount of protease which liberates 1 µg of tyrosine min^{-1} under experimental conditions.

Process optimization for maximum protease production

Inoculum concentration

Effect of inoculum concentration on protease production was determined by inoculating the production medium with different concentrations ranging from 2 to 7% of overnight grown selected bacterial culture. The inoculated medium was incubated for 24 h. The culture medium was centrifuged at 5000 rpm at 4°C for 15 min. Protease activity was determined in the cell free supernatant.

Incubation time

The effect of incubation time on protease production was determined by incubating the culture medium at different time intervals (24 – 168 h) with an interval of 24 h.

pH

The effect of initial pH of the medium on protease production was studied by adjusting the pH of the production medium in the range of 5 to 11 using 1 N HCl or 1 N NaOH. After the incubation time, the culture medium was centrifuged at 5000 rpm for 15 min in a refrigerated centrifuge at 4°C. Protease activity was determined in the supernatant.

Temperature

The yeast extract casein medium at pH 9 was inoculated with 2% overnight grown selected bacterial strain. The broth was incubated at different temperatures ranging from 27° - 77°C at 10°C interval for 24 h. Protease activity was determined after 24 h.

Carbon sources

The effect of various carbon sources such as starch, wheat bran, rice bran, maltose and sucrose at a concentration of 1% was examined by replacing glucose in the production medium.

Nitrogen sources

Various nitrogen sources like beef extract, tryptone, glycine, peptone and casein were examined for their effect on protease production by replacing 0.5% of yeast extract in the production medium.

Metal ions

Influence of various metal ions on protease production was determined by incubating the yeast extract casein medium with different metal ions such as $BaCl_2$, $CaCl_2$, $MgSO_4$, K_2HPO_4 and $CuSO_4$ at a concentration of 0.2%.

All the experiments were carried out in triplicates and results presented are the mean of three values.

RESULTS AND DISCUSSION

Among the 58 isolates, 14 strains showed good zone of clearance. Protease activity was determined for these 14 strains and the results were depicted in Table 1. From the table, it is clear that bacterium BC_8 showed maximum protease activity (0.9388 U/ml/min). Using morphological and biochemical characteristics the selected isolate was identified as *P. fluorescens.* The results of gram staining, motility and biochemical tests of selected bacterium BC_8 were given in Table 2. Maximum protease production was achieved at 2% inoculum concentration. The enzyme activity gradually decreases from 3 to 7% (Figure 1). These results were in accordance with Elibol et al. (2005) who reported that 2.5% inoculum level gives higher protease production.

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Maximum protease production was achieved at pH 9.0 (Figure 2) by *P. fluorescens*. The production of protease increased as pH of the medium increases and reaches maximum at pH 9.0. After pH 9.0 there was a decrease in enzyme production. Results suggest that there is a stimulation of enzyme production at alkaline pH. The obtained results coincide with Kumar et al. (2002) who has reported that protease production was maximum at pH 7 and 9 for *Bacillus* sp. strain S_4 and *Pseudomonas* sp. strains S_{22} respectively. Similarly Borriss (1987) reported maximum alkaline protease production at pH 9 -13.

P. fluorescens was capable of producing protease in the range of 27 - 57°C with production maximum at 37°C

Table 1. Protease activity of isolated bacteria.

S/N	Bacterial colonies	Protease activity (U/ml/min)
1	BC_1	0.3557
2	BC_2	0.1539
3	BC_3	0.1875
4	BC_4	0.3109
5	BC_5	0.5688
6	BC_6	0.1875
7	BC_7	0.1763
8	BC_8	0.9388
9	BC_9	0.0417
10	BC_{10}	0.4118
11	BC_{11}	0.2884
12	BC_{12}	0.1539
13	BC_{13}	0.2324
14	BC_{14}	0.3221

BC = Bacterial colony.

Table 2. Biochemical tests of selected bacteria.

