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Aspects of protease from isolated bacteria from bovine milk

Mahmoud Sahar G.[a*], Ali S. G.[a], Salman Kh. H.[b]

[a]*Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assuit, 71524, Egypt*
[b]*Dairy Science Department, Faculty of Agriculture, Al-Azhar University, Assuit, 71524, Egypt*

Abstract

Ninety-five isolates were screened for produce of protease enzymes. The most potent isolate was identified as Bacillus sp. The aim of study is production and characterization of protease from selected isolate. Protease production was increased by optimizing the culture conditions, these included physical parameters like incubation time, pH, and inoculum size. Lactose and yeast extract were the best carbon and nitrogen sources. The purified enzyme was found to be stable at pH 5–9. The activity of protease increased under an alkaline condition with the optimum pH estimated at pH 9 and thermostability at 50°C. Partially purified protease enzymes were inhibited with EDTA and SDS. EDTA concentrations show a strong inhibitory effect on protease activity. The effect of various metal ions at concentrations of 1, 5, 10, and 15 mM on crude protease was investigated Protease enzyme activated with Ca, Ni, Co, and Mn. Result shows a strong positive correlation between Ni concentration and protease activity.

Keywords: isolation, protease enzyme, optimization condition, enzyme activity, characterization.

*Corresponding author: Mahmoud Sahar G.,
E-mail address: saharyousif556@gmail.com

1. Introduction

It is well recognized that, microorganisms are essential to the technology used to produce intracellular and extracellular enzymes on a large scale (Gupta *et al*., 2002; Moo-young *et al*., 1994). In nature, proteases are found in large quantities. Microorganisms are the primary source of extracellular enzymes because of their rapid development and extremely small growth environment. Microbes are ideal for this purpose because they can be genetically manipulated to create modified enzymes with desired properties. Currently, certain companies want new microbial enzymes with modified properties to meet various applications (Kocher and Mishra, 2009). Yeast, actinomycetes, bacteria, fungi and other types of microbes create these extracellular enzymes (Devi *et al*., 2008). First on the list of organisms that create alkaline proteases are bacteria. Proteases are most commonly obtained from the genus *Bacillus* (Gupta *et al*., 2002). Proteases are enzymes that catalyze the hydrolysis of hydrolytic bonds in proteins, releasing peptides and/or amino acids (Louwrens and Benoit, 2014). Alkaline proteases account for 60-65% of the world market for industrial products (Sawi *et al*., 2008; Zanphorlin *et al*., 2000). The only class of enzymes commonly utilized in the food and agriculture sectors, leather, detergents and pharmaceuticals are these proteases (Azura*et al*., 2009; Rao*et al*., 1998). When treating skin ulcers locally, proteolytic enzymes effectively remove necrotic debris from the wound, promoting the natural healing process (Sjodahl *et al*., 2002). Studies have shown that, nutritional factors, including sources of carbon and nitrogen, can influence protease enzyme production by bacteria. Besides these nutritional factors, physical factors such as inoculum concentration (Kaur *et al*., 1998), temperature, pH (Tobe *et al*., 2005), and incubation time (Yossan *et al*., 2006) can also significantly affect protease production. The growth medium composition affects the enzyme's purification procedure. For fermentation to produce a high production of desired enzymes, it is therefore essential to choose the appropriate medium ingredients and optimize their concentrations after choosing a suitable microbe. The aim of the study is to isolate the characterization protease enzyme from the selected strain that was isolated from bovine milk.

2. Materials and methods

2.1 Collection and isolation of sample

Bovine milk samples were collected from different sources in Assuit governorate, Egypt. Samples were collected using sterilized sample bottles and brought to the laboratory with an icebox for microbiological investigation. Samples were kept in a refrigerator (around 4°C) until the analysis began. The collected samples were serially diluted, and 10^{-3}, 10^{-6} and 10^{-9} dilutions were spread over the surface of nutrient agar plates, followed by incubation at 37°C for 48-72 h. All the isolated colonies having different culture characteristics were subcultured several times by repeated streaking at the same condition to get pure colonies (Kader *et al*., 1999).

2.2 Screening of strains for extracellular protease activity

Ninety-five pure bacterial isolates were screened for their proteolytic activities using the skim milk agar plate method (Abdel Galil, 1992). The strain exhibiting the largest zone of hydrolysis was selected for further experimentation. The stock culture of the organism was maintained at -20 °C in 50% glycerol.

