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MICROBIOLOGY

Production and Optimization of an Alkaline Protease from *Acinetobacter variabilis* Isolated from Soil Samples

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ABSTRACT

Protease enzymes have immense commercial value and play a pivotal role in application of various industrial sectors. Microbial proteases are one of the important groups of industrially and commercially produced enzymes which have several applications. In this study 148 bacterial strains were isolated from 50 different soil samples of slaughter house, fish market and sewage wastes of Lucknow, Uttar Pradesh, India. Out of which thirty eight strains competent of secreting extracellular alkaline protease. In preliminary screening the isolate SSB2 showed highest ability to hydrolyzed casein and skimmed milk which was done on skim milk agar media. Based on biochemical test the isolate showed positive for casein, starch, catalase and negative for gram staining, indole, methyl red, voges proskauer, gelatin, urea, oxidase, hemolysis and triple sugar iron test and found to be non motile. Strain SSB2 with the maximum yield alkaline protease was identified as *Acinetobacter variabilis* based on nucleotide homology and phylogenetic analysis (16S rDNA sequencing). Protease production was enhanced by optimizing the culture conditions. Many physical parameters were studied to optimize the maximum yield of alkaline protease. The maximum enzyme activity were observed with optimum incubation time, temperature, pH, carbon, nitrogen sources, NaCl and metallic ions were determined as 36 h, 37°C, pH 11.0, 1% glucose, 1% yeast extract, 1M NaCl, and 1mM Zn^{2+}, respectively for protease production. The study revealed that the strain of *A. variabilis* is a potent source of alkaline protease. In consequence, such additions can supplement alkaline protease production and their application in various industries.

Highlights

- A new potent alkaline protease producing strain *Acinetobacter variabilis* was isolated from the soil sample of slaughter house, Lucknow region, Uttar Pradesh, India
- The study concluded that *A. variabilis* has a wide scope for the industrial production of alkaline protease. Further studies needed to ascertain the potential applications

Keywords: Alkaline protease enzyme, 16S rDNA, *Acinetobacter variabilis*, soil samples

Proteases (EC 3.4.21) are a large group of hydrolytic enzymes that catalyze the hydrolysis of the proteins by cleavage of the peptide bonds between the amino acid residues in other proteins (Shankar *et al.*, 2011). These enzymes are very important in the world market where they account for about 59% (Deng *et al.*, 2010). The microbial proteases constitute approximately 40% of the total worldwide production of enzymes (Raval *et al.*, 2014). Alkaline proteases, with high activity and stability in high alkaline range, are interesting for several bioengineering and biotechnological applications. However, their main application is in the detergent industry, accounting for approximately 30% of the total world enzyme production (Haddar *et al.*, 2009). The Alkaline proteases (EC 3.4.21–24, 99)

are active in a neutral to alkaline pH range (Jisha *et al.,* 2013). They also have a serine center or are of metal- type and the alkaline serine proteases are the most considerable group of enzymes so far exploited (Gupta *et al.,* 2002). Microbial proteases are advantageous in that they can be produced easily at lower cost and in large quantities in industrial scale. Genetic engineering techniques are readily applicable to microbial strains; they improve the efficiency of enzyme production. Bacterial species are the main producers of extracellular proteases, and industrial sectors frequently use these bacteria for the production of proteases. Proteases are the main enzymes produced from microbial sources, of which only a few are recommended as commercial producers. There are plenty of literature available in worldwide on the production, optimization and applications of alkaline protease (Anwar and Saleemuddin 2000; Banerjee *et al.,* 1999; Kuddus and Ramteke 2008, 2009, 2011; Shankar *et al.,* 2011; Kuddus *et al.,* 2013. Sawant and Nagendrans 2014; Abdelnasser *et al.,* 2015. Shine *et al.,* 2016; Patil and Jadhav 2017). But scanty literature is available on the *Acinetobacter* spp. Salwan and Kasana (2013) first reported the purification and biochemical characterization of extracellular peptidase from genus *Acinetobacter* and also revealed that this is a novel extracellular peptidase showing optimum activity at 40 °C and potential for detergent formulations. In this backdrop, the present study was conducted to isolate the bacterial strain with alkaline protease activity from the soil samples and to characterize alkaline protease from *Acinetobacter variabilis with* an optimization for maximum production.

