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Purification and Characterization of Protease From *Bacillus sp.* TBRSN- 1

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Abstract

Potato Cyst Nematode (PCN), *Globodera rostochiensis*, is one of the important potato's pests and caused economic looses up to 70% in the several centrals of potato plantations in Indonesia. PCN's shell component of egg shell containing chitin (inner layer) and viteline/ protein (outer layer). The purpose of this research was to purify of protease *Bacillus* sp. TBRSN-1, isolate from tomato's rhizosfer in Yogyakarta province. The purified protease could be used for cutting the life cycle of PCN. Results showed that *Bacillus* sp. TBRSN-1 could produce extracellular protease and purification using DEAE-cellulose ion-exchange chromatography and Sephacryl S-300 gel filtration chromatography resulted in specific activity 4.31 fold and 1.68% recovery. Analysing using SDS-PAGE 12.5% and molecular weight 48.1 kDa. Km and Vmax values of the protease for casein substrate were 7.83 mg/ml and 4.03 µg/h, respectively. The optimum activity at the temperature 30°C and pH 7.0.

Keywords : protease, purification, indigenous *Bacillus* sp. TBRSN-1

Introduction

Plant-parasitic nematodes cause serious losses to a variety of agricultural crops worldwide. Since the traditional methods based on the use of nematicides and antihelminthic drugs are associated with major environmental and health concerns, the development of biocontrol agents to control nematodes is of major importance (Duncon, 1991). The potato cyst nematode *Globodera rostochiensis* is the major pests for the potato cultivars in several countries in the world (Margino *et al*., 2009). Soil treatment with nematocides for controlling *Globodera*, it is very expensive for farming community. In recent years biological control agents such as chitinolytic and proteolytic bacteria, fungi, actinomycetes were applied to control potato cyst nematode (PCN) eggs shell, for cutting their life cycle. Previous research succeded in controlling PCN up to 60% using mixed cultures inoculum (Margino *et al*., 2009) and succeded in purifying chitinases of selected bacteria and actinomycetes (Margino *et al*., 2010, Margino *et al*., 2012). Because of the fast breeding, easy cultivation and production compared to fungi, nematophagous bacteria have been used extensively as bioinsecticides against nematodes in soil, and levels of control equivalent to those of chemical pesticides development (Zhou *et al*., 2002). Egg cell of PCN containing vitelin (protein) and chitin layers so that protease can be used for controlling PCN through the namatode's egg.

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications for example in biological control (bionematicide). Although there are many microbial sources are available for producing proteases, only a few are

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recognized as commercial producers, that is strains of *Bacillus* sp. (Gupta *et al*., 2002[b]). In this work, we present the purification and characterization protease of *Bacillus sp*. TBRSN-1, isolated from tomato's rhizosfer.

Material And Methods

Microorganism and inoculum preparation

A culture of *Bacillus sp*. TBRSN-1 previously isolated from soil and identified by standard method for bacterial identification. Stock cultures were maintained in nutrient broth medium (Difco) with 70% glycerol, cultures were preserved at -20°C. One loopful of bacteria strain (*Bacillus* sp. TBRSN-1) was transferred to a tube of sterile containing of nutrient broth and allowed to grow overnight at 37°C (Shafee *et al*., 2005; Sharmin *et al*., 2005) before being used to inoculation.

Protease activity

The protease enzyme activity was determined as previously mentioned by Secades and Guijarro (1999) using casein as a substrate. Briefly, 120 µL of a suitable dilution of enzyme solution was added to 480 µL of casein (2% wt/vol) in reaction buffer, and the mixture was incubated at 30^{0}C for 30 min. The reaction was terminated by adding 600 µL of 10% (v/w) TCA and left for 30 min on ice, followed by centrifugation at 15,000 x g, at 4^{0}C for 10 min. Eight hundred µL of the supernatant was neutralized by adding 200 µL of 1.8 N NaOH, and the OD value was measured using spectrophotometer at 420 nm (λ_{420}). One units of enzyme activity was defined as the amount of enzyme which required to produce an increase in OD value at 420 nm equal to 1.0 in 30 min, at 30^{0}C.