2.3 Identification of bacteria

The bacteria isolates were identified based on microscopic morphology, growth condition, gram staining, endospore staining, and biochemical tests (Sneath and Halt, 1986).

2.3.1 Gram staining of bacterial isolates

All purified bacterial isolates were stained with Gram stain and microscopically examined with an oil immersion lens to determine the bacterial cell forms and to check and confirm their purity.

2.4 Production of protease

Alkaline protease was produced in an optimized production medium of pH 8.0 containing 1% galactose, 0.5% casein, 0.55% peptone, 0.2% KH_2PO_4, 1% $Na_2CO_3$and 0.2% $MgSO_4$ $7H_2O$; 4% inoculum was added to the production medium and incubated with continuous shaking. Shaker fermentations were carried out at 37°C for 72 h with controlled agitation at 150-200 rpm. At the end of the fermentation period, the whole culture broth was centrifuged at 5000 rpm for 30 min to remove debris, and the supernatant was collected and used for further experiments (Pant *et al*., 2015).

2.5 Protease assay

Using casein as the substrate, the crude enzyme extract's protease activity was assessed using methodology of Cupp-Enyard (2008) as follow: 1 ml of 0.65% casein solution (prepared in 50 mM potassium phosphate buffer, pH 7.5) and 0.2 ml of crude enzyme supernatant were added to the reaction mixture, which was then incubated for 30 min at 37°C. The reaction was then halted by adding 1 ml of trichloroacetic acid solution (110 mM). After 15 min, this mixture was filtered through a Whatmann No. 1 filter paper. A 500 mM sodium carbonate solution was combined with 0.4 ml of the filtrate, and 0.2 ml of a Folin & Ciocalteu's phenol reagent that had been diluted twice was added. The final combination was left to develop its blue color for half an hour at room temperature in the dark. Using a tyrosine standard, the concentration of released tyrosine was measured at 660 nm in comparison to a reagent blank. The tyrosine was measured at 660 nm in comparison to a reagent blank. Using a tyrosine standard (Lowry *et al*., 1951). One unit of protease is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min under the standard conditions of the supernatant. The enzyme

activity (U/ml¬) was calculated using the following formula:

$$\text{Protease activity (units/mL)} = \frac{\mu \text{ mole tyosine equialent releases } \times \text{ 2.2 (totalvolum of assay)}}{\text{volume of enzyme taken (0.2ml) } \times \text{ incubation time 30}}$$

2.6 Effect of different carbon source

To fifty ml quantities of standard growth broth containing 1 percent casein, various carbon sources, viz., starch, glucose, fructose, maltose, sucrose, and sorbitol, at 0.5 percent levels, were added in 100 ml Erlenmeyer flasks and incubated at 30°C without adding carbon sources. They were sterilized and inoculated for 2 days, and at the end of incubation, the cell-free filtrate served as the enzyme source (Boominadhan *et al*., 2009).

2.7 Effect of different nitrogen source

To fifty ml of standard broth containing one percent casein, various organic nitrogen sources, viz., beef extract, yeast extract, peptone, tryptone, and inorganic nitrogen sources, viz., ammonium nitrate, ammonium sulfate, and ammonium chloride, 0.5 percent were added in separate 100 ml Erlenmeyer flasks without adding nitrogen sources. Sterilized, inoculated, and incubated at 30°C for 48 hours, the cell-free extract served as the enzyme source (Boominadhan *et al*., 2009).

2.8 Characterization of alkaline protease

2.8.1 Effect of pH on activity and stability of protease

Protease activity was investigated at different pH values (pH 5-10). The enzyme was preincubated with a variety of pH buffers, including acetate buffers (5), citrate phosphate buffers (pH 6), Tris-HCl buffers (pH 7-8), and carbonate buffers (pH 9-10), at 37°C for 30 min in order to determine the ideal pH for protease activity. Under conventional assay conditions, the residual activity of protease was calculated (Usharani and Muthuraj, 2010).

2.8.2 Impact of temperature on activity and stability of protease

The temperature impact on the purified protease enzyme activities was explored by incubating the reaction mixtures at distinct temperature ranges (30, 35, 40, 45, 50, 55, 60, 65 and 70°C) with 1% casein as a substrate. The activities were checked as discussed by Ullah *et al*. (2022).

