UNIVERSITY OF SINDH JOURNAL OF ANIMAL SCIENCES
Vol. 2, Issue 4, Pp: (3-11), December, 2018
Email: editor.usjas@usindh.edu.pk
Website: http://sujo2.usindh.edu.pk/index.php/USJAS
ISSN (P): 2521-8328
ISSN (E): 2523-6067
Published by University of Sindh, Jamshoro
(USJAS)

CHARACTERIZATION OF CRUDE PROTEASE PRODUCED BY *PLEUROTUS ERYNGII* ATCC 90888

Safia Bano, Muhammad Umar Dahot, Syed Habib Ahmed Naqvi, and Abdul Sattar Qureshi

Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan.

ARTICLE INFORMATION

Article History:
Received: 07. Nov.2018
Accepted: 23rd March 2019
Published online: 5th July 2019

Author's contribution
SB performed the experiments, MUD designed the study, SHAN analysis the data & ASQ complied the data.

Key words:
Protease, ATCC, pH , temperature, substrate, Mercaptoethano

ABSTRACT

In this study, research was carried out to characterize protease produced by *Pleurotus eryngii* ATCC 90888. Maximum protease production was noted at 22 °C, pH was adjusted to 6.5 after 96 hours. Sucrose and casein were used as carbon and nitrogen sources. Protease activity was characterized in terms of reaction time, enzyme and substrate concentration, pH, temperature, stability of enzyme (pH & temperature), activator and the inhibitors. Proteases produced by *Pleurotus eryngii* shown highest activity when 0.5 mL and 1.5% enzyme volume and substrate concentration respectively were used and reaction was incubated for 60 min. However, protease was active at 60 °C and pH 8, but enzyme was found to be stable at about 80 °C. Enzyme was also activated with divalent metal ions as well as with sulphur group (Cysteine, Mercaptoethanol).

1. INTRODUCTION

Proteases are large group of hydrolytic enzymes which split peptide bond of protein into amino acids [1]. Proteases are the one of the largest group of industrial enzyme among three industrial enzymes including lipase, amylase, and protease [2]. Proteases accounting for approximately 60% of industrial enzyme market due their applications mainly in detergent, leather and other industries [1]. Alkaline proteases are most demanded due to their specificity, stability toward pH, salt tolerance, thermostability and stability in presence of organic solvents, metal ions and surfactants. Hence alkaline proteases producing organisms have gained more attention of the scientific community [3].

Corresponding Author: sattar.qureshi@usindh.edu.pk

Demand of heat stable proteases is continuously increasing due to high industrial processing temperatures that enhance the rate of reaction and solubility of non-gaseous reactants. Recently, protein engineering and genetic engineering are employed in enzyme technology to alter the characteristics of enzyme according to industrial needs [2]. Additionally, protease gene overexpression also improved enzyme yield in the production medium [2]. Efficiency of protease is influenced by various factors including pH, ionic strength of buffer solution, temperature, detergents etc. Thus, suitability of enzyme for any application specially detergents needs protease of specific characteristics. This is why need for newer enzymes with unique characteristics keeps researchers busy in searching new microbial strains those could secret novel enzymes and other bioproducts [2, 4].

Now a days edible mushrooms (higher fungi) also considered as a protease producing organism. Many mushrooms have been reported for protease production for example *Agaricus bisporus*, *Armillariella mellea*, *Cordyceps sinensis*, *Flammulina velutipes*, *Grifola frondosa*, *Helvella lacunosa*, *Lyophyllum cinerascens*, *Pleurotus eryngii*, *Pleurotus ostreatus*, and *Pleurotus citrinopileatus* of protease enzyme under submerged fermentation. In present study I used *Pleurotus eryngii* ATCC 90888.

2. MATERIALS AND METHODS

Microorganism

Pleurotus eryngii ATCC 90888 was gifted from the Edible Fungi I. S. A. Shanghai, China. . This strain was used in our previous studies for xylanase production [5] Culture was maintained on potato dextrose agar medium [6].

Inoculum development

50 mL pre fermentation media was used for inoculum preparation (mushroom) in 250 mL Erlenmeyer flasks on a rotary shaker at 120 rpm and 27 ± 2°C. Medium contained 20 g of glucose, 10 g of peptone, 3mL of corn steep liquor , 1.0 g of NH_4NO_3 and 0.5 g of $MgSO_4 \cdot 7H_2O$ (per liter). pH of medium was adjusted at 5.5 by using 0.1 N HCl or NaOH. After 4 days, pallets were collected and homogenized to use as inoculum for enzyme production medium.