The protein content of protease was determined by the method of Lowry *et al.*, (1951) as mentioned in Bradfort (1976) using bovine serum albumin as a standard and during the course of enzyme purification by measuring at OD value at λ280 nm.

The specific activity of the protease protein was expressed in terms of units/ mg protein/ml^{-1} according the following equation: Specific activity = enzyme activity/ protein content (mg/ml^{-1}).

Protease production (Singh *et al*., 1999)

Protease crude enzyme was produced by fermentation. *Bacillus sp*. TBRSN-1 was cultivated in minimal medium consisting of (g/l): K_2HPO_4 0.7, KH_2PO_4 0.3, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, $ZnSO_4$ 0.001, $MnCl_2$ 0.001, skim milk 1%, and distilled water 1 L, pH 7.0. Media were autoclaved at 121^{0}C for 20 min. Cultivations were performed on different condition (inoculum concentration, pH, substrate (skim milk) concentration, agitation, temperature, and incubation period) in 250 ml erlenmeyer flasks with a working volume of 20 ml. The cultures were centrifuged and the supernatants were used for estimation of proteolytic activity.

Optimization of growth conditions: Optimization was done under the inoculum concentration (2.5, 5.0, 10,and 15% (v/v); different pH values (50 ml of selected medium of different pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 respectively; different substrate (skim milk) concentrations (g/l^{-1}) was performed using 0.5, 1.0, 2.0, 3.0 and 4.0% (w/v), then incubated 48 h at 30^{0}C**;** different agitation (150, 200, and 250 rpm); incubation temperature (30°C and 37°C); The best results of these condition teatments were applaied to the fermentor for looking best conditions of aeration, desolved oxsygen, and time of proteasevproduction, during incubation for 96 hours.

Purification of the protease

The culture supernatant was first subjected to ammonium sulphate precipitation (Scopes, 1994). Poteins presents in culture broth were extracted by ammonium suplhate 40%, 50%, 60%, 70%, 80%, and 90% (w/v). Extractants were collected by centrifugation at 10,000 g, 4^{0}C for 45 min, and the pellet was suspended in 20 mM buffer phosphate, pH7.0. The 40% (w/v) ammonium sulphte fraction was subjected to gel filtration on Sephacryl S- 300 column (1.5x 60 cm) equilibrated with

20 mM Tris-HCl, pH 8.0 containing 0.2 M NaCl and 0.02% NaN3. Fractions of 1.5 ml were collected at a flow rate of 46 ml/h with the same buffer. Protein content and protease activity were determined. All purification steps were conducted at temperatures not exceeding 4°C.

Polyacrylamide gel electrophoresis (Laemmli, 1970)

Sodium Dodecyl Sulfate-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular weight of the enzyme as described by Laemmli (1970) using a 5% (w/v) stacking gel and 10% (w/v) separating gel. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers consisting of: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and bovine α-lactalbumin (14.2 kDa). Protein bands were visualized by staining with Coomassie Brilliant blue 0.25% (w/v) and nitrate silver 0.1% (w/v).

pH optimum and pH stability **(Harman *et al.*, 1993)**

The optimum pH of the purified protease was studied over a pH of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10 with casein as a substrate. For the measurement of pH stability, the enzyme was incubated for 30 min at 30°C in different pH buffers and the residual proteolityc activity was determined under standard assay conditions. The following buffer system were used: 100 mM phosphate buffer, pH 6.0-7.5; 100 mM Tris-HCl buffer, pH 8.0-8.5; and 100 mM glycine-NaOH buffer, pH 9.0-12.

Temperature effect on protease activity and stability

To investigate the effect of temperature, the activity was measured using casein as a substrate at the temperature range from 10°C to 50°C in 100 mM phosphate buffer, pH 7.0. Thermal stability was examined by incubating the purified enzyme at different temperatures. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 7.0 and 30°C. The non-heated enzyme was considered as control (100%).

Enzyme kinetics determination

K_m and V_{max} were determined by the kinetics of Michaelis-Menten Model reactions as described by Wilson and Walker (2005).

