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Isolation and Purification of Chitinase *Bacillus* sp. D2 Isolated from Potato Rhizosfer

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Abstract

Potato Cyst Nematodes (*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. Potato Cyst Nematodes (PCN) shell component of egg shell containing chitin (inner layer) and vitelline/protein (outer layer), so the purpose of research was to find out of chitin degrading bacteria for controlling of egg's PCN by cutting of their life cycle. The results showed that *Bacillus* sp. D2 isolated from potato rhizosphere could produce extra cellular chitinase in the medium containing of 0.20% colloidal chitin and fermented for 72 hours. Result of chitinase purification using ammonium sulphate precipitation and *DEAE-Cellulose* ion-exchange chromatography showed a specific activity 2691,052 U/mg and analyzing using SDS-PAGE 12.5% resulted in molecular weight 30 kDa. The apparent K_m and V_{max} of chitinase towards colloidal chitin were 2 mg/ml and 2.2 µg/h, respectively.

Keywords : chitinase, purification, *Bacillus* sp. D2, potato rhizosphere

Introduction

Chitin, a polysaccharide consisting of linear 1,4-*N*-acetyl-*D*-glucosamine moieties, the second most abundant biopolymer. It can be found mainly in the cuticles of insects, shells of crustaceans, nematodes, and cell walls of most fungi (Aranaz *et al*., 2009; Gohel *et al*., 2006). The Potato Cyst Nematodes (*Globodera rostochiensis)* had been found in Indonesia since 2003 and could cause economic looses estimated up to 70% for potato's farming in the several regions in Indonesia (Mulyadi *et al*., 2003). The egg shell of Potato Cyst Nematodes (PCN) has already been known containing chitin and protein (Brodie and Marks, 1998), and in case of tylenchoid nematodes, chitin is located between the outer vitelline (protein) and inner lipid layer and may occur in association with proteins (Bird and Bird, 1991).

Biodegradation of chitin is performed by chitinases and appears to occur in two steps. An endochitinase (EC 3.2.1.14) degrades the polymer to oligomers, which subsequently be degraded to monomers by exochitinase (β-N-acetylhexosaminidase [EC 3.2.1.52]). These enzymes are found out in a wide variety of organisms such as bacteria (Yong *et al*., 2005; Annamalai *et al*., 2010; Toharisman *et al*., 2005; Wen *et al*., 2002), fungi (Matsumoto, 2006), insects (Bansode and Bajekal, 2006), actinomycetes (Margino, 2010), plants and animals.

The recent studies focused on an increasing attention towards the biological control potential of rhizosphere bacteria from several plant species against various plant pathogens including nematodes. Chitinase-

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producing bacteria have been considered to be used in reducing numbers of plant-parasitic nematodes in soil (Margino *et al.,* 2009)., Various species of *Bacillus* have been known could secrete chitinase, including *B. circulans* , *B. licheniformis,* and some others (Toharisman *et al*., 2005; Wen *et al*., 2002; Sakai *et al*., 1998; Woo and Park, 2003; and Cody, 1989).

During the last decade, chitinases have received remarkable attention due to their wide range of applications. Efforts are going on throughout the world to enhance the production and purification of bacterial chitinase. Some novel characteristics of bacterial chitinase have been reported (Bhattacharya *et al.,* 2007). However there has been no report about purification of bacterial chitinase from Indonesia in terms of biological control agents of PCN. We found out a novel chitinase-producing bacteria isolated from potato rhizosphere in Batur, Banjarnegara, Central Java and this study succeed in purification and characterization of an extra cellular chitinase isolate *Bacillus* sp. D2.

This paper describes the isolation and selection of chitinolytic bacteria from potato rhizosphere, with purification and characterization of chitinase producing selected isolate.

