Open Access Journal | Journal of Progressive Research in Biology (JPRB)

ISSN 2454-1672

SCITECH RESEARCH ORGANISATION

Volume 2, Issue 1

Published online on July 15, 2015

Journal of Progressive Research in Biology
www.scitecresearch.com/journals

Characterization of the Protease Produced by Rhizophus Stolonifer Purified from Bread

Ranganathan Kapilan
Department of Botany, University of Jaffna, Sri Lanka.

Abstract

Rhizophus species are capable of producing diverse enzymes. Proteases are one of the enzyme types that are produced by large number of microbes and they break up the peptide bonds of proteins to release amino acids. The study was aimed to determine the types of enzymes produced by Rhizophus stolonifer strain purified from bread and to characterize the proteases produced by this fungal strain. A Rhizophus stolonifer strain was isolated and purified from 48 hours old moist bread and screened for the production of diverse group of enzymes. Selected Rhizophus stolonifer strain grew well and produced proteases, amylases and xylanases at room temperature and pH 7.0 and a pure form of this strain was used to study the kinetic properties of the crude protease. The protease was extracted with 0.1M phosphate buffer of pH 7.0 and at 25^{o}C and it showed zero order kinetics for 10 minutes. The crude protease activity was higher at pH 8.0 and 30^{o}C. Michaelis constant for the crude enzyme to soluble substrate was 624.9µM and Vmax was 136.4 pmol/min at pH 8.0 and 30^{o}C.

Keywords: Rhizophus Stolonifer; Protease; Michaelis Constant; Vmax.

Introduction

Enzyme proteases are produced by wide range of animals, plants and microorganism. On the basis of various economical, technological and ethical issues, microorganisms are the best sources of proteases (Kelly et al. 1976). Proteases account about 60% of total enzyme market and among the valuable commercial enzymes, are the single largest class of enzymes occupying a pivotal position due to their wide range of application in the industrial processes. Microbes serve as the preferred source of proteases because of their rapid growth, limited space required for their cultivation and the ease of producing enzymes with altered properties (Rao et al. 1998). Protein is catabolised by hydrolysis of the peptide bonds that link adjacent amino acids together in the polypeptide chain that forms protein (Paudel and Parajuli, 1999). Consistent with these important contributions of proteases in cell and survival and death of all organisms, alterations in proteolytic enzyme systems underlie multiple pathological diseases (Christeller, 2005). Rhizophus species produce a huge variety of extracellular enzymes such as protease, amylase and xylanase which are of great industrial importance. There are a lot of factors that influence metabolic processes and protease synthesis and function. There is a huge expectation on the thermophilic and extremely thermophilic microorganisms and their enzymes (Grubb and Lofberg, 1982). Rhizophus stolonifer is a widely distributed thread-like Mucoralean mold. It is commonly found on bread surfaces, it takes food and nutrients from the bread and causes damage to the surface where it lives. The Black Bread Mold causes rotting of fruits and infects humans. Rhizopus stolonifer work as decomposers in soil, dung, and in many foods. They grow inside food and absorb nutrients and dissolve the substrate with extracellular enzymes. The Objective of this research was to test the enzymes produced by Rhizopus stolonifer and to characterize the extracellular protease enzyme.

ISSN 2454-1672

Materials and Methods

Culture of Rhizophus Stolonifer

The 24 hour old moist bread was kept in the laboratory for 24 hours and the colonies were streaked on a PDA plate and grown at 48 hours at room temperature. Colonies grown were transferred on to new sterile PDA plates and incubated. Selection of Rhizophus stolonifer was done by streak plate technique, on PDA plates. The pure fungal cultures were streaked uniformly over the PDA media and incubated for 48 hours at room temperature. Eight pure colonies of Rhizophus were selected based on the observed fungal structures such as sporangium, stolen and rhizoids seen through light microscope.

Screening of Rhizophus Stolonifer

The selected pure strains were screened for the production of extracellular protease, amylase and xylanase enzymes.

Analytical Methods

Testing the Protease Production by Rhizophus Stolonifer

The pure cultures were streaked as a line on the skim milk agar plates and plates were incubated at 25^{o}Cfor 48 hours. The isolates producing clear zones of hydrolysis were considered as protease producers.