Culture conditions

Protease enzyme was produced in optimized fermentation medium including 2.0% glucose, 1% peptone,0.3% corn steep liquor and 0.1%, NH_4NO_3 which was prepared 50 mL in 250 mL conical flask and initial pH of the production medium was set at 6.5. The media was sterilized at 15 pounds/inch pressure for 20 min. The each sterilized flask containing medium was introduced with 1.0 mL inoculum of mushroom (*Pleurotus eryngii)* and incubated at 25±2ºC in an orbital shaking incubator (gallenkamp). After incubation time period which was set at 48 hours' time of interval biomass was filter from culture broth through Whatman filter paper No.1 and culture broth was used for further analysis.

Enzyme Assay

Protease activity was determined according to Penner method. 0.5 mL of 1% w/v casein solution as substrate was taken and 0.5mL crude enzyme was added. The reaction was mixed well then 1 mL of phosphate buffer (pH 7.6) was added and reaction was incubated at 35 ºC for 1 h. The reaction was stopped by adding, 2 mL of 15% trichloroacetic acid TCA solution. after the completion of incubation time reaction mixture was centrifuged for 10 minutes at 4000 rpm then aliquot 1 mL of supernatant , 4 mL of 0.5 N sodium hydroxide, and 1 mL diluted Folin-Ciocalteu reagent (1:1 v/v with water) were mix with 4 mL double distilled water then reaction was incubated for 30 min 37 °C [7]. Optical density was checked after completion of reaction against blank at 625 nm.

3. RESULT

In previous study we focused on optimization of cultural conditions for protease production using *Pleurotus eryngii* ATCC 90888. Maximal *Pleurotus eryngii* growth was attained when cultured on potato dextrose agar medium (24 ºC, pH 5.6, 7 days). Highest protease secretion was noted after 4 days incubation in mineral medium containing 5 g/L sucrose and 10 g/L of casein as carbon and nitrogen sources, at pH 6.5 and 22 ºC. Crude enzyme was extracted from fermented medium that was used for characterization. Characterization of protease was investigated in terms of reaction time, substrate and enzyme concentration, pH and temperature, pH and thermostability and effect of metal ions. Protease reaction with substrate was checked for different time period ranging from 10 to 150 min. Protease activity increased with reaction time upto 60 min then declined, results are shown in figure-1.

Effect of substrate concentration was investigated on the rate of enzymatic reaction by using different concentration of casein (as substrate) ranging from 0.5-2.5%. Figure 2 shows that 1.5% casein gave maximal protease activity while below and above this concentration lower activities were noted. Effect of enzyme volume (0.1-0.5 mL) was also checked on

protease activity produced from *Pleurotus eryngii*. Enzyme activity was found directly proportional to enzyme concentration, highest protease activity was found using 0.5 mL crude enzyme, results are depicted in figure 3.

Enzyme remains active and stable in suitable conditions are also known as optimum conditions for that enzyme. Optimum conditions varies from enzyme to enzyme and also varies with microorganisms used. pH is one of the important physical parameter that affects the activity and stability of protease. Figure 4 shows the effect of pH and pH stability on protease activity using glycine sodium hydroxide and sodium phosphate buffer ranging from pH 1.0-12.0. Protease activity increased with increase in pH upto pH 8.0 and one further increase in pH decreased the protease activity. Protease also shown similar pattern in the pH stability. More than 70% activity was observed at pH 9.0 after 1 h.

Temperature is an essential parameter for enzyme activity and stability. Rate of enzymatic reaction increases with temperature due to increase in collision of molecules and increase in activation energy. Crude protease produced from *Pleurotus eryngii* was characterized in different temperature ranges 30-80 °C. Protease activity increased with temperature upto 60 °C on further increase in temperature decreased the enzyme activity due to inactivation of enzyme. Thermostability of protease was also checked by incubating the crude enzyme at different temperature (30-80°C) for 10 min and nearly 100% stability for enzyme was observed upto 60 °C, results are presented in figure 5.