MATERIALS AND METHODS

Sample collection and isolation of bacterial strain

Soil samples from slaughter house (30), fish markets (15), sewage wastes (05) were collected at Lucknow, Uttar Pradesh, India and brought to the laboratory in sterile containers. The collected samples were serially diluted in sterile physiological saline (0.85% NaCl) and the diluted sample were plated on nutrient agar plates (pH 8) and incubated at 37°C for 48h. Colonies from the agar plates were selected based on divergence in morphology, size and color and were stored at -20 ˚C for further processing.

Screening of protease producing bacteria

Primary screening was done on nutrient agar plates supplemented with 1% casein by spot inoculation. The plates were incubated at 37°C for 48 h. The protease producing strains were selected based on the zone of clearance and after that individual colonies were further screened onto skim milk agar plates (Chu 2007). A clear zone of skim milk hydrolysis was considered as an indication of the protease producing organism.

Proteolytic activity assay

Protease assay was performed by a modification of the casein digestion method of Kunitz (1947). The assay was performed at alkaline range of 8-10 pH. Protease production was assayed in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Proteolytic activity with azocasein used as substrate was assayed by the modification method of Secades and Guijarro (1999). In briefly, 120 µl of the enzyme solution or cell-free supernatant was added to 480 µl azocasein (1% wt/vol) in reaction buffer (0.1 M Tris buffer, pH 9) and the mixture was incubated at 30°C for 30 min. The reaction was terminated by adding 600 µl of 10% (wt/vol) trichloroacetic acid and resulting precipitate was allowed to settle for 30 min on ice, followed by centrifugation at 15000 x g at 4 °C for 10 min. A suitable control was run simultaneously, in which trichloroacetic acid was added into the incubation mixture at zero time used as a blank. For the neutralization 200 µl of 1.8 N NaOH was added to 800 µl of supernatant solutions. The final absorbance was measured at 420nm (A_{420}) using the 20 UV-Visible spectrophotometers. The protease activity is expressed in the unit as one unit of enzyme activity was defined as the yielded an increase in A_{420} of 0.01 in 30 min at 30 ° C. Protein was determined by the method of Folin Lowry method (Lowry *et al*., 1951).

Identification of the bacterial strain

For identification of SSB2 isolate, both physiological and biochemical tests were performed as per Holt *et al.* (1994). The potential bacterial strain was identified using 16S rDNA gene sequence analysis as per the standard protocols. The bacterial isolate was grown overnight in 5 ml alkaline broth medium. Total DNA was extracted using D Neasy

Kits (Qiagen, USA) according to the manufacturer's instructions. Gene-specific forward primer: 16s27F (5'-AGA GTT TGA TCC TGG CTC AG-3'), and reverse primer: 16s1115R (5'-AAG GAG GTG ATC CAG CCG CA-3') were used to amplify 16S rDNA gene. The purified 16S-rDNA was sequenced using an ABI 3730xl Genetic Analyzer (Eurofins Genomics India Pvt Ltd. Bangalore), and the obtained sequence was aligned with reference 16S rDNA sequences available in NCBI homepage (National Center for Biotechnology Information) using the BLAST algorithm.

Enzyme production

The basal culture medium for the protease production broth medium contained (g^{-1}): glucose 0.5, yeast extract 0.5, peptone 0.5, KH_2pO_4 0.1, $MgSO_47H_2O$ 0.01, pH 11. Desired pH was obtained by adding sterile 10% Na_2CO_3 solution after autoclaving. The medium (100ml) was inoculated with 1 ml of a 24 h old culture in 500 ml conical flask, and incubated at 37 °C with shaking at 250 rpm for 48h. The cell free supernatant was recovered by centrifugation at 10,000Xg for 15 min at 4 °C and used for determining the protease activity.

Optimization of incubation time, temperature, pH for protease production

To determine the various physical parameters for maximum production of protease, the 24h grown culture were subcultures (@1:100) in 250 ml flask contain 100 ml protease production broth. For optimizing pH, the medium was prepared by varying the pH from 4.0 to 13.0 at 1.0 unit interval incubate in shaker (150 rpm) at 30 to 50°C for 24-120 hours. Then 10 ml of the culture samples withdrawn from the medium were collected every 24 hours after incubation. The sample cultures were filtered, followed by centrifugation at 7000 rpm for 30 min. The culture filtrate solutions obtained were used to determine protease activity. All the experiments were conducted in triplicates.