Testing the Amylase Production by Rhizophus Stolonifer

Starch agar plates were prepared and inoculated with 5mm diameter cork bore inoculum of the selected isolate. Amylase production was detected by the development of clear zone with iodine after 2 days of incubation at room temperature (Vengadaramana et al., 2011).

Testing the Xylanase Production by Rhizophus Stolonifer

Liquid xylan broth (490 mL) was prepared and 10 mL of overnight liquid culture of Rhizophus stolonifer was inoculated and maintained at 25^{o}C for 48 hours in a shaker incubator (Kapilan, 2015). Xylanase activity was determined by measuring the amount of xylose produced. Here the xylose produced was measured by Dinitrosalicylic acid (DNSA) method (Miller, 1959). One unit of xylanase activity is defined as the amount of enzyme that produces one µmol of reducing sugar in one minute at pH 7.0 and 30^{o}C with 20 gL^{-1} xylan.

Crude Protease Production by Rhizophus Stolonifer and Enzyme Assay

10 mL of overnight liquid culture of Rhizophus stolonifer was used to inoculate 0.5L Erlenmyer flask containing 490 mL aliquots of liquid production medium consist of glucose 0.5% (wt/vol), peptone 0.75% (wt/vol), KH_2PO_4 0.5% (wt/vol), $MgSO_4 4.7H_2O$ 0.05% (wt/vol), $FeSO_4.5H_2O$ 0.01% (wt/vol), pH–7.0 and maintained at 25^{o}C for 48 hours in a shaker incubator. The pH of the medium was adjusted by 1N NaOH or 1N HCl. After the completion of fermentation, the whole fermentation broth was centrifuged at 15,000 rpm at 4^{o}C for 15 minutes and the clear supernatant was recovered. The crude enzyme supernatant was used for further studies. The Protease activity in the crude enzyme extract was assayed by using 1% casein in citrate buffer (pH 7). The reaction mixture contains1ml casein and 1ml crude enzyme extract and allowed to stand for 1hr at the room temperature. After 1hr, 5 ml TCA solution was added to stop the enzymatic reaction. After addition of the TCA, the tubes were shaken and then contents were centrifuged at 10000 rpm for 15mins for the sedimentation of the pellet. The supernatant was collected from the centrifugal tubes, and to this supernatant 5ml of NaOH solution was added and allowed to stand for another 15mins. Finally 0.5ml of FC reagent was added and the intensity of blue color was measured at 700nm within half an hour. One unit of enzyme activity was defined as the amount of enzyme that released 1µg of tyrosine mL–1 of crude enzyme per hour.

Kinetic Studies of the Crude Protease from Rhizophus Stolonifer

Effect of pH

For which different pH from 3 to 10 were prepared for the use as buffer. 30µl of enzyme extract was mixed with 50 µl of BSA standard solution (1mg/ml) and the volume was maintained to 200 µl by adding buffer of different pH and after incubation of 30 min, 2.3ml of Bradford reagent was added. The absorbance was noted after 5 min of extra incubation with UV-ray spectrophotometer at 595 nm. The activity was calculated with reference to two controls one is enzyme control having 30 µl of enzyme only and the next one is BSA control having 50µl BSA only.

Effect of Temperature

The effect of temperature on protease activity of the crude extracts was characterized on different temperature. For different temperature fridge (10^{o}C), room temperature (20^{o}C), incubator (40^{o}C), oven of 60^{o}C, 80^{o}C, 100^{o}C were used.

ISSN 2454-1672

30 µl of enzyme extract was mixed with 50µl of BSA standard solution and the volume was maintained to 200 µl by adding phosphate buffer and after incubation of 30 min, 2.3ml Bradford reagent was added and the absorbance was noted after 5 min of extra incubation with UV-ray spectrophotometer at 595nm. The activity was calculated with reference to two controls one is enzyme control having 30 µl of enzyme only and the next one is BSA control having 50µl BSA only.