Materials and Methods

Cultivation condition of isolates

Colloidal chitin was prepared from purified chitin according to the method of Vessey and Pegg (1991) with few modification. Medium composition was the minimal medium supplemented with 0.2% (w/v) colloidal chitin. The minimal medium containing of : 0.7 g/l K_2HPO_4; 0.3 g/l KH_2PO_4; 0.5 g/l $MgSO_4.5H_2O$; 0.01 g/l $FeSO_4.7H2O$; 0.001 g/l $ZnSO_4$; 0.001 g/l $MnCl_2$, pH value was arranged to be 6, and sterilized at 121^{0}C for 15 min. Five percent of culture broth was inoculated onto minimal medium and incubated for 96 h. Biomass of cells were separated by centrifugation at 3000 rpm, 4^{0}C, for 20 min. Supernatant was collected for the next analysis.

Chitinase activity assay

Chitinase activity was defined as the turbidity relatively reduction percentage towards the colloidal chitin without enzyme (control) (Harman *et al*., 1993). The five hundred microliters of enzymes solution was added to 500 µl of 1% (w/v) colloidal chitin (made with 50 mM Phosphate buffer saline=PBS, pH 6.5); This solution was incubated at 28^{0}C, in shaker incubator 150 rpm, for 24 h. After incubation, the reaction was added with 3 ml dH_2O. The reduction of turbidity was measured by UV-vis spectrophotometer at 510 nm**.** As control, the enzyme solutions were substituted with dH_2O. One unit of chitinase activity was defined as the amount of enzyme needed to reduce 5% turbidity of colloidal chitin. Protein concentration was measured according to Bradford (1976) method using bovine serum albumin as a standard.

Bioassay of crude enzyme towards G. rostochiensis eggs

Cysts of *G.rostochiensis* were pooled and separated from all impurities with 250 mesh filter and washed with dH_2O. Eggs were collected in Syracuse plate (200-300 eggs) and treated with 500 µl crude enzymes, mixed with 500 µl of 10 mM PBS (pH 6,5) then incubated at 28^{0}C. As a control, crude enzymes was substituted with dH_2O (Tikhonov *et al*., 2002). During 3 days, the broken eggs caused by enzymes activity was counted under stereo microscopes. Reduction of the number of egg in the first day to third day, counted as number of broken eggs, then the ratio between number of broken eggs and number of eggs in the early incubation were defined as percentage of broken eggs.

Purification of chitinase

After cultivation in optimal condition, the cells were harvested by centrifugation at 3000 rpm, at 4^{0}C for 20 min. Free-cell supernatant (5000 ml) were precipitated with

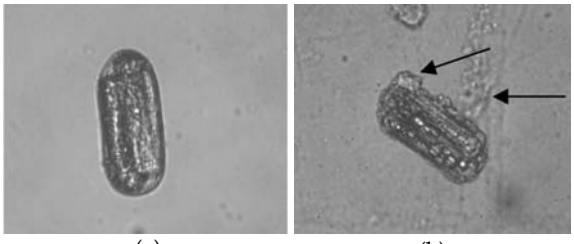
40-90%(w/v) $(NH_4)_2S0_4$ and stored overnight at low temperature. Precipitates collected by centrifugation at 10000 rpm, at 4^0C for 45 min. The precipitates of protein were dissolved in 50 mM of PBS, pH 6.5 (volumes of PBS were 2-3 times according of the volumes of precipitates). The insoluble materials in supernatant were separated by centrifugation at 15.000 rpm for 5 min before the derived supernatant (with the highest enzymes activity) applied on to a DEAE-Cellulose column (1 by 20 cm, Pharmacia-Biotech). The column was pre-equilibrated with 50 mM of PBS, pH 6.5 and then washed with 1-2 bed volumes of 0.2 M NaOH, 2-3 bed volumes of 50 mM PBS pH 6.5, and then eluted with distilled water. The flow rate was maintained at 0.3 ml/min. The fractions with chitinase activity were pooled, concentrated and kept at -20°C, for the next analysis. During chromatographic separation, protein was determined by measuring the absorbance at 280 nm. Enzymatic activity was assayed using method according to Harman *et al* . (1993).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass of purified chitinase was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel according to Laemmli (1970) method. The gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 and followed by staining with 0.1% (w/v) Silver nitrate.