Protease activation or inhibition was observed in presence of 5 mM different metal ions and other compounds. Enzyme was incubated with 0.1 mL of different compounds for 10 min at optimized temperature then substrate was added and enzyme activity checked according to reported protocol. Protease activity was reduced to 33% in presence of mercuric chloride and 69% in presence of copper sulphate whereas protease activity was highly stimulated by addition of cobalt chloride and triton X-100 more than 240% as compared to control.

4. DISCUSSION

In this study, edible mushroom specie (*Pleurotus eryngii*) was used for protease production and strain was found highly capable of secreting proteolytic activity in submerged condition using simple medium. Protease production was observed during the early stationary phase of growth in concomitance with the sporulation process [8]. Protease production from mushroom was characterized for its applications. Enzyme was characterized in terms of time, substrate and enzyme concentration, pH, temperature, surfactants, oxidizing and bleaching agents, and organic solvents is required for the local detergents formulations, leather industry, and synthetic biotechnology.

Proteolytic activity increased with reaction time upto 60 min then enzyme activity declined. This happened probably due to product inhibition [9, 10] or due to bye-products of enzymatic reaction those possibly inhibit protease activity or due to denaturation of enzyme structure [11]. Inhibitory action of other metabolite present in fermented medium could also be the reason for decline in enzyme activity [12, 13].

Figure 2 shows the effect of substrate concentration, protease activity reduced after certain range of substrate concentration. This happened probably due to changes in enzyme and substrate ratio [12, 14]. Similar patterns are observed by [15] they obtained maximum enzyme activity at 1.5% (15g/L). Actually performance of all enzyme present in sample, became saturated with the substrate and the activity reached a maximum value. So when we add more substrate that inhibited the enzyme and hence the observed decline in relative activity.

Enzyme activity is directly proportional to enzyme concentration. Figure 3 shows 5-fold increase in enzyme concentration only enhances the enzyme activity to 20% suggesting that low enzyme concentration could be economical for industrial application [15].

Protease was stable in wide pH range from 3 to 8 and retained 70% of its original activity at pH 9.0 after 60 min, maximum activity was noted at pH 8 by [16] also reported similar results and 60% of activity was retained at pH 9.

Effect of temperature and thermostability of crude protease was investigated. Enzyme activity increased with increase in temperature upto 60 °C on further

increase in temperature decreased the enzyme activity due to inactivation of enzyme. It is characterized by [15] the crude protease from *Bacillus subtilis* EFR01 and enzyme activity increased upto 50 ^{o}C. Protease produced from Bacillus sp BBXS-2 showed maximum enzyme activity upto 60 °C. Characterized the crude protease from *Bacillus subtilis* EFR01 and enzyme activity increased upto 50 ^{o}C [15]. Protease produced from Bacillus sp BBXS-2 showed maximum enzyme activity upto 60 °C. Qureshi et al. found thermostable protease from Bacillus sp and enzyme retained 95.22, 75.46 and 26.67% of its initial activity after 1 h incubation at 60, 70 and 80°C, respectively [17]. The present results are in agreement with the results of Lakshmi, et al where enzyme shown maximal activity at 70 °C [18] and Cui, et al., ., (cui et al reported protease stability in wide temperature ranges between 20 and 70∘ C). It has been observed maximum activity at 55 ºC [19, 20]. Thermostability of protease was also checked by incubating the crude enzyme at different temperature (30-80°C) for 10 min and nearly 100% stability for enzyme was observed upto 60 °C, results are presented in figure 5. Purified protease from *Cordyceps sobolifera* after purification the maximum enzyme activity was noted at at 65^{o}C by [21] so thermophilic microorganisms could produce thermostable enzyme at high temperature and this might eliminate the chances of contamination during fermentation. However, isolation of thermophilic microorganisms is certainly required to meet increasing demands for thermostable proteases. Our study provides a suitable source of thermostable protease production from newly isolated thermophilic bacterial strains, this enzyme could be a good candidate for various industrial processes

Protease activity was checked for activation and inhibition. Protease activity reduced to 33% in presence of mercuric chloride whereas protease activity was highly stimulated by addition of cobalt chloride and triton X-100 more than 240% as compared to control. In present study addition of EDTA activity reduced to 91%. Similar results were reported by Femi-Ola et al. enzyme activity was inhibited by addition of EDTA [22]. In our study, addition of $CaCl_2$ enhanced enzyme activity to 104% as compared to control. Similar results were observed by [20] where enzyme activity increased by the addition of $CaCl_2$ in reported assay method.