Optimization of carbon, nitrogen sources, NaCl and metallic ions for protease production

The effect of various chemical parameters on protease production was studied by varying the carbon sources (glucose, fructose, maltose, sucrose, and lactose (1% w/v)) and nitrogen sources (peptone, yeast extract, beef extract, ammonium sulphate and sodium nitrate (1% w/v)). The NaCl salt concentrations various from 0 to 2 M was supplemented. Divalent metal ions such as Zn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} each 1mM were used to evaluate the impact on alkaline protease production. After 48 hours incubation at 37°C, 150 rpm, the cell-free supernatants were quantified for protease production.

Statistical analysis

All the experiments, enzyme assay, and cells growth measurement were carried out in triplicate, and the standard deviation for each test was calculated using SPSS 20. The standard deviations are indicated as error bars.

RESULTS AND DISCUSSION

Isolation, Screening and identification of the bacterial strain

One hundred forty eight bacterial strains were isolated from 50 different soil samples of slaughter house, fish market and sewage wastes of Lucknow, Uttar Pradesh, India. Out of which thirty eight strains competent of secreting extracellular alkaline protease at pH 11. The isolates were then purified, preserved and tested for their proteolytic ability. In preliminary screening the isolate SSB2 showed highest ability to hydrolyzed casein and skimmed milk (Fig. 1) and was finally selected for further optimization of the extracellular alkaline proteases production.

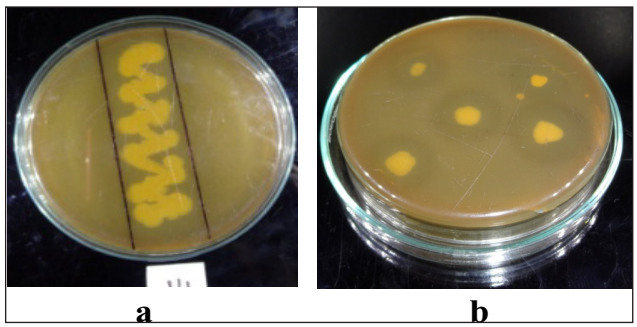


Fig. 1: Proteolytic activity of SSB2 **(a)** Spread inoculation **(b)** Spot inoculation

The isolate showed positive for casein, starch, catalase and negative for gram staining, indole, methyl red, voges proskauer, gelatin, urea, oxidase, hemolysis and triple sugar iron test and found

to be non motile. Based on nucleotide homology and phylogenetic analysis, SSB2 was identified as *Acinetobacter variabilis*. (Fig. 2-5).

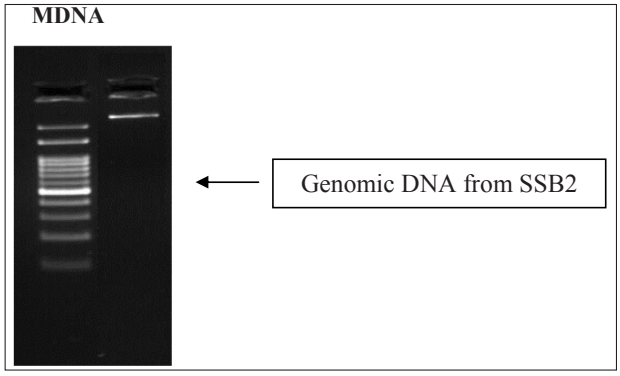


Fig. 2: Agarose gel electrophoresis of *A.variabilis* (SSB2) genomic DNA. Lane M: is 1 kb DNA ladder. Lane DNA: is bacterial genomic DNA marked by an arrow

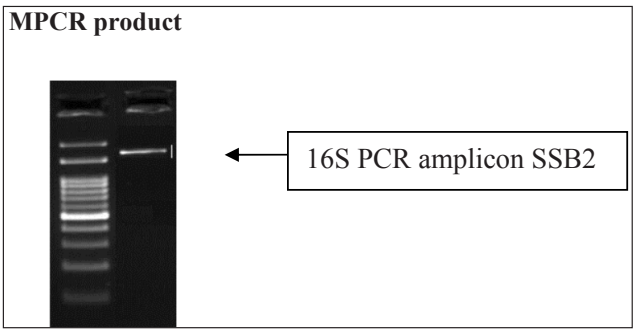
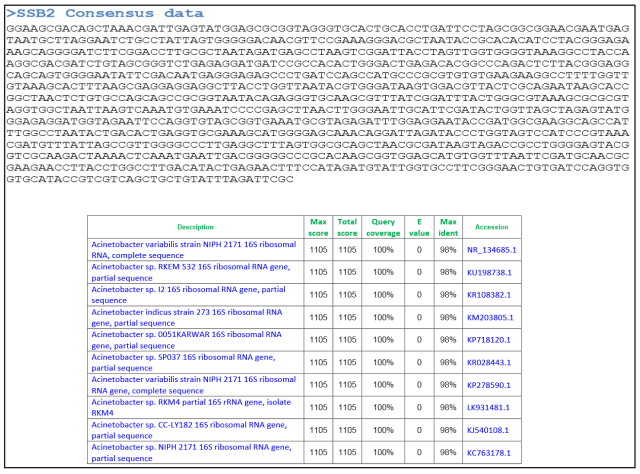