Effect of pH and temperature on the chitinase activity

The effect of pH on the purified chitinase was determined at different pH values (3-10) under standard assay conditions. The used buffer systems were as follows: 0.1 M citrate-phosphate buffer, pH 3.0-6.5; 0.1 M phosphate buffer, pH 6.5-8.5; 0.1 M carbonate buffer, pH 8.5-11.0. The effect of temperature for the chitinase activity was determined in the range of 0^0C to 70^0C under standard assay conditions.



(a) (b)

Figure 1. Bioassay towards eggs of PCN. (A)=normal eggs; (B)= eggs of PCN treated with *Bacillus* sp. D2 crude enzyme resulted in a small scars at surfaces until breakage of the egg continued with the moved out of eggs liquid; (M=40x10).

Results and Discussion

Bioassay towards eggs of PCN

Figure 1 showed that *Bacillus* sp. D2 had highest consistencies of hydrolytic activity (data unshown) and could degrade egg shell of PCN. *Bacillus s*p. D2 was isolated from rhizosphere of potato farm in Dieng Plateau, Batur, Banjarnegara, Central Java, which known as one of endemic PCN regions in Java Island. Chitinolytic bacteria may contribute to biological control of plant-parasitic nematodes such like PCN by causing decreases of hatch ability through degradation of egg shells. Chitinase may attack nematode egg shells which possesses a chitinous layer and can caused premature hatch, resulting in few number of viable juveniles. It has been reported that *S. maltophilia* reduced hatching of egg of the PCN *Globodera rostochiensis*, both *in vitro* and in soil (Cronin *et al*., 1999).

Based on the highest consistencies of hydrolytic activity, the highest enzyme specific activity and percentage of PCN eggs damage in bioassay, *Bacillus* sp.D2 was chosen to be the potential candidates for biological control agents for PCN and used for further study of chitinase in this research.

Production of chitinase and precipitation using ammonium sulphate

Production of the extracellular chitinase by the new strain of *Bacillus* sp. D2 based on the optimization of growth condition (5% (v/v) of inoculums; 0.2% (w/v) of colloidal

chitin; pH 6; 150 rpm of agitation; and 72 h incubation time). The results of precipitation using ammonium sulphate 40%, 50%, 60%, 70%, 80% and 90% (w/v) showed that at concentration of 60% it was able to produce a maximum chitinase specific activity 285.348 U/mg and followed at concentration of 70% was 211.640 U/mg (Figure 2). Furthermore, ammonium sulphate at 60% (w/v) was used for precipitation of protein in crude enzyme.

Addition of ammonium sulphate leads to protein precipitation and reduced its solubility. While the solubility of proteins decreased, the interaction between hydrophobic regions formed aggregates, then aggregates of proteins which contained of big molecules suddenly precipitated and resulted in more precipitates until its optimum concentration. This optimum concentration also called saturation levels (Scopes, 1994). As it showed in Figure 2, the optimum concentration for precipitated chitinase of *Bacillus* sp. D2 were at 60% (w/v). Recent study about chitinase of *Bacillus circulars* WL-12 showed that its chitinase precipitated at 40% (w/v) concentration of ammonium sulphate (Watanabe *et al.*, 1994).

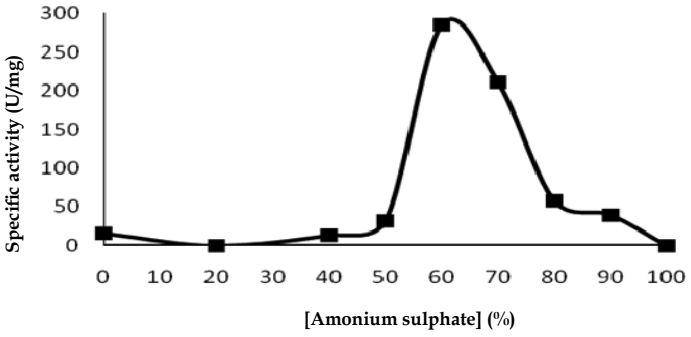


Figure 2. Effect of ammonium sulphate concentration on purification of *Bacillus* sp. D2 chitinase