Effect of time was check on protease activity with 30 min time of interval. Enzyme was mixed with substrate and incubate at different time. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation Figure 1. Effect of substrate concentration on enzyme activity. Enzyme and with different concentrations were incubate at 35^{o}C for 60min. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation Figure 2. Effect of enzyme volume on activity of protease .Different volume of enzyme mix with 1.5% substrate and incubate at 35^{o}C for 60min. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation Figure 3. Effect of pH on protease activity and stability. Percentage of relative activity was measured by incubating enzyme with substrate at different pH values (1-12). pH stability of crude protease was determined by measuring protease activity at 35^{o}C for 60min. prior addition of substrate, and % of relative activity was determined. Results are the average of triplicate experiment Figure 4. Effect of temperature on protease activity. Enzyme was mixed with substrate and incubated at different temperature for 60min. % of relative activity was measured under assay conditions. Thermostability of protease was determined by incubating enzyme at different temperature without addition of substrate for 10min. Percentage of relative activity was checked under assay conditions. Results are the average of triplicate experiment Figure 5. Effect of metal ions/compounds on crude protease at 60°C, pH 8. Results are shown as percentage of relative activity comparing to that of control (no additive). The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation Figure 6.

5. CONCLUSION

In this study, crude enzyme produced by *Pleurotus eryngii* was characterized and enzyme was pH and thermostable in nature that makes it suitable for industrial uses. Proteases produced by *Pleurotus eryngii* shown highest activity when 0.5 mL and 1.5% enzyme volume and substrate concentration, respectively were used and reaction was incubated for 60 min. However, protease production was active at 60°C and pH 8, but enzyme was found to be stable at about 80 °C. Enzyme was also activated with divalent metal ions as well as with sulphur group (Cysteine, Mercaptoethanol) also. It could be concluded that protease appropriate for industrial application due to its pH and thermostability.

REFERENCES

1. Sales, A.E., T.P. Nascimento, C.S. Porto, A.L.F. Porto, and T.S. Porto,(2015) *Purification and cherecterization of Novel Protease with Fibronolytic activity from Mucor subtilissimus UCP 1262.* Blucher Chemical Engineering Proceedings. **1**(2): p. 1216-1223.
2. Gupta, R., Q. Beg, and P. Lorenz,(2002) *Bacterial alkaline proteases: molecular approaches and industrial applications.* Applied Microbiology and Biotechnology. **59**(1): p. 15-32.
3. Imrana Khushk , S.B., Abdul Sattar Qureshi , Muhammad Aqeel Bhutto, Altaf Ahmed Simiar and Abdul Nabi Jatt (2017) *Study of nutrient media components and cultivation conditions of Bacillus licheniformis BCC-02-50 for protease production using molasses as energy source* **Volume 04** (Issue 06): p. 552 -562
4. Rao, M.B., A.M. Tanksale, M.S. Ghatge, and V.V. Deshpande,(1998) *Molecular and biotechnological aspects of microbial proteases.* Microbiology and Molecular Biology Reviews. **62**(3): p. 597-635.
5. Altaf, S.A., D.M. Umar, and M.S. Muhammad,(2010) *Production of xylanase enzyme by Pleurotus eryngii and Flamulina velutipes grown on different carbon sources under submerged fermentation.* World Applied Sciences Journal. **8**: p. 47-49.
6. Lin, J., L.M. Ndlovu, S. Singh, and B. Pillay,(1999) *Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, Thermomyces lanuginosus-SSBP.* Biotechnology and Applied Biochemistry. **30**(1): p. 73-79.
7. Penner, D. and F.M. Ashton,(1967) *Hormonal control of proteinase activity in squash cotyledons.* Plant Physiology. **42**(6): p. 791-796.
8. Safia Bano, M.U.D.a.S.H.A.N.,((2016)) *OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF PROTEASE BY Pleurotus eryngii.* Pak. J. Biotechnol. . **Vol. 13 (3)**: p. 193-198
9. Galliard, T.,(1971) *The enzymic deacylation of phospholipids and galactolipids in plants. Purification and properties of a lipolytic acyl-hydrolase from potato tubers.* Biochem. J. **121**: p. 379-390.
10. Gatt, S.,(1968) *Purification and properties of phospholipase A 1 from rat and calf brain.* Biochimica et Biophysica Acta (BBA)-Enzymology. **159**(2): p. 304-316.
11. Bruzik, K.S. and M.-D. Tsai,(1994) *Toward the mechanism of phosphoinositide-specific phospholipases C.* Bioorganic & Medicinal Chemistry. **2**(2): p. 49-72.
12. Lakshmi , P.V.R.s., K. Ambika Devi , KP.J.Hemalatha,,(2014) *Screening , Optimization of production and partial cherecterization of Alkaline protease from Haloalkaliphilic Bacillus sp.* International Journal of Research in Engineering and Technology **eISSN: 2319-1163** p. pISSN: 2321-7308
13. Cui, H., L. Wang, and Y. Yu,(2015) *Production and Char acterization of Alkaline Protease from a High Yielding and Moderately Halophilic Strain of SD11 Marine Bacteria.* Journal of Chemistry. **501**: p. 798304.
14. Sandhya, T. and D. Tambekar,(2013) *Optimization of the production and partial characterization of an extracellular alkaline protease from thermohalo-alkalophilic Lonar lake bacteria.* Bioscience Discovery. **4**(1): p. 30-38.
15. Qureshi, A.S., I. Khushk, Y. Chisti, M.A. Bhutto, A.A. Simiar, and M.U. Dahot,(*Characterization of crude proteases of Bacillus subtilisEFRL 01.*
16. Maruthiah, T., P. Esakkiraj, G. Prabakaran, A. Palavesam, and G. Immanuel,(2013) *Purification and characterization of moderately halophilic alkaline serine protease from marine Bacillus subtilis AP-MSU 6.* Biocatalysis and Agricultural Biotechnology. **2**(2): p. 116-119.