Result And Discussion

Production of protease and precipitation using ammonium sulphate

Bacillus sp. TBRSN-1 was originally isolated from soil around rhizosphere of tomato plant in (Sleman, Yogyakarta) and had high protease and chitinase activities in the culture broth medium. This isolate was also one of the mixed cultures inoculum candidate of bionematicides (data Margino *et al.*, 2009). Production of extracellular protease by new strain *Bacillus* sp. TBRSN-1 was done in the two liter fermentor, based on the optimization of growth conditions (5%, v/v) of inoculums, substrate concentration (1%, w/v), pH value 7, agitation 150 rpm, temperature at 30° C, and incubation time 60 hours.

Enzyme precipitation and purification

Present tproteins in the culture filtrate were extracted by ammonium sulfate 40, 50, 60, 70, 80, and 90% (w/v). Result showed that 40% saturation was able to produce a maximum protease activity, 324.13 U/mg, and followed 277 U/mg for saturation 50% (w/v) (Figure 1). Furthermore, ammonium sulphate in this 40% saturation level was used for precipitating protein in crude enzyme. The added ammonium sulphate will press out of water molecule from protein and cause the hydrophobic condition of protein compounds (Harris and Angal, 1990). In addition to, ammonium sulphate also leads to the protein precipitation and reduces its solubility. While the solubility

of protein decrease, interaction between hydrophobic regions formed agregates, then agregates of proteins which contained of big molecules suddenly precipitated and resulted in more precipitates until its optimum concentration, Figure 1 (Scope, 1994). Ammonium sulphate purification increased the protease activity 1.46 fold. The precipitation step also decreased the overall protein concentration compared to the protein in the crude enzyme. Icreasesing of the *Bacillus* sp. TBRSN-1 protease activity using ammonium precipitation 1.46 fold are consistent with published literature, which shows a purification (fold) up to 9,6 (Liao *et al.*, 1998). The prcipitation also gave lower result compared to precipitation of protease *Burkholderia* strain 2.2 N, using ammonium sulphate 40-60% (w/v) produce protease activity 20 fold (Jewell, 2000). A little bit different result showed by protease activity from *Bacillus* sp. PS719 which precipitated using ammonium sulphate 80%, resulted in 1.5 fold (Towatana *et al.*, 1999).

The results of ammonium sulphate precipitation in saturation level 40% (w/v) as much as one ml (formerly been dialyzed with 0.05 PBS pH 7.0) applied into the colomn ion exchange chromatography containing DEAE Cellulostein. The 100 fractions were measured in early experiment to find out protein concentration of each fractions (based on the absorpton value at 280 nm). Results showed that two peak of protein but only one had protease activity, that fraction numbers 7-14 and 15-25. After collecting the samples and then be run using SDS-PAGE was found out two lines with a little bit far of their distance (data unshown). Furthermore analysis was done using gel filtration chromatography method by Sephacryl S-300 (Figure 3). This experiment has measured 70 fractions to find out the protein contents of each fraction (based on absorption value at 280 nm). In the gel filtration, one protein peak was observed, which formed by fraction number 51, but fraction numbers 50 and 52 have closed protein peaks and they

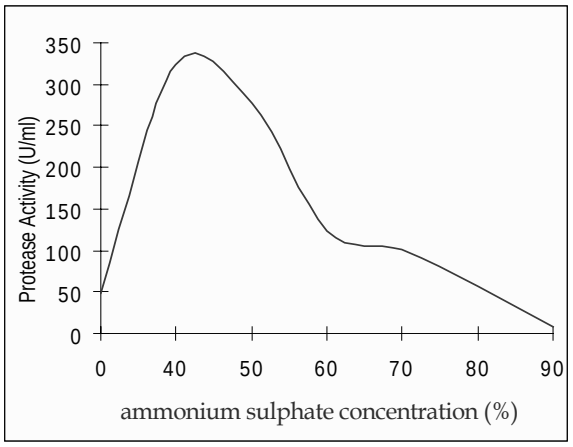

Figure 1. Different effect of ammonium sulphate concentration on purifying protease protein of *Bacillus* sp. TBRSN-1