Fig. 3: Agarose gel electrophoresis of PCR amplicon *A.variabilis* (SSB2) Lane M: is 1 kb DNA ladder. Lane DNA: is PCR amplicon marked by an arrow



Description	Max score	Total score	Query coverage	E value	Max ident	Accession
Acinetobacter variabilis strain NIPH 2171 16S ribosomal RNA, complete sequence	1105	1105	100%	0	98%	NR_134685.1
Acinetobacter sp. RKEM 532 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KU198738.1
Acinetobacter sp. I2 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KR108382.1
Acinetobacter indicus strain 273 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KM203805.1
Acinetobacter sp. 0051KARWAR 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KP718120.1
Acinetobacter sp. SP037 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KR028443.1
Acinetobacter variabilis strain NIPH 2171 16S ribosomal RNA gene, complete sequence	1105	1105	100%	0	98%	KP278590.1
Acinetobacter sp. RKM4 partial 16S rRNA gene, isolate RKM4	1105	1105	100%	0	98%	LK931481.1
Acinetobacter sp. CC-LY182 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KJ540108.1
Acinetobacter sp. NIPH 2171 16S ribosomal RNA gene, partial sequence	1105	1105	100%	0	98%	KC763178.1

Fig. 4: Sequences producing significant alignments *A. variabilis* (SSB2) carry out BLAST with the nrdatabase of NCBI GenBank database

Optimization of parameters for maximum enzyme production

Incubation time: Enzyme production is connected to cell growth and therefore there is a co-relation between incubation period and enzyme production. The enzyme production varies with incubation time. Maximum enzyme production was obtained after 36 h of incubation (Fig. 6). These results are in accordance with observations made by Durhams *et al.* (1987), Gashaw and Gessesse (1997) and Qadar *et al.* (2009), where maximum enzyme production was observed during continuous growth of the culture at the late exponential phase and early stationary phase of the growth. The number of viable cells decreased due to depletion of readily available carbon source and other nutrients.

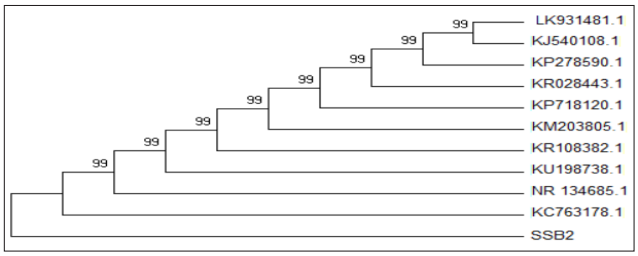


Fig. 5: Phylogenetic tree *A. variabilis* (SSB2) was constructed using MEGA 4

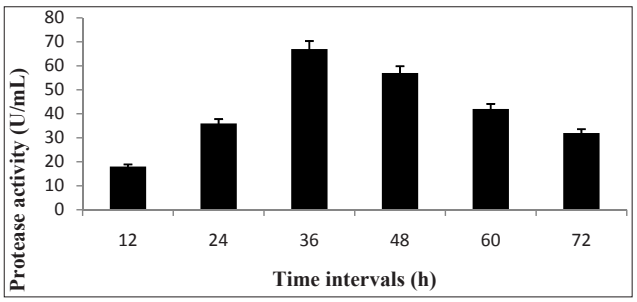


Fig. 6: Incubation periods for protease production in *A. variabilis* isolated. The bars indicate the standard deviation (± S.D) of three replicates (n = 3).