Characteristic	Bacterial colony BC_8
Gram staining	Gram negative
Motility test	Motile
Indole production test	Negative
Methyl red test	Negative
Voges Prokaurer test	Negative
Citrate utilization test	Positive
Catalase test	Positive
Oxidase test	Positive
Urea hydrolysis	Positive
Nitrate reduction	Positive
Starch hydrolysis	Negative
Gelatin hydrolysis	Positive
Fluorescence on King's B medium	Positive
Growth at 4°C and 41°C	Good
Levan formation	Positive

(Figure 3). However, increase in temperature beyond 37°C led to decline in production of enzyme proving that temperature plays a major role in protease production. These results are in accordance with Fujiwara and Yamamoto (1987) who reported that protease activity was high at 30°C for *Bacillus* sp.

Protease production was found to be maximum at 24 h (Figure 4). The enzyme activity gradually decreases from 48 to 168 h. This finding is in partial agreement with findings of Kumar et al. (2002) who reported that *Pseudomonas* sp. S_{22} showed a peak for protease production at 24 h of incubation and again peaks at 108 h which was against the present study.

The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzyme (Sudharshan et al., 2007). Among the carbon sources, wheat bran and maltose were found to support protease production. The isolated strain showed high enzyme yield (0.389 U/ml/min), when wheat bran was used as carbon source (Figure 5). These results are in agreement with Naidu and Devi (2005) who reported that wheat bran supported the maximum production of protease in *Bacillus* sp. Uyar and Baysal (2004) examined wheat bran and lentil husk, in that wheat bran showed highest protease production in *Bacillus* sp.

Among the nitrogen sources, peptone produced maximum protease (Figure 6). This finding is in agreement

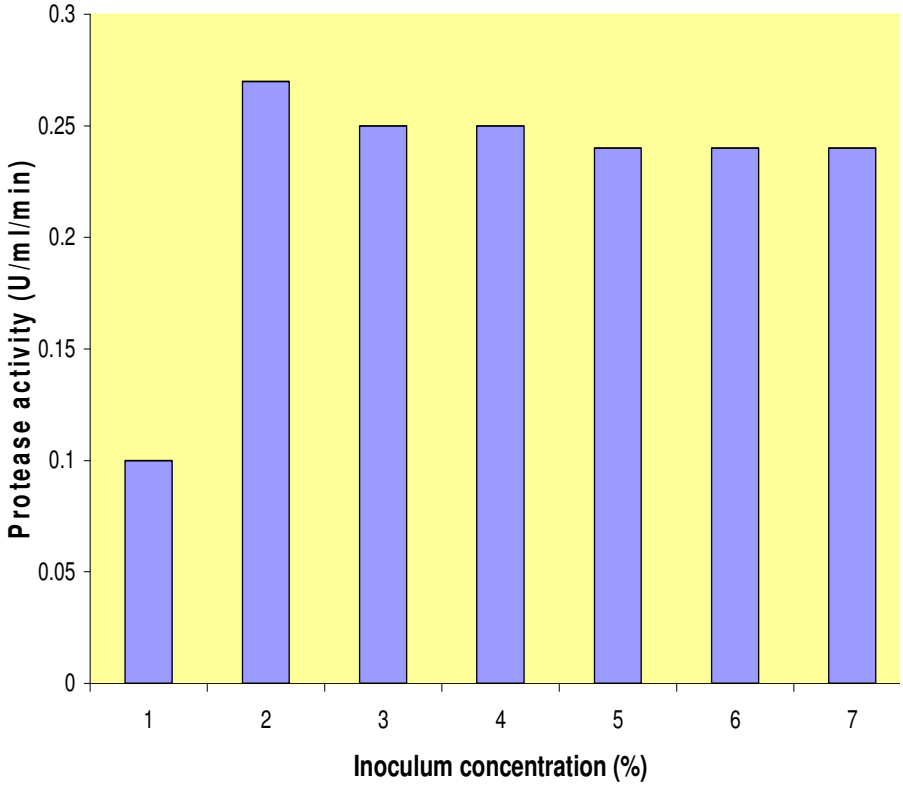

Figure 1. Effect of inoculum concentration on protease production by *Pseudomonas fluorescens.*