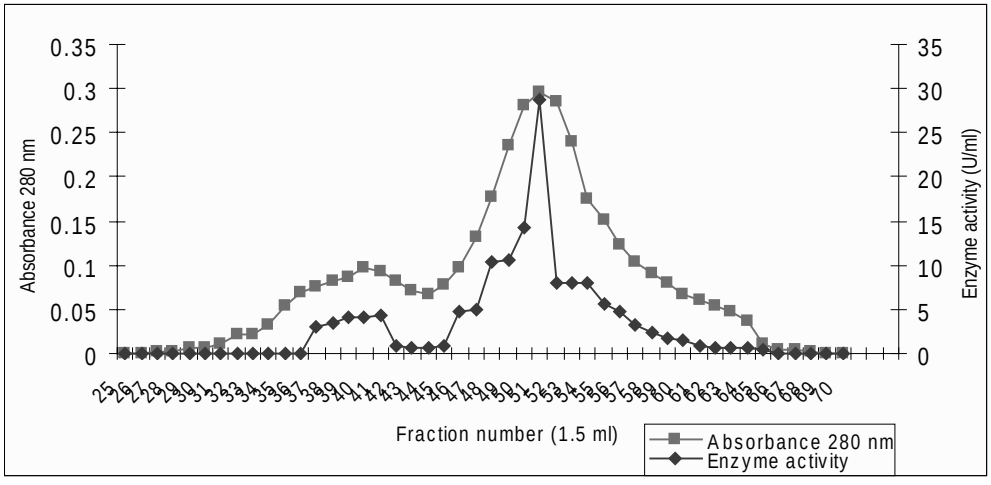

Figure 3. Elution profile of protease purification with gel filtration on Sephacryl S-300

Table 1. Summary of the purification of *Bacillus sp*. TBRSN-1 protease

	Total Protein (mg)	Total activity (10^3 U)	Specific activity (10^3 U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	116.45	24761.90	212.64	1.00	100.00
40% ammonium sulfate fraction and dialysis	38.37	11901.67	310.16	1.46	48.06
DEAE Cellulose	0.82	533.33	653.60	3.07	2.15
Sephacryl S-300	0.45	416.67	916.76	4.31	1.68

had high protease activities. Futhermore, the samples were collected into one tube, then to be freezed-dried for characterization of enzyme. Enzyme purification using Sephacryl S-300 may increased enzyme purity as much as 4.31 times. The yield and purity for each purification steps were summarized at Table 1.

The protease purification using DEAE-Cellulose at Table 1, showed 3.07 fold compared to crude enzyme, its specific activity 653.60 U/mg protein, and its recovery was 2.15%. This result a little bit different with purification of extracellular protease from *Bacillus subtilis* EAG-2, using DEAE-Cellulose, the overal recovery 29% and the purity level was 11 fold (Ghafoor and Hasnain, 2010). The extracellular protease was purified on Sephacryl-300 had purification value 4.3-fold with a recovery 1.68% and a specific activity 916.76 U/mg of protein.

All operations were carried out 4^0C. Only 40% ammonium sulphate was subjected to gel filtration on Sephacryl S-300.

Characterization of protease

The characterization of protease were done including SDS-PAGE analysis, molecu;lar weight, pH value and temperature effect.