17. Qureshi, A., A. Simair, C. Ali, I. Khushk, J. Khokhar, A. Ahmad, M. Danish, and C. ChangruiLu,(2018) *Production, Purification and Partial Characterization of Organo-Solvent Tolerant Protease from Newly Isolated Bacillus sp*. BBXS-2. Ferment Technol. **7**(151): p. 2.
18. Lakshmi, B., P.R. Sri, K.A. Devi, and K.J. Hemalatha,(2014) *Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic Bacillus sp*.
19. Nilegaonkar, S., V. Zambare, P. Kanekar, P. Dhakephalkar, and S. Sarnaik,(2007) *Production and partial characterization of dehairing protease from Bacillus cereus MCM B-326.* Bioresource Technology. **98**(6): p. 1238-1245.
20. p Pathak, A. and M.G. Rathod,(2013) *Bioprospecting an alkaline ecosystem of Lonar Lake for novel alkaline protease producer*. Innovative Research in Chemistry. **1**(1): p. 22-26.
21. Wang, S.-X., Y. Liu, G.-Q. Zhang, S. Zhao, F. Xu, X.-L. Geng, and H.-X. Wang,(2012) *Cordysobin, a novel alkaline serine protease with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom Cordyceps sobolifera*. Journal of bioscience and bioengineering. **113**(1): p. 42-47.
22. Femi-Ola, T.O., O.P. Akinsanmi, and O.S. Bamidele,(2014) *Production and Characterization of Protease from Serratia marcescens.* AU Journal of Technology. **18**(1).