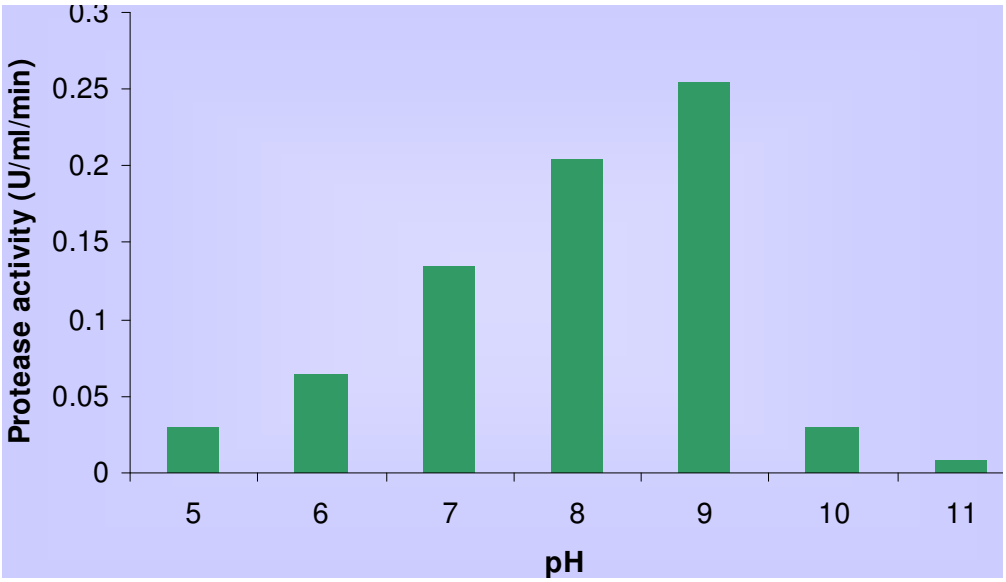

Figure 2. Effect of pH on protease production by *Pseudomonas fluorescens.*

with findings of Wang and Hsu (2005) who found out that casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicolo* 23. However, production medium enriched with soyabean meal has been reported as best nitrogen source for protease production as stated by Sinha and Satyanarayana (1999).

A metal ion in media is an important factor that affects enzyme production. $CaCl_2$ at a concentration of 0.2%

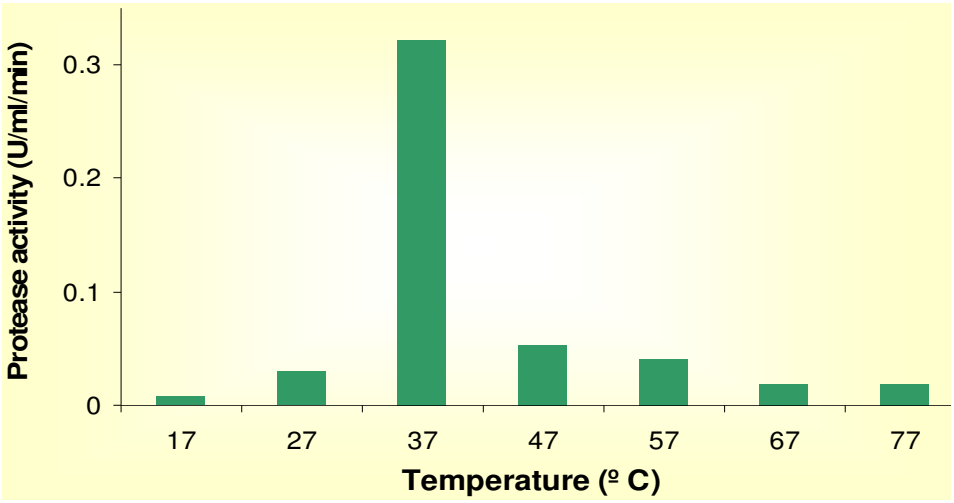

Figure 3. Effect of temperature on protease production by *Pseudomonas fluorescens.*