The character of protease protein produced by *Bacillus* sp. TBRSN-1 could be

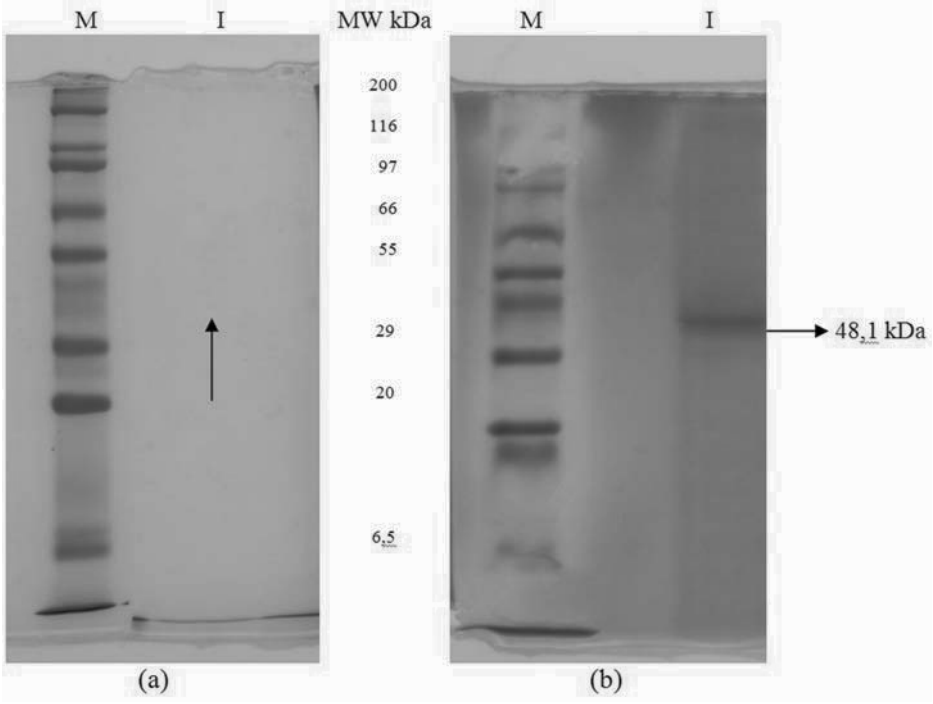

Figure 4. SDS-PAGE of the purified protease from *Bacillus sp*. TBRSN-1. (a) Staining with coomassie brilliant blue 0.25% (w/v); (b) Staining with nitrate silver 0.1% (w/v). M = Markers; I = purified protease.

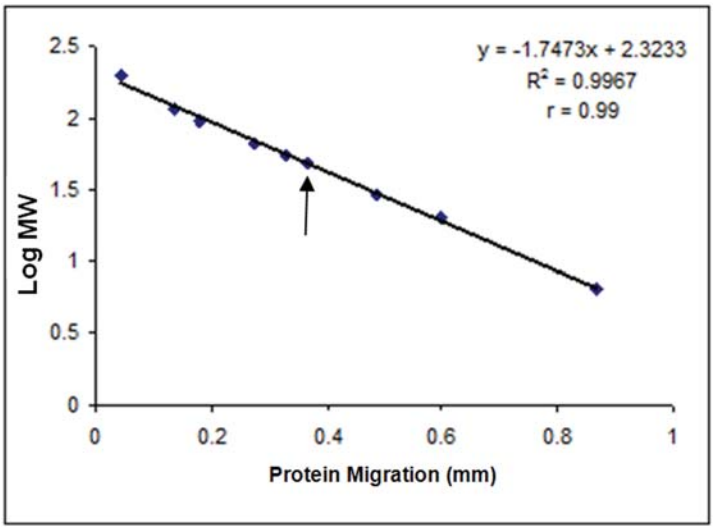

Figure 5. Determination of molecular weight of protease at pH optimum

performed on 10% SDS-PAGE (Figure 4). This figure showed that one band of protease protein was found in this experiment. The molecular weight of single protein that resulted in Sephacryl S-300 isolation was determined using relative mobility calibration curve of standard polypeptide (Figure 5). The molecular weight estimation was determined using regression equation Y= 2.3235 – 1.7473 X (with r=0.99), Figure 5.

The purified protease was homogenous on SDS-PAGE and its molecular weight was estimated to be 48.1 kDa (Figure 4).