Effect of Protease Concentration

The characterization was done by using 16.07, 32.14, 48.21, 64.28, 80.35, and 96.42µg of enzyme for determining the effect of enzyme concentration on protease activity of crude extract. The different concentration of enzymes were mixed with 50 µg of BSA standard solution and the volumes were made to 200µl and after incubation of 30 min the Bradford reagent of 2.3ml were added in each. After again incubation for 5 min and the absorbance was noted with spectrophotometer at 595nm.

Effect of Substrate Concentration

This was characterized using 20, 40, 60, 80, and 100µg of BSA as a substrate. 48.21µg of enzyme extract was mixed with different amount of BSA (20, 40, 60, 80, and 100µg) and phosphate buffer (pH 7.0, 0.1M) was added to make final volume of 200µl. The reaction mixture was incubated for 30 min and then 2.3ml of Bradford reagent was added. Then after 5 min of incubation time absorbance was taken by spectrophotometer at 595nm.

Results

Screening of Colonies

Among the eight purified colonies, colony numbers 1 and 2 were selected first because of the production of three types of enzymes and then it was decided to proceed with colony number 2 for its increased amount of enzymes based on the diameter/length of the clear zones on the skim milk agar and starch agar plates.

Table 1: Enzyme production pattern of the eight colonies of Rhizophus stolonifer purified strain

	Colony Numbers							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Protease	+	+	+	+	+	+	+	+
Amylase	+	+	+	-	-	-	NC	NC
Xylanase	+	+	NC	+	+	NC	-	+

Positive - + **Negative- -** **Not very prominent clear zone – NC**

Enzyme Production of the Rhizophus Stolonifer Strain

The selected Rhizophus stolonifer strain was screened for the production of amylase, protease and xylanase by growing with the appropriate substrate. On the basis of these screening tests, the examined strain produced all the enzymes tested. As the protease activity was prominent among the other enzymes, it was decided to study the kinetic properties of the proteases first.

Protease, Amylase and Xylanase Activities of the Crude Enzyme Mixture

The activity of crude protease, amylase and xylanase of the selected colony number 2 were 79.4, 54.2 and 31.5 pmole/min/m respectively.

Effect of Time

Purified protease showed zero order kinetics for 10 minutes (**Figure 1**).