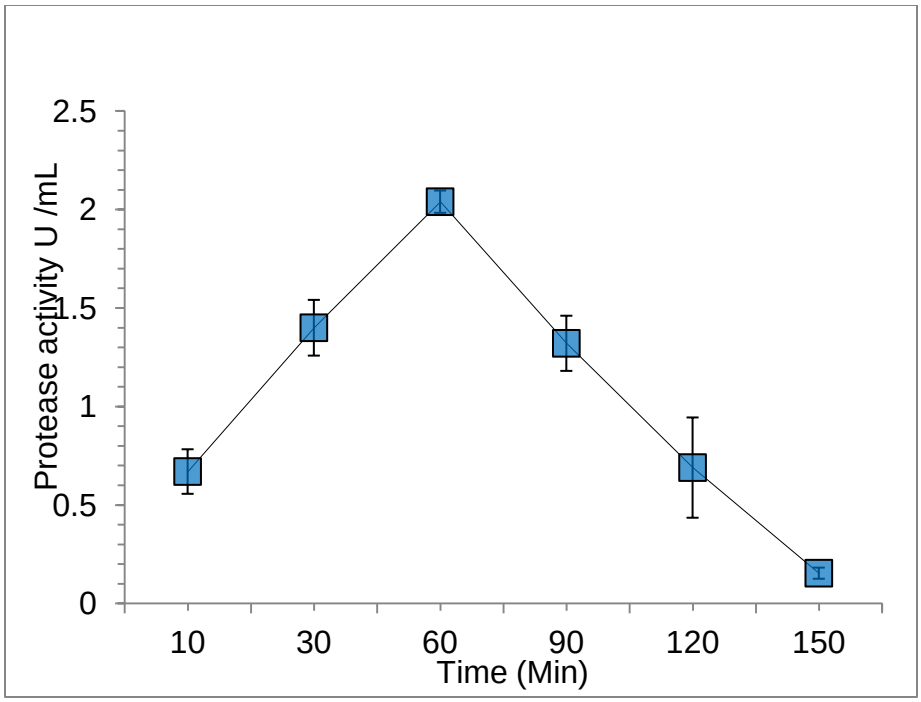
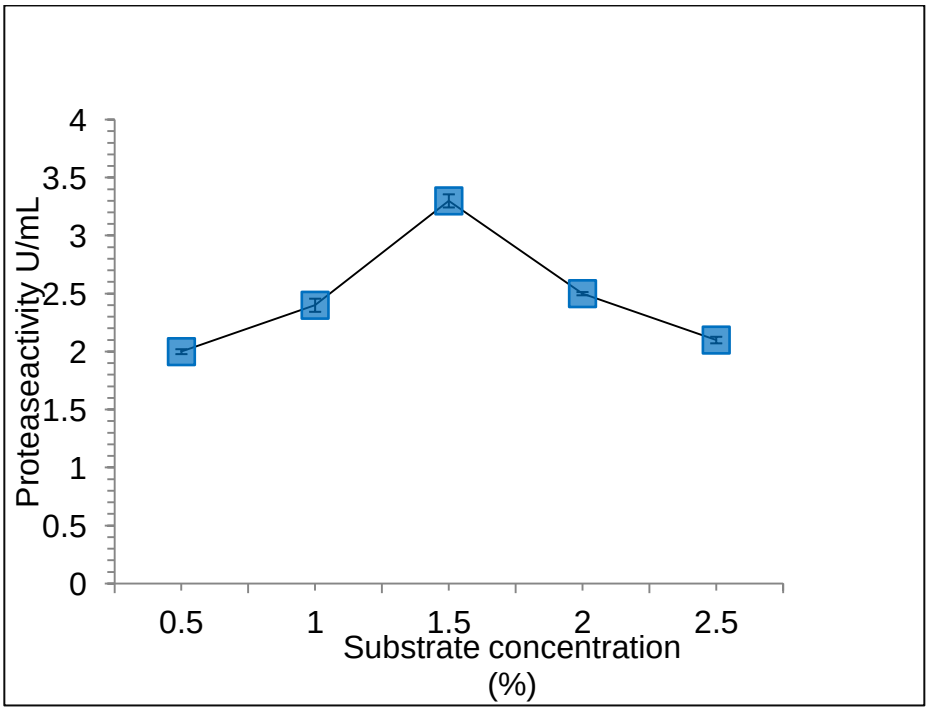


Figure. 1. Effect of time



.