The estimation of protease molecular weight of *Bacillus* sp.TBRSN-1was about 48,1 kDa (Figure 5). It was supposed that this band is protease, this was approved by increasing of protease activity and its enzyme purity. Padmapriya and Williams (2012) reported that the purified neutral protease of Bacillus subtilis had molecular weight 50 kDa, while purified serine protease from Bacillus sp. from marine had molecular weight 37 kDa (Padmapriya *et al.*, 2012). Towatana *et al.* (1999) reported that purified an extracellular protease from alkalophilic thermophile Bacillus sp. PS 719 using DEAE-Cellulose ion-exchange chromatography had molecular weight 42 kDa. Yang *et al.* (2000) reported that Bacillus subtilis isolated from soil in Taiwan, which purified using DEAE-Sepharose ion-exchange chromatography and Sephacryl S-200 gel-permeation chromatography showed that protease had molecular weight 44 kDa.

Based on the several of researches showed that molecular weight of protease from *Bacillus* sp. TBRSN-1 had the similar types of them.

Effect of pH on enzyme activity

The pH profile of the purified enzyme was determined using different buffers of varying pH values. The purified enzyme was active in the pH range 3.5 – 9.0, with an optimum activity at pH 7.0 (Fig. 6a) with protease activity was 24.58 U/ml. Similarly, Abdul-Rouf (1990) reported that the optimum pH for all purified 4 proteases enzymes in their reaction mixture was found to be 7.2. Purified protease of *Bacillus* sp. isolated from soil samples around the Bungalore had specific activity at pH neutral (Josephine *et al.*, 2012), other purified protease of *Bacillus subtilis* also had activity at pH 7.0 (Padmapriya and Williams, 2012).

Effect of temperature on the enzyme activity

The effect of temperature on the activity of protease enzyme was examined at various

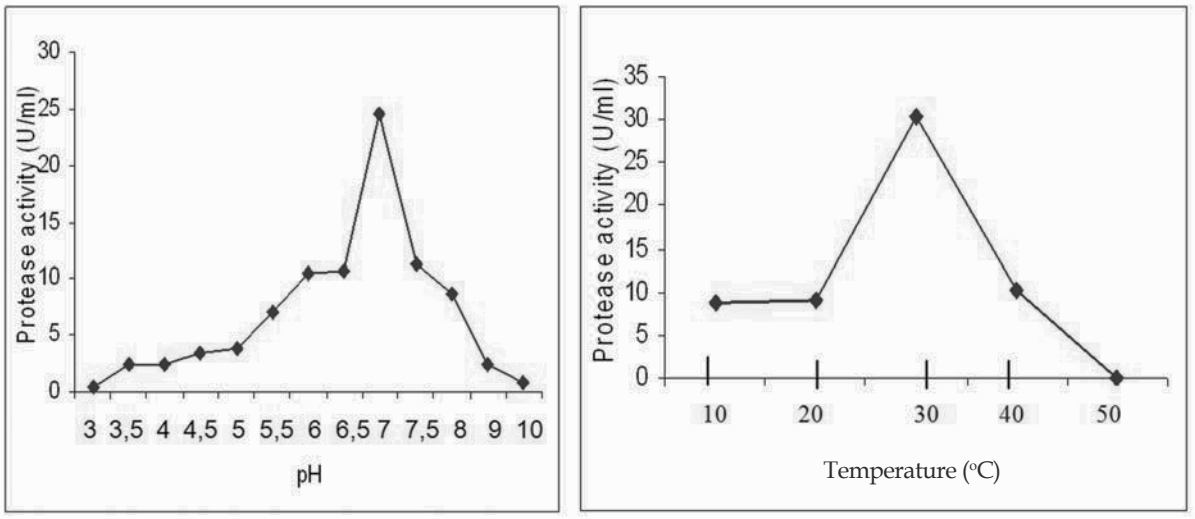

Figure 6. (a) Effect of pH on the activity of the purified protease; (b) Effect of temperature on the activity of the purified protease from *Bacillus sp*. TBRSN-1.

temperatures. The purified protease was active between 10°C to 40°C, with an optimum around 30°C (protease activity 30.36 U/ml) (Figure 6b). The protease activity at 10°C and 40°C were about 8.75 U/ml and 10.24 U/ml, respectively. The enzyme was completely inactivated after 30 min incubation at 50°C. While the temperature below or above 30°C exhibited lower activities of protease. Secades and Guijarro (1999) reported that a novel exoprotease, that was purified from the culture supernatant of *Yersinia ruckeri* (fish pathogen), had more activity in the range of 25 to 42°C and had an optimum condition at 37°C. Asker *et al*. (2013) reported that protease of Bacillus megaterium had the optimum activity at 50° C, while other purified protease from Bacillus subtilis had optimum activity at 37°C. This illustration showed that optimum temperature of protease from several *Bacillus* sp. had the large variation.