Temperature: Every organism possesses a minimal temperature, optimum temperature and maximum temperature for the growth and metabolism. Temperature below optimum is bacteriostatic and above optimum is bactericidal. Enzyme activity recorded at different temperatures revealed that the isolate yielded maximum protease production at 37°C (Fig. 7). El-Safey *et al.* (2004) reported the same findings in production, purification, and characterization of protease enzyme from *Bacillus* sp. Related studies also reported that protease production by *B. laterosporous* was best at 37°C (Usharani and Muthuraj 2010).

pH: Among physical parameters, pH of the growth medium plays an important role by

inducing morphological changes in microbes and in enzyme secretion. The pH changes were observed throughout the growth of microbes also affects product stability in the medium. The optimal pH varies with different microorganisms and enzymes. The isolate was allowed to grow in media of different pH ranging from 4.0 to 13.0. Maximum enzyme production was observed in the medium of pH 11.0 in *A. variabilis* (Fig. 8). Thangam and Rajkumar (2002) also reported the optimum pH for protease production by *Alcaligenes faecalis* as 8. pH optima for protease production in various *Bacillus sp*. were in the range 7 and 10 (Kaur *et al*., 2001; Singh *et al*., 2001; Puri *et al*., 2002; Kumar 2002).

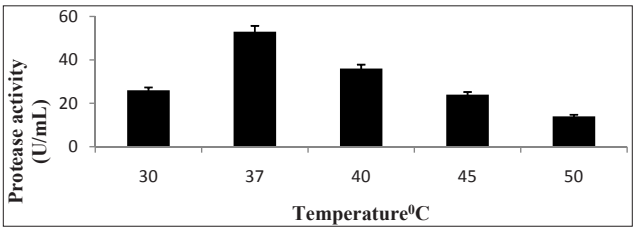


Fig. 7: Effect of incubation temperature on protease production in *A. variabilis*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

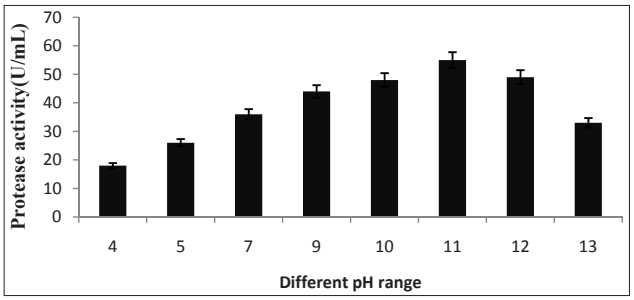


Fig. 8: Effect of pH on protease production in *A. variabilis*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

Carbon: Various sources of carbon such as glucose, fructose, maltose, sucrose, and lactose were used to the original carbon source in growth media. Results obtained were showed that Glucose brought the highest protease production compared to other carbon sources at 36 h of incubation in *A. variabilis* (Fig. 9). Similar findings were observed by Bhatiya and Jedeja (2010) in the optimization of environmental and nutritional factors for alkaline protease production. Analogous findings were also reported by Pastor *et al*. (2001) and Santhi (2014), where glucose and starch gave maximum protease production, respectively. These results are in good accordance with previous studies showing maximum protease production when glucose was used as carbon source in the culture broth.

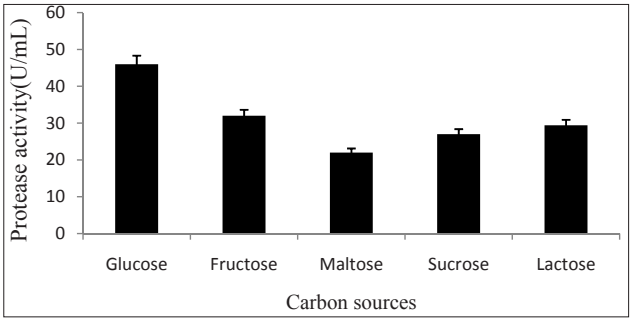


Fig. 9: Effect of carbon sources on protease production in *A.variabilis* isolated. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

Nitrogen sources: The nitrogen sources like peptone, yeast extract, beef extract, ammonium sulphate and sodium nitrate were used. Protease production is believed to be affected by the presence of complex nitrogen sources in the growth medium and each organism varies from the other in terms of its requirement for a specific nitrogen source. The result revealed that the best nitrogen source was yeast extract for *A. variabilis* (Fig. 10). Respectively this showed the highest level of protease activity compared to other sources of organic nitrogen. Ashour *et al*. (1996) also suggest that yeast extract and peptone is a suitable nitrogen source, which stimulates protease production by using various microbial species.