ISSN 2454-1672

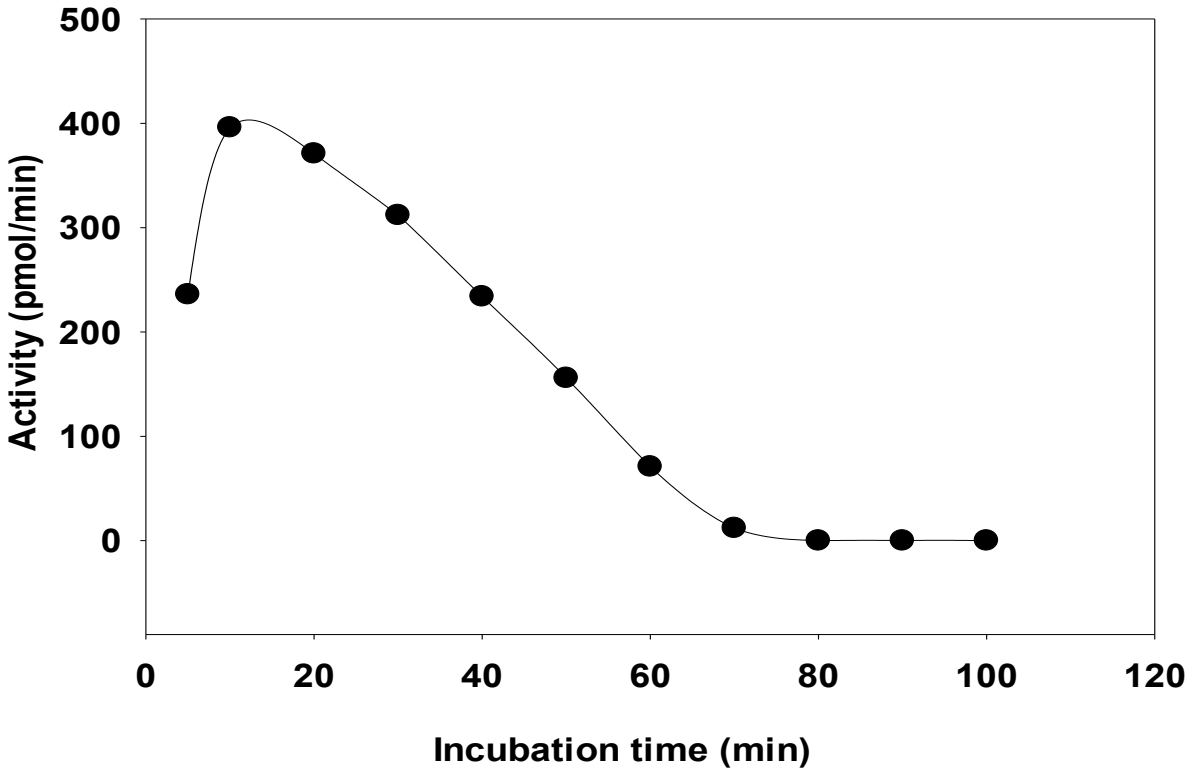

Figure 1: Effect of time of incubation on crude protease activity

Effect of pH

The highest proteolytic activity was obtained at pH 8.0 (**Figure 2**).

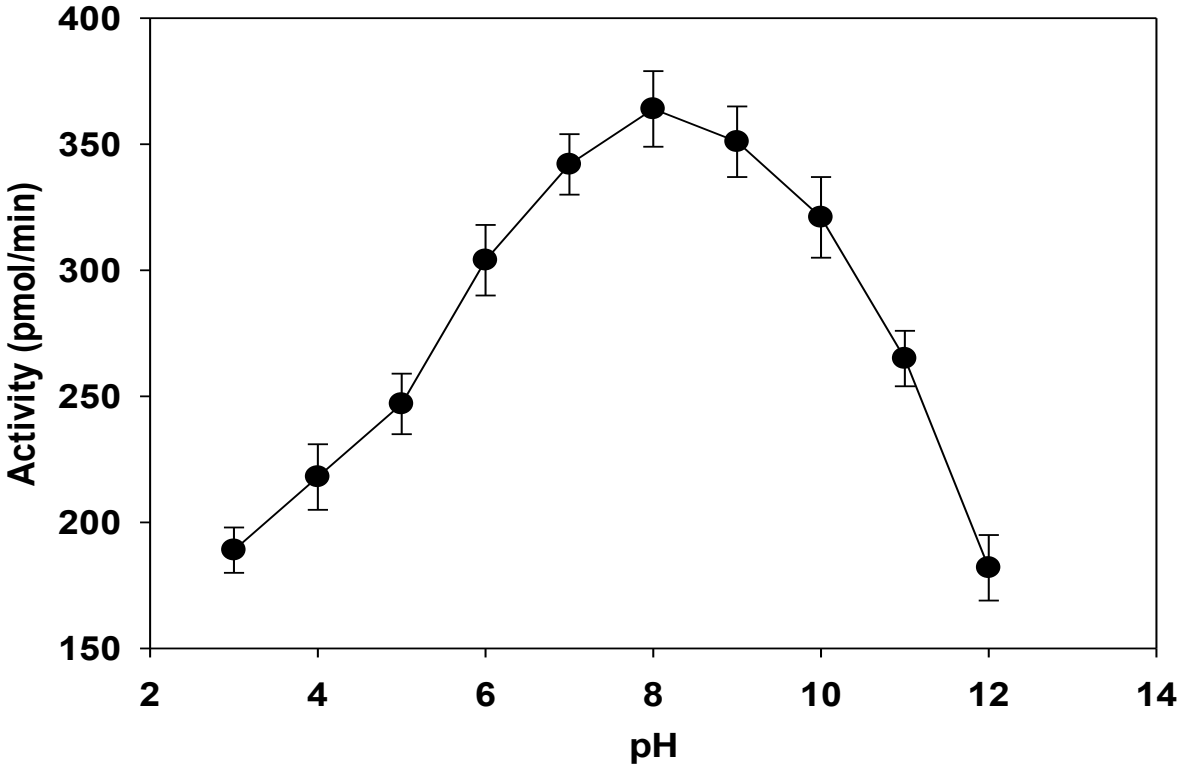

Figure 2: Effect of pH on the crude protease activity

ISSN 2454-1672

Effect of temperature

As shown in figure 3, the optimum temperature of extract was found to be 30°C, while protease was still active at 80°C too (**Figure 3**).