Determination of K_m and V_{max} value of protease

The result of enzyme kinetic analysis (K_m and V_{max}) are shown in Table 2. Determination of Km and Vmax values were based on the pH condition and optimum temperature that have been procured. In this event, the increasing of substrate saturation will increase enzyme activity to achieve a certain limitations at the certain substrate saturation as well, so that the with the increasing of substrate after optimum limitations will not increase the enzyme activity.

Michaelis Menten's (Km) constant value analysis and maximum speed (Vmax) can be seen at Table 2. According to above computation, there was quantitative relation between the speed (Vi) with substrate saturation (S). Michaelis Menten's (Km) constant was procured about 7.83 mg/ml and maximum speed (V_{max}) was 4.03 µg/h with regression equation; Y=1942.7X – 248.29 and have correlation value about r = 0.998. Wilson (2005) explained that enzyme activity more higher, if the its K_m values was small. The correlation between of reaction velocity (v) with substrate concentration as shown at Figure 7.

Conclusion

The best conditions for protease production by *Bacillus sp*. TBRSN-1 were: substrate concentration (skim milk) 1.0% (w/v), inoculum concentration 5.0% (v/v), growth temperature 30°C, pH 7.0, agitation at 150 rpm, and incubation time for 60 hours.

Purification of *Bacillus* sp. TBRSN-1 protease using DEAE-Cellulose and Sephacryl

Table 2. Data of K_m and V_{max} values of protease

No	[S]	1/[S]	Vi	1/Vi	Regression equation
1.	0,5	2,00	0,000280	3570,400	Y = 1942,7 X – 248,29
2.	1,0	1,00	0,000530	1887,000	K_m = 7,83 mg/ml
3.	1,5	0,67	0,000930	1075,500	V_{max} = 4,03 µg/h
4.	2,0	0,50	0,001739	575,000	

[S] : Substrate concentration
Vi : reaction velocity

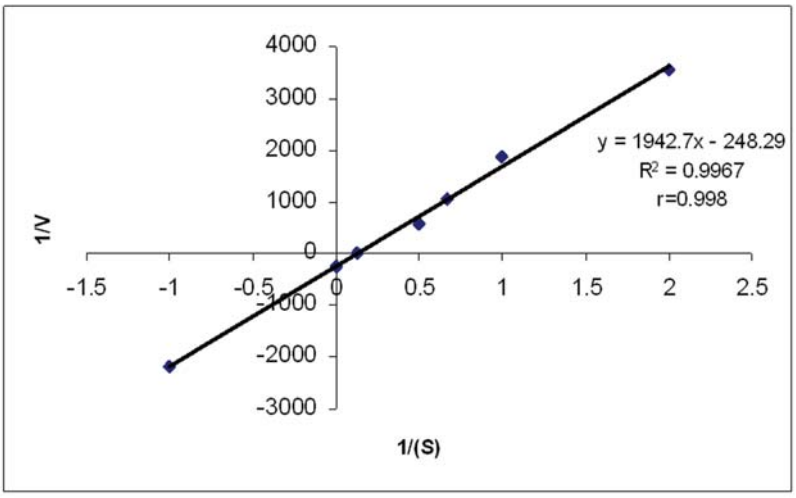

Figure 7. Lineweaver – Burk plot

S-300 gel filtration, increased a 4.31-fold in specific activity and 1.68% recovery. The molecular weight of the purified protease was estimated to be 48.1 kDa.

The optimum conditions of pure protease activity were on temperature 30°C, pH 7.0, K_m and V_{max} values were 7.83 mg/ml and 4.03 µg/h, respectively.

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