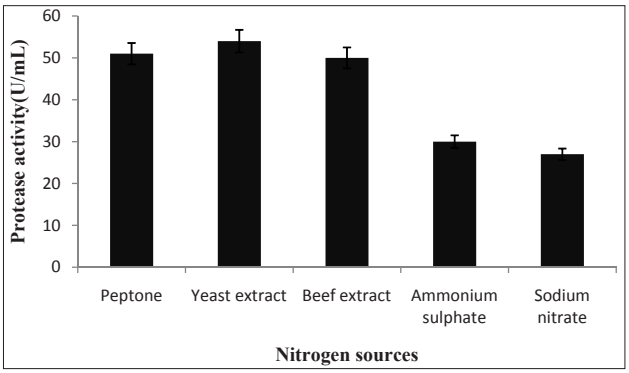


Fig. 10: Effect of nitrogen source on protease production in *A.variabilis*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

NaCl: Various NaCl concentrations (i.e. 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, and 2.0M) were used to determine optimum level required for the production of protease by the selected isolate. It was observed that the growth medium containing 1.0 M yielded

the maximum activity in *A. variabilis* (Fig. 11). The bacterium was showed NaCl tolerance up to 2 M. In a previous report, a similar NaCl concentration was found to be optimum for the production of protease (Shivanand and Jayaraman 2009).

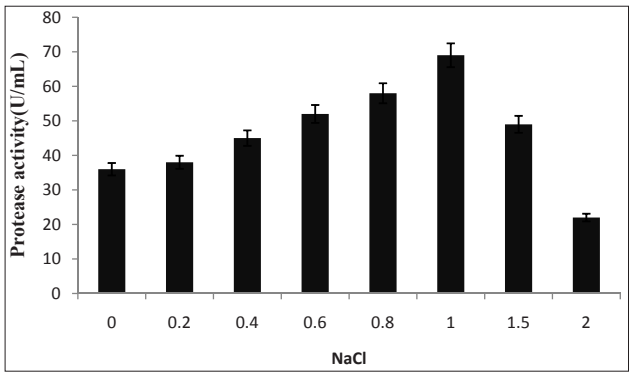


Fig. 11: Effect of NaCl concentration on the production of protease in *A. variabilis*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

Metallic ions: Divalent metal ions such as Zn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} are required in the fermentation medium for growth and optimum production of alkaline proteases. The influence of various metal ions on cell growth and alkaline protease production was evaluated, and the results are demonstrated in Fig.12. Among the cations tested Zn^{2+} and Cu^{2+} ions significantly enhanced the protease production compared to the control, respectively. However, most of the other tested ions caused a significant reduction of the enzyme yield, particularly at high concentration. These results corroborate the earlier findings of metal ions such as Zn^{2+} enhancing the activity of protease in *Bacillus subtilis*. Mcconn *et al*. (1964) and Cu^{2+} enhancing the activity of protease in *Bacillus cereus.* Doddapaneni and Mangamoori (2009).

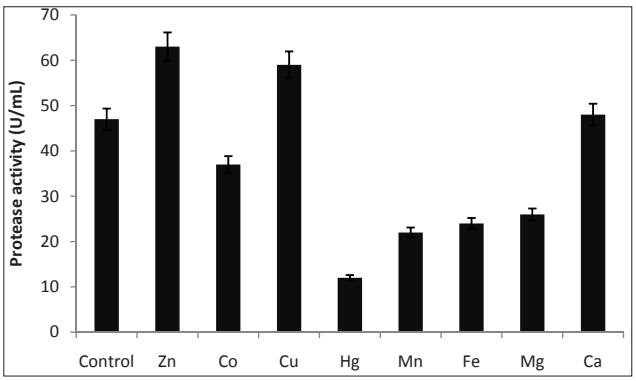


Fig. 12: Effect of metallic ions on the production of protease in *A.variabilis*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

CONCLUSION

A new potent alkaline protease producing strain *A. variabilis* was isolated from the soil sample of slaughter house, Lucknow region, Uttar Pradesh, India. The organism was affiliated to *A. variabilis* based on biochemical tests and 16S rDNA gene analysis. The characterization of *A. variabilis* indicated that this bacterium is alkaliphilic in nature. Optimization of various fermentation parameters resulted in an increase of enzyme production, that indicating the significance of optimization of the process to obtain a commercial yield of the enzyme. Alkaline protease secretion was consistent with the growth pattern, showing maximum enzyme production in a growth phase. The optimum incubation time, temperature, pH, carbon, nitrogen, sources, NaCl and metallic ions for protease production were determined as 36 h, 37°C, pH 11.0, 1% glucose, 1% yeast extract, 1M NaCl, and 1mM Zn^{2+}, respectively. Based on the present study, it is concluded that *A. variabilis* has a wide scope for the industrial production of alkaline protease. Further studies are necessary to ascertain the potential applications of the protease obtained from this study.

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