2.8.3 Effect of metal ions and inhibitors on the protease activity

The effects of various metal ions (5 mM) on enzyme activity were investigated by pre-incubating the enzyme preparation for 30 min at 25°C in the presence of Ca, Mn and Ni^{2+}). Thereafter, sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) were used to study the effects of protease inhibitors. The enzyme

was pre-incubated with inhibitors for 15 min at 20°C, and casein was used as a substrate to test the residual activity (Ghorbel *et al*., 2003).

2.9 Statistical methods

All statistical calculations were done using SPSS (statistical package for social science; SPSS Inc., Chicago, IL, USA) version 25. Quantitative data were statistically described in terms of mean ±SD when they were normally distributed. A comparison of quantitative variables was done using a one-way ANOVA (analysis of variance) test for normally distributed data. For significant data, a post-hoc Duncan test was run for multiple comparisons. The correlation between protease activity and other variables was determined by the Pearson coefficient of correlation. *P*-value was set to be significant at the 0.05 level (Fisher, 1925).

3. Results

The isolated bacteria were identified based on cellular morphology, Gram staining, and biochemical tests (Table 1). The results showed that most isolates were Gram-positive, spore-forming bacteria that were presumptively identified as *Bacillus* spp. (Figure 1).

Table (1): The isolated bacteria were identified based on cellular morphology, gram staining, and biochemical tests.

Strain No.	Colony color	Colony surface	Gram staining	Catalase test
Al7	Red	Smooth	+ve	+ve
A18	White	Smooth	+ve	+ve
A9	Of white	Smooth	+ve	+ve
B7	Of white	Smooth	+ve	+ve
B8	Of white	Smooth	+ve	+ve
B9	Of white	Smooth	+ve	+ve
A27	Of white	Smooth	+ve	+ve
A1N	Of white	Smooth	+ve	+ve
A1E	Of white	Smooth	+ve	+ve
B1E	Of white	Smooth	+ve	+ve
A82	Of white	Smooth	+ve	+ve
AS2	Of white	Smooth	+ve	+ve
Ae2	Of white	Smooth	+ve	+ve
An2	Of white	Smooth	+ve	+ve
E8	Of white	Smooth	+ve	+ve
E8R	Of white	Smooth	+ve	+ve
Ged1	Of white	Rough	+ve	+ve
Ged2	Of white	Rough	+ve	+ve
G21	Of white	Smooth	+ve	+ve
G22	Of white	Smooth	+ve	+ve
Ma1	Of white	Smooth	+ve	+ve
M201	Of white	Smooth	+ve	+ve
T222	Of white	Smooth	+ve	+ve

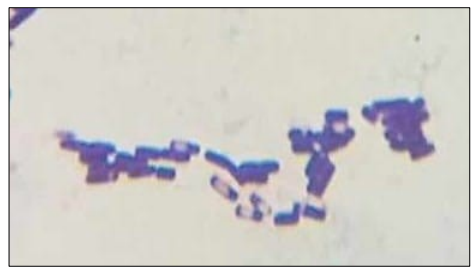

Figure (1): Gram-positive rod-shaped spore-forming bacterium.

The isolates were screened for protease production on skim milk agar. The strain exhibiting the largest zone of hydrolysis was selected for further study (Table 2).

Table (2): Proteolytic bacterial activities were noted as diameter (mm) of clearing zone on skim milk agar.

Strain No.	Proteolytic activity on skim milk	Diameter of clear zone (mm)
E8	+ve	18
E8R	+ve	15
Ged1	+ve	17
Ged2	+ve	16
G21	+ve	10
G22	+ve	9
Ma1	+ve	8
M201	+ve	20
A17	+ve	15

The selected strain, M201, produced a zone of hydrolysis of 20 mm in diameter (Figure 2). The strain was further characterized using biochemical tests (Table 3). The results confirmed that strain M201 was a *Bacillus* sp.

Figure (2): Show zone of hydrolysis around the colony on skim milk agar plate for isolate M201.

Table (3): Biochemical tests for isolate M201.

Biochemical test	Response
Indole	-ve
MR (Methyl red)	-ve
VP	+ve
Starch hydrolysis	-ve
Nitrate reduction	-ve
Pectin hydrolysis	-ve
Sugar fermentation	+ve
Motility	+ve
Blood hemolysis	+ve
Citrate	+ve

The effect of different carbon and nitrogen sources on protease production by *Bacillus* sp. M201 was investigated. The results showed that lactose was the best carbon source, supporting a maximum protease activity of 172 U/ml (Figure 3). The best nitrogen source was yeast extract, which supported a maximum protease activity of 36.8 U/ml (Figure 4).