Figure. 2. Effect of substrate concentration

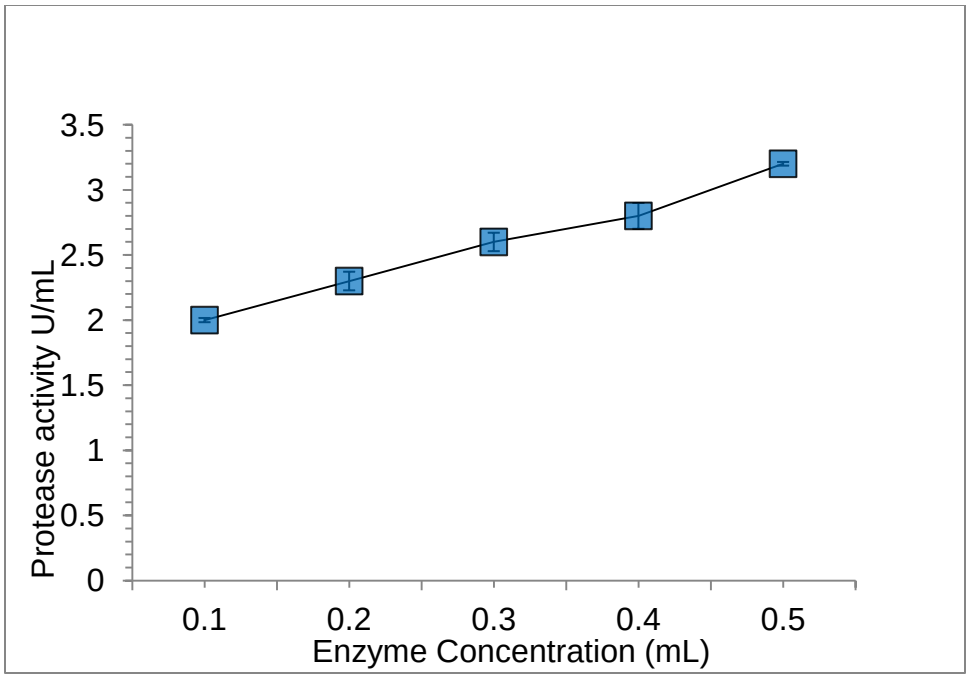


Figure. 3. Effect of enzyme concentrations

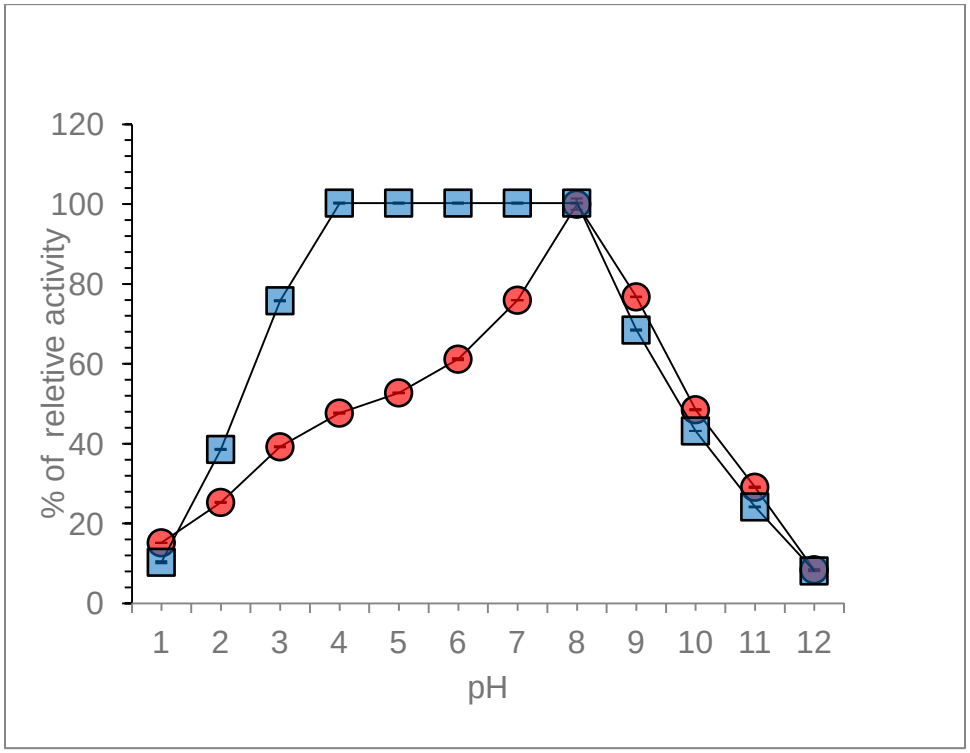


Figure. 4. Effect of pH and pH stability