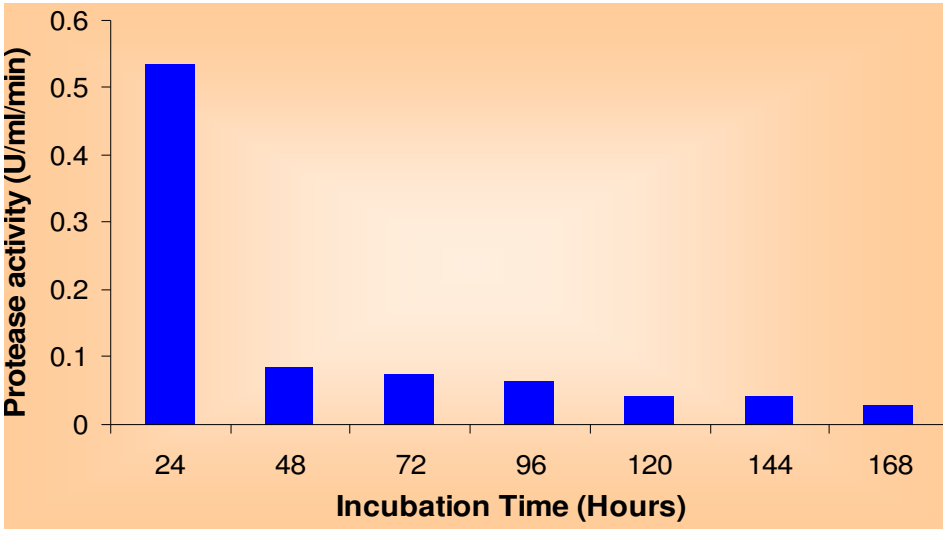

Figure 4. Effect of incubation time on protease production by *Pseudomonas fluorescens.*

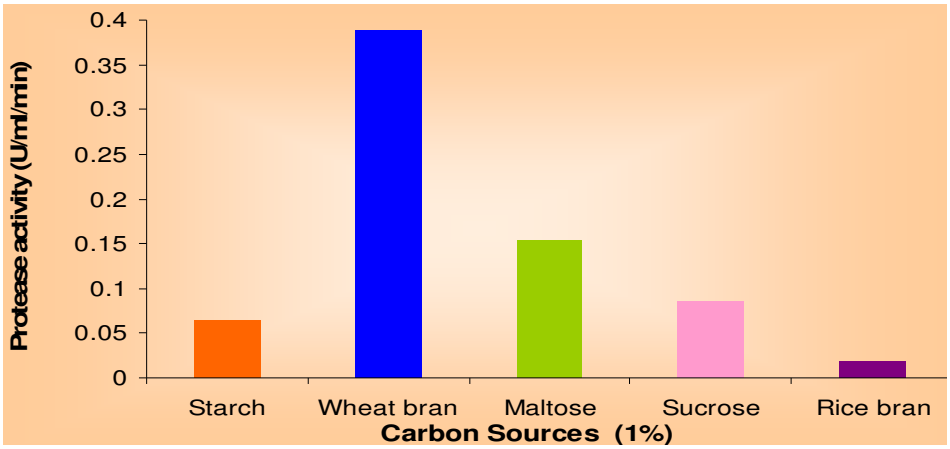

Figure 5. Effect of carbon source on protease production by *Pseudomonas fluorescens.*