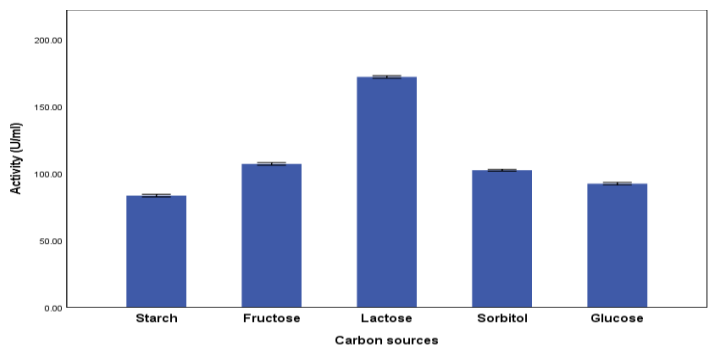

Figure (3): Effect of carbon source on protease activity for isolate M201.

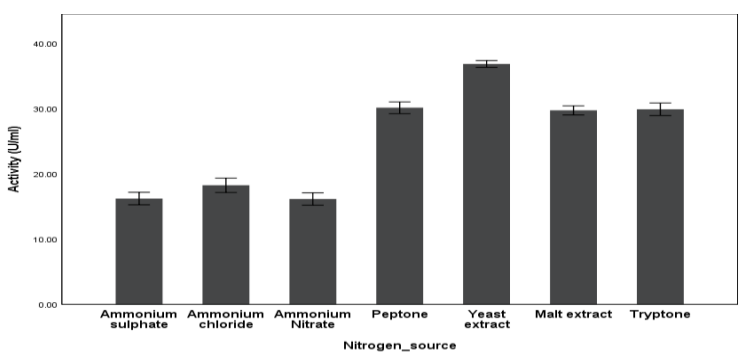

Figure (4): Effect of nitrogen source on protease activity for isolate M201.

The effect of pH and temperature on protease activity was also investigated. The optimum pH for protease activity was found to be 9 (Table 4), and the optimum temperature was 50 °C (Table 5). The results showed a strong positive correlation between pH and protease activity (Figure 5). The effects of various metal ions and inhibitors on protease activity were also studied.

Table (4): Effect of different pH on crude protease activity for isolate M201.

pH	Activity (U/ml) (Mean ±SD)	*P* value
5	32.42±1.9^{c}	< 0.001
6	55.23±0.85^{b}	
7	58.17±2.18^{ab}	
8	58.6±2.62^{ab}	
9	60.89±1.06^{a}	

Data expressed as mean ±SD. *P* value was significant if < 0.05.

Table (5): Effect of different temperature on protease activity for isolate M201.

Temperature (°C)	Activity (U/ml) (Mean ±SD)	*P* value
30	67.7±4.3^{bc}	0.001
40	69.63±4.25^{b}	
50	82.63±2.68^{a}	
70	61.5±2.51^{c}	

Data expressed as mean ±SD. *P* value was significant if < 0.05.

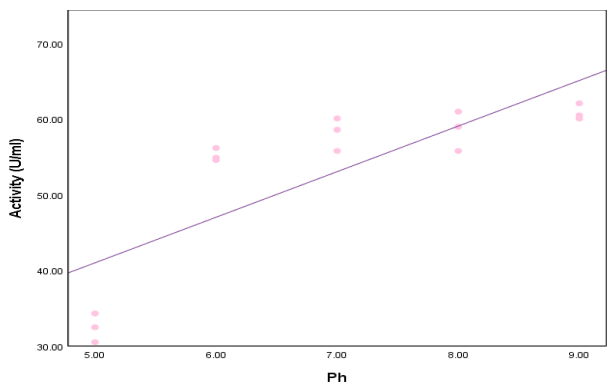

Figure (5): Scatter plot showing strong positive correlation between pH and protease activity.

The results showed that the enzyme was activated by Ca^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} at all concentrations tested. The stimulatory effect on enzyme activity was highest for Ni^{2+} (Table 6). The enzyme activity was not significantly affected by Mg^{2+}.

Table (6): Effect of different concentrations of metals on protease activity for isolate M201.