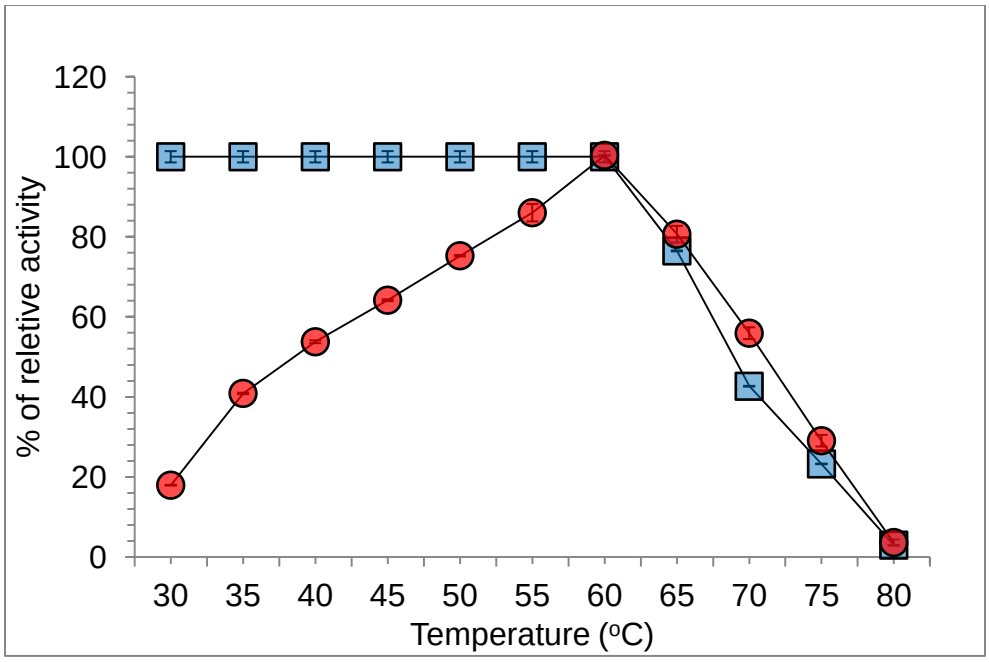


Figure. 5. Effect of temperature and temperature stability

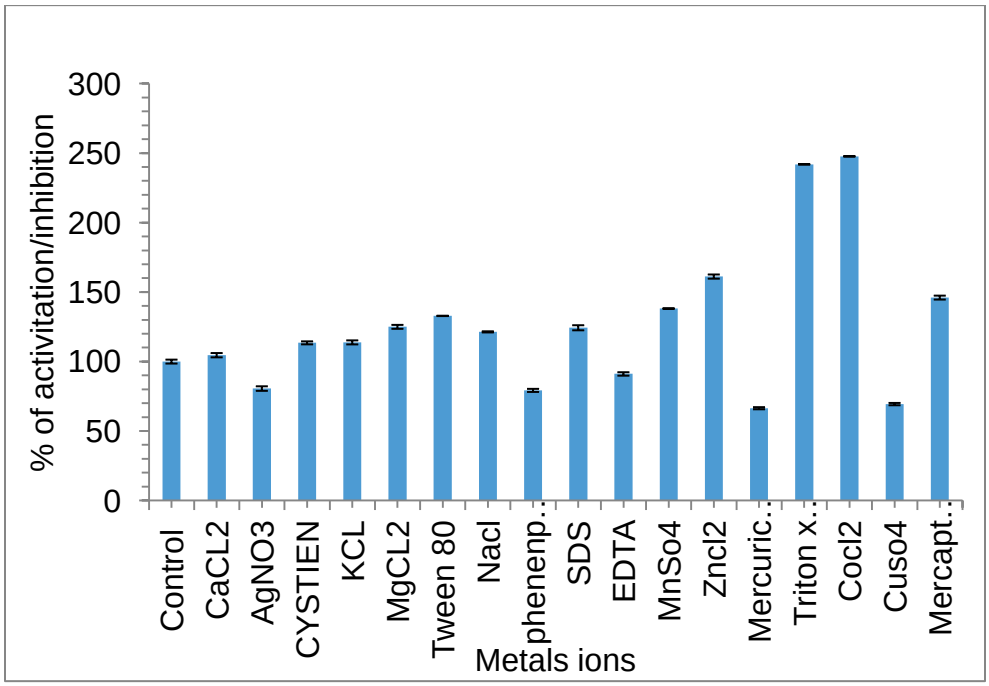


Figure. 6. Effect of metal ions /com