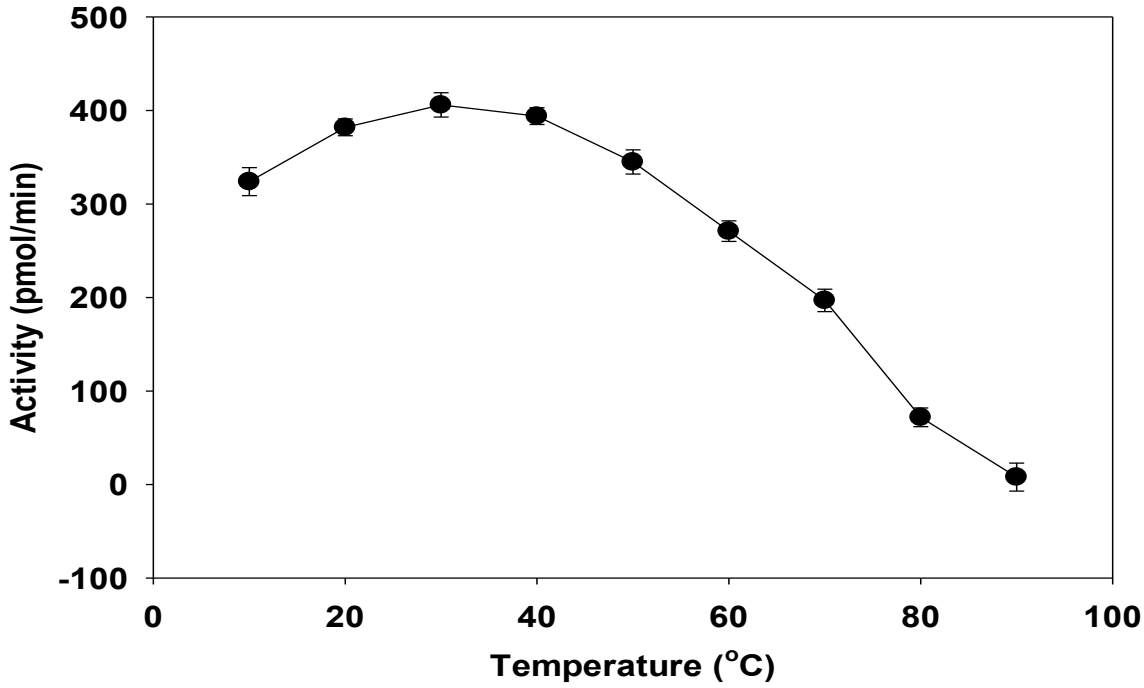

Figure 3: Effect of temperature on the crude protease activity

Effect of Enzyme Concentration

The effect of enzyme concentration on protease activity was found to be increasing linearly up to 40g. (**Figure 4**)