Metal	Concentration	Activity (U/ml) (Mean ±SD)	*P* value
Co	1	46.93±1.16^{b}	< 0.001
	5	38.3±3.05^{c}	
	10	44.16±0.45^{b}	
	15	53.1±2.56^{a}	
Mg	1	36.16±1.06	0.774
	5	38.6±4.03	
	10	38.4±4.06	
	15	39±4	
Mn	1	11.46±2.3^{c}	< 0.001
	5	21.76±0.62^{a}	
	10	14.03±0.95^{b}	
	15	13.33±0.47^{bc}	
Ca	1	16.5±4.1^{a}	0.006
	5	17.4±2.42^{a}	
	10	26.43±0.87^{b}	
	15	20.03±1.79^{a}	
Ni	1	26±0.89^{b}	< 0.001
	5	51.53±4.3^{a}	
	10	51.8±3.86^{a}	
	15	51.67±4.3^{a}	

Data expressed as mean ±SD. *P* value was significant if < 0.05.

The results showed a strong positive correlation between Ni^{2+} concentration and protease activity (Figure 6). The enzyme was inhibited by EDTA and SDS at all concentrations tested. EDTA showed a strong inhibitory effect on protease activity (Figure 7). The results showed a strong negative correlation between EDTA and SDS concentration and protease activity (Figures 7 and 8).

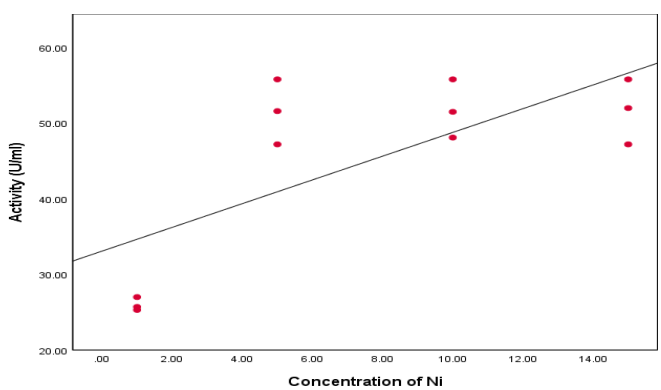

Figure (6): Scatter plot showing strong positive correlation between Ni concentration and protease activity.

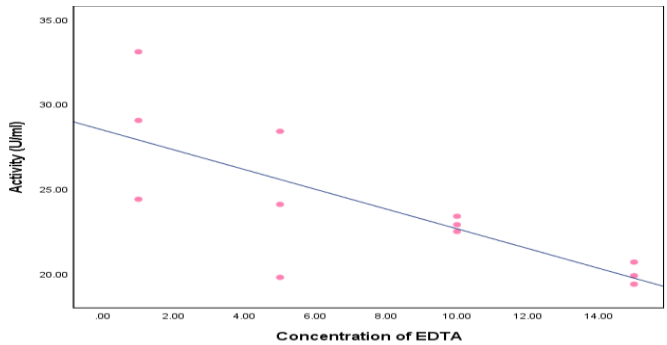

Figure (7): Scatter plot showing strong negative correlation between EDTA concentration and protease activity.

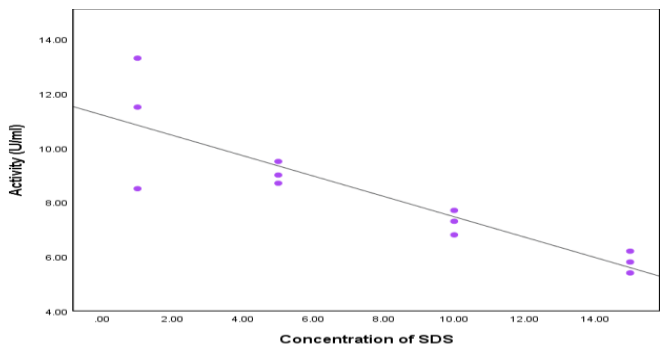

Figure (8): Scatter plot showing strong negative correlation between SDS concentration and protease activity.

The protease enzyme was inhibited by EDTA and SDS at all concentrations tested (Table 7). EDTA showed a strong inhibitory effect on protease activity. The results showed a strong negative correlation between EDTA and SDS concentration and protease activity (Table 8).

Table (7): Effect of different concentrations of inhibitors on protease activity for isolate M201.