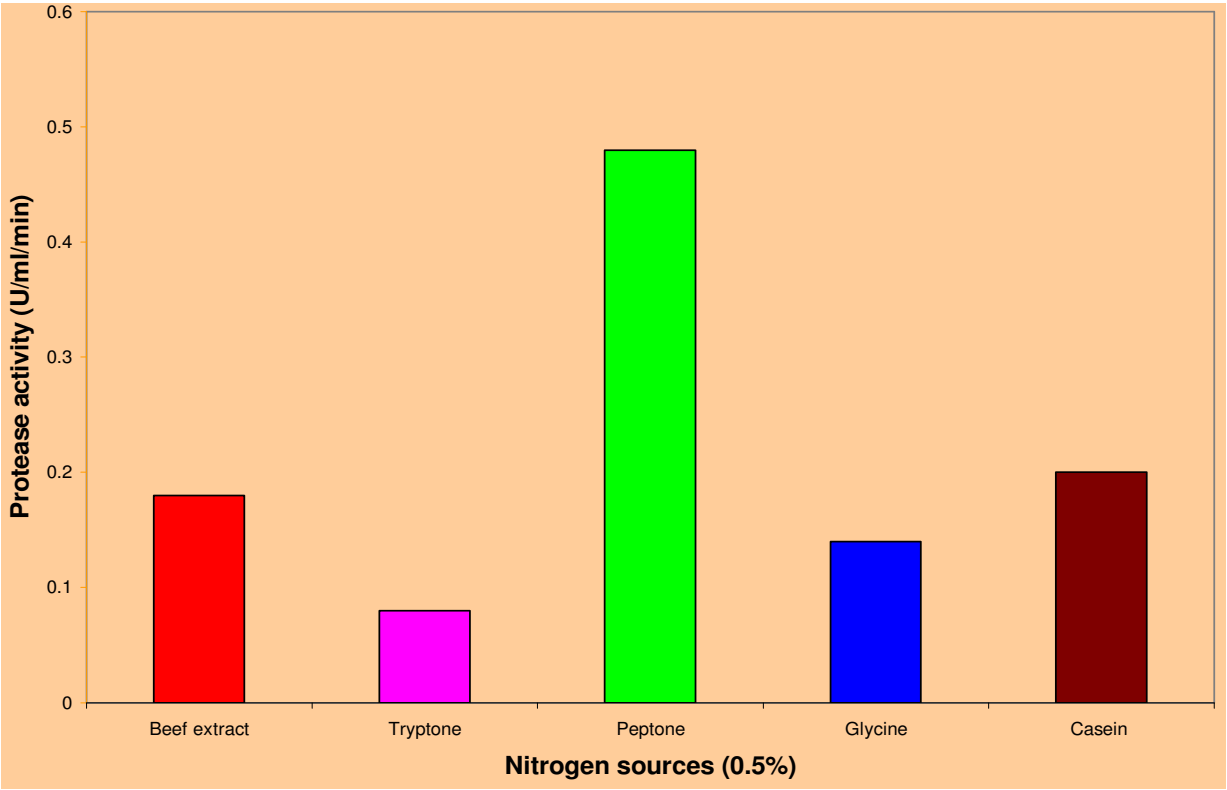

Figure 6. Effect of nitrogen for protease production by *Pseudomonas fluorescens.*

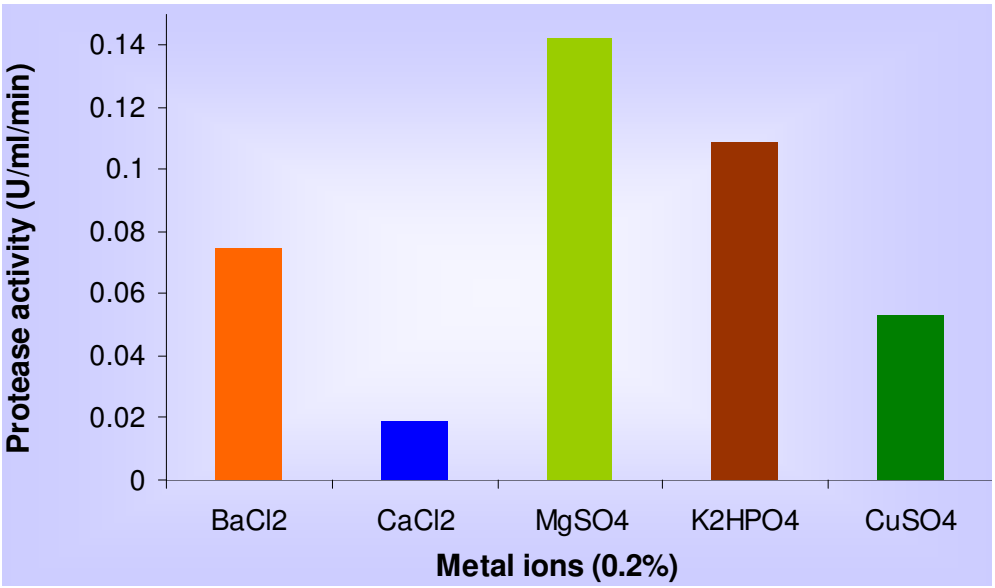

Figure 7. Impact of metal ions on protease production by *Pseudomonas fluorescens.*

inhibits protease production followed by $CuSO_4$ and $BaCl_2$ (Figure 7). Magnesium sulphate has less inhibitory effect on the production of protease. These results are in accordance with Wang et al. (2008) who reported that the optimized metal ion for protease production by *Chryseo* bacterium was 0.05% $MgSO_4$.

In conclusion, the appreciable high enzyme activity at alkaline pH suggested that *P. fluorescens* is a potential producer of alkaline protease which can find application in detergent and textile industries.

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