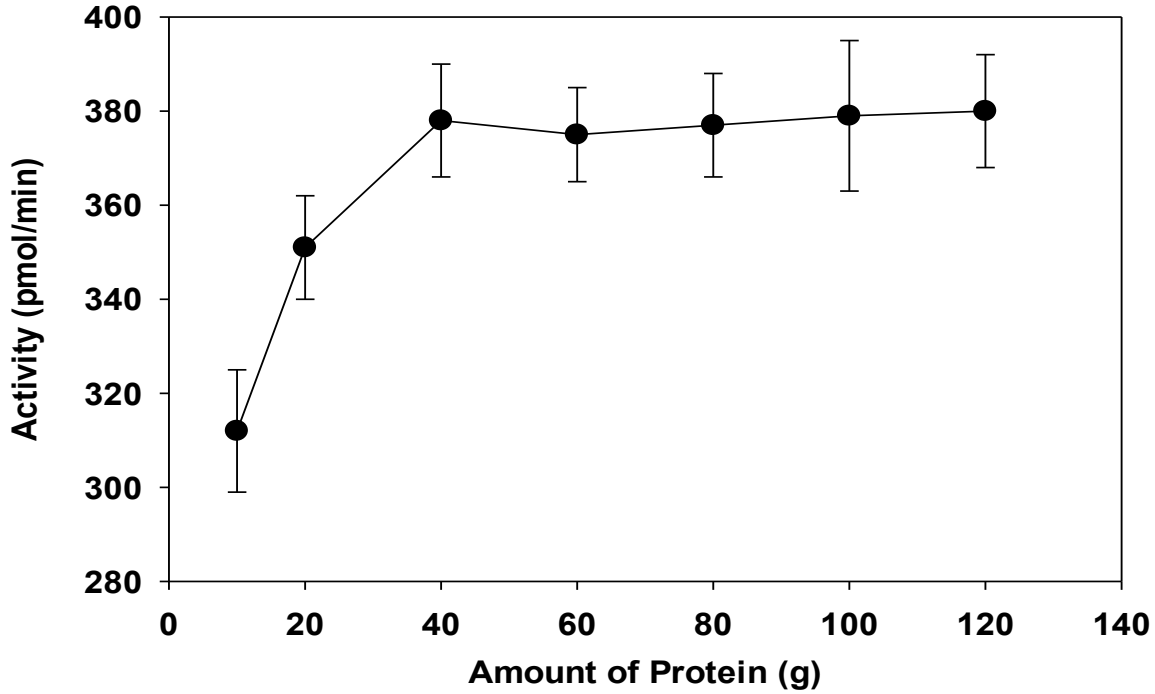

Figure 4: Effect of enzyme concentration on the crude protease activity

ISSN 2454-1672

Effect of Substrate Concentration

Line weaver-Burk plot (**figure 5**) was used to determine the Km and Vmax of the crude extract of protease enzyme and is shown in Figure 5. Michaelis constant for the crude enzyme to soluble substrate was 624.9µM and Vmax was 136.4 pmol/min at pH 8.0 and 30°C.