Inhibitors	Concentration	Activity (U/ml) (Mean ±SD)	*P* value
EDTA	1	28.84±4.34^{a}	0.045
	5	24.1±4.3^{ab}	
	10	22.93±0.45^{ab}	
	15	20±0.65^{b}	
SDS	1	11.1±2.42^{a}	0.005
	5	9.07±0.4a^{b}	
	10	7.26±0.45^{bc}	
	15	5.8±0.4^{c}	

Data expressed as mean ±SD. *P* value was significant if < 0.05. Values followed by different letters are significantly differed.

Table (8): : Correlation between activity and other factors.

Factors		
pH	R	.806
	P	< 0.001
Temperature	R	-.240
	P	.453
Concentration of metals		
Co	R	.529
	P	.077
Mg	R	.285
	P	.369
Mn	R	-.105
	P	.745
Ca	R	.489
	P	.107
Ni	R	.718
	P	.008
Concentration of Inhibitors		
EDTA	R	-.753
	P	.005
SDS	R	-.881
	P	< 0.001

Date expressed as *r* value (*p* value). *P* value was significant if < 0.05.

4. Discussion

In the present study, a total of thirty bovine milk samples were collected and processed by serial dilution and the spread plate method. Ninety-five different bacterial strains were isolated from different samples of bovine milk from Assuit governorate, Egypt. Proteolytic activity was performed for isolates bacterial separated on skim milk agar. Strain M201 was chosen for further investigation because of the maximum diameter of the clear zone (Figure 2). Strain (M201) was identified based on morphological characterization (Table 1) and biochemical tests (Table 3). The results showed that, strain M201 is a motile, Gram-positive, rod-shaped, spore-forming bacterium that was identified as a *Bacillus* strain (Figure 1) (Kim *et al*., 1998). The standard media was optimized with different carbon and nitrogen sources. Different carbon sources, such as glucose, fructose, lactose, starch and sorbitol, were tested to evaluate their effect on extracellular protease production by the bacterial strain M201. There is a significant effect on protease activity with a carbon source. Lactose supports maximum protease production of 172 U/ml (Figure 3), similar to El-Safey and Abdul-Raouf (2004), Ananthan (2014), and Asha and Palaniswamy (2018). Carbon sources influenced protease production considerably, and different bacteria utilized different carbon sources for their growth and metabolism. Tambekar and Tambekar (2013) reported a halophilic bacterium, *Bacillus odysseyi,* which utilized lactose as a carbon source for maximum protease

production. Production of protease has been studied in the presence of different organic and organic nitrogen sources (Figure 4). Among all, yeast extract showed maximum enzyme activity of 36.8 U/ml, in agreement with Hamza and Woldesenbet (2017). Reaction media pH can affect protease activity. To evaluate this information, the enzyme activity over the pH range (5-9) was assayed. The activity of protease increased under an alkaline condition. The maximum activity of the protease enzyme is at pH 9 (Table 4). Enzyme activity increased with increasing temperature, as shown in Table 5. There was a significant increase in enzyme activity between 30°C and 50°C. Enzyme activity decreases when the temperature rises to 70°C. The optimum temperature was found to be 50 °C, in agreement with Gupta *et al.* (2005). The effect of various metal ions at concentrations of 1, 5, 10 and 15 mM on crude protease was investigated (Table 6). Stimulatory effects were observed for Ni, Mn, Ca and Co at each concentration. This phenomenon indicates that, the enzyme requires metal ions as cofactors. The stimulatory effect on enzyme activity was found to be Mn and Ni in agreement with Deng *et al*. (2010). The enzyme activity was not significantly affected by Mg. The result shows a strong positive correlation between Ni concentration and protease activity (Figure 6). The effects of various enzyme inhibitors on enzyme activity were studied and reported in Table (7). Protease activity was inhibited by EDTA and SDS (1, 5, 10 and 15 mM). EDTA concentrations show a strong inhibitory effect on protease activity (Figure 7).

5. Conclusion

It is well recognized that, microorganisms are essential to the technology used to produce intracellular and extracellular enzymes on a large scale. Proteases are enzymes that catalyze the hydrolysis of hydrolytic bonds in proteins, releasing peptides and/or amino acids Proteases are most commonly obtained from the genus *Bacillus.* Protease production was increased by optimizing the culture conditions, these included physical parameters like incubation time, pH, and inoculum size. The purified enzyme was found to be stable at pH 5–9 and the activity of protease increased under an alkaline condition with the optimum pH estimated at pH 9 and thermostability at 50°C. Therefore, the protease enzyme produced from isolate M201 can be used for various industrial, commercial, and environmental purposes.

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