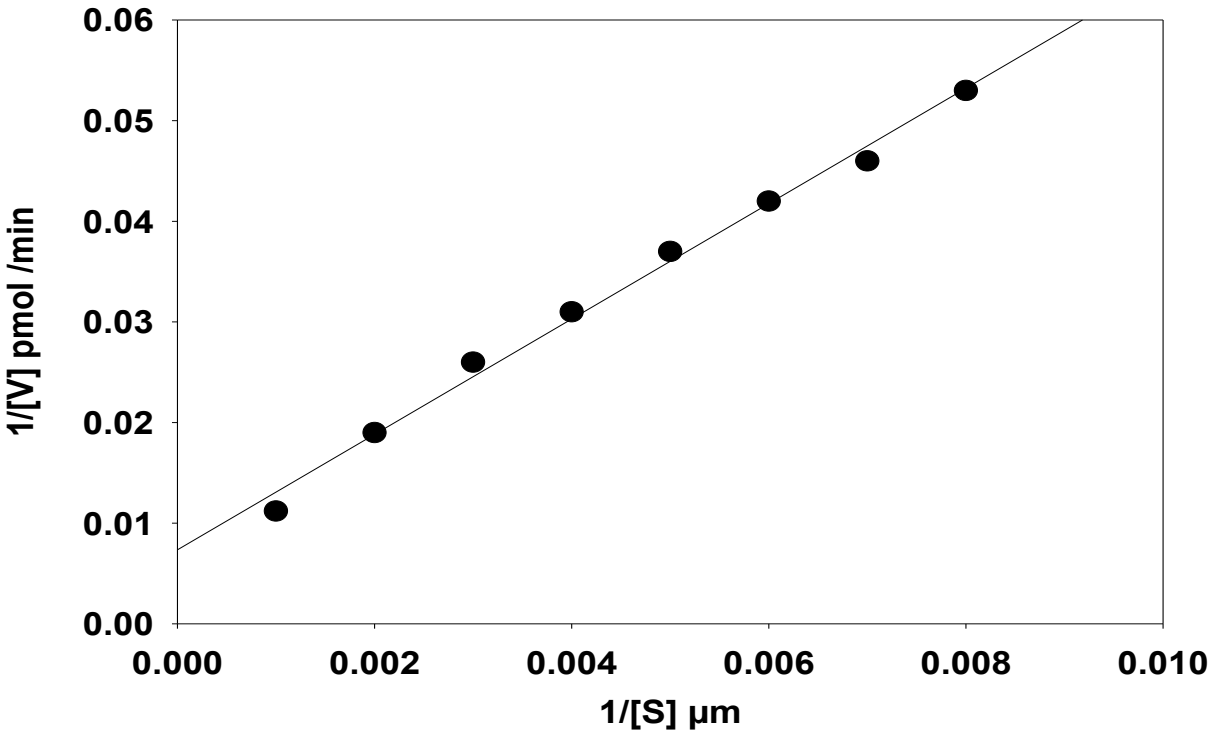

Figure 5: Line weaver-Burk plot of the crude protease activity at pH 8.0 and 30°C using different concentrations of substrate

Discussion

The optimal pH of protease was 8.0 that suggest it can be classified as alkaline protease. The optimal temperature for the protease activity was 30°C. The activity remained at 90°C, so protease is thermos table, this may be due to the presence of higher amount of cysteine residues (Murray et al, 1999). The initial increase in protease activity with temperature is due to collision between enzyme and substrate. The increase in temperature causes increase in collision between enzymes and substrates (Mehrato et al, 1999). Beyond these optimal temperatures the activities were decreased due to denaturation of proteases due to heat (Karki et al. 2009). The incubation time was found to be most effective only for 100 minutes. The enzyme activity gradually fell down indicating inactivation of enzyme with time. Slightly linear line was obtained for the effect of enzyme concentration on protease activity indicated that the enzyme concentration enhances the rate of reaction. The linear line obtained for the effect of substrate concentration on protease activity indicated that the rate of reaction increases with the increase in substrate concentration. The low Km value of proteases indicated that the substrate is tightly bound to the enzyme.

Conclusion

The crude protease from Rhizophus stolonifer was characterized. The activity of crude protease was higher at pH 8.0 and temperature of 30°C. Protease showed zero order kinetics for 10 minutes and it was active even at 90°C.

Acknowledgements

The author is sincerely grateful to the MRC, Chiang Mai, Thailand for the Financial support and laboratory facilities.

References

[1] Christeller, J,T, (2005). Evolutionary mechanisms acting on proteinase inhibitor variability. FEBS Journal, 272, 5710–572.
[2] Grubb, A, and Lofberg, H, (1982). Human adenophysis. Proc Natl Acad Sci, U.S.A, 79, 3024–3027.

ISSN 2454-1672

[3] Kapilan, R, (2015). Purification of xylanase from Bacillus subtilis BS166, Journal of Science, 5(7),511-515.

[4] Karki, S, Shakya, R, and Agrawal, V,P, (2009). A Novel Class of Protease from Choreospondias axillaris (Lapsi) Leaves. Int J Life Sci, 3, 1-5.

[5] Kelly, C,T, Fogarty, W,M, (1976). Microbial alkaline enzymes. Process Biochem, 11,3-9.

[6] Mehrato, S, Pandey, P,K, Gaur, R, and Darmwa,l, N,S, (1999). The production of alkaline protease by a Bacillus species isolate. Bioresour Technol, 67, 201-203.

[7] Miller, G,L, (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-428.

[8] Murray, M,T, and Pizzorno, J,E, Bromelain. In, Pizzorno, J,E, and Murray, M,T, (eds.), (1999). Textbook of Natural Medicine, Vol 1. (2nd ed.), Churchill Livingstone, Edinburgh, 619-622.

[9] Paudel, K,C, and Parajuli, D,P, (1999). Domestication and Commercialization of Lapsi tree, A potential income source agroforestry in the middle hills of Nepal. In, Ministry of Science and Technology, Scientific World, Kathmandu, Nepal, 1(1), 116-120.

[10] Rao, M,B, Tanksale, A,M, Ghatge, M,S, Deshpande, V,V, (1998). Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev. 62, 597-635.

[11] Vengadaramana, A, Balakumar, S and Vasanthy Arasaratnam, (2011). Purification and comparison properties of crude enzyme with purified α-amylase from Bacillus licheniformis ATCC 6346. European Journal of Experimental Biology, 1 (3), 58-69.