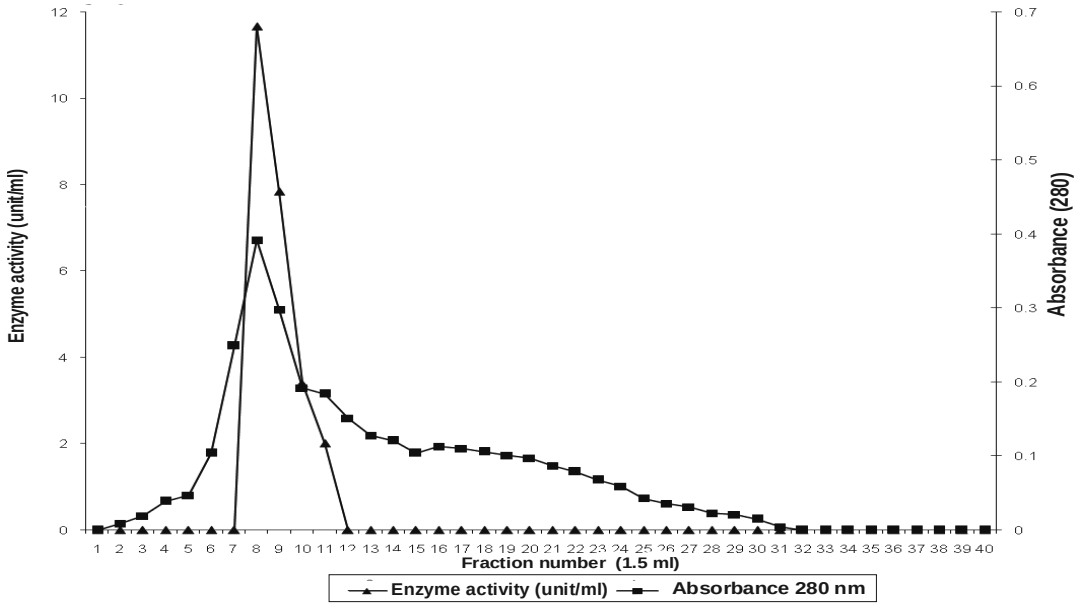


Figure 3. Elution profile of chitinase on DEAE-Cellulose of anion-exchange chromatography

Table 2. Summary of chitinase purification produced by *Bacillus* sp. D2

Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude enzyme	31	10500	338.709	1.00	100
60% ammonium sulphate precipitation	13.9	5950	428.057	1.26	56.67
DEAE cellulose	0.19	511.3	2691.052	7.94	4.87

Chitinase of *Bacillus brevis* precipitated at 50% (w/v) concentration of ammonium sulphate (Sheng *et al*., 2002).

Enzymes fractionation using anion exchange chromatography

The result of ammonium sulphate precipitation in saturation level 60% (w/v) as much as one ml (formerly have been dialyzed with 0.05 M PBS pH 6.5) applied into column containing DEAE-Cellulose (Figure 3).

The 100 fractions were measured in early experiment to find out protein concentration of each fractions (based on absorption value at 280 nm). Result showed that the only one peak **(**at fraction number 9) between fractions 7 to 12 (Figure 3). According to assays of enzymes activity for fraction number 8-11, their showed the highest enzyme activity. Furthermore, all of the fractions with higher enzyme activity were collected, then to be freeze-dried for characterization of enzyme. Enzyme purification using DEAE-Cellulose may increased enzyme purity as much 7.94 times. The yield and purity for each purification steps were summarized in Table 2

Table 2 showed that precipitation using 60% ammonium sulphate and continued with dialysis resulted in the enzyme purity levels as much 1.26 times compared with the crude enzyme. Precipitation using ammonium sulphate also reduced the amount of protein from 31 mg to 13.9 mg (± 2.23 times). DEAE-Cellulose was able to increase the purity of *Bacillus* sp. D2 chitinase at recovery level up to 4.87%. The results from all steps of purification, showed that chitinase was the protein which expressed in relatively great amount compared to other proteins produced by *Bacillus* sp. D2 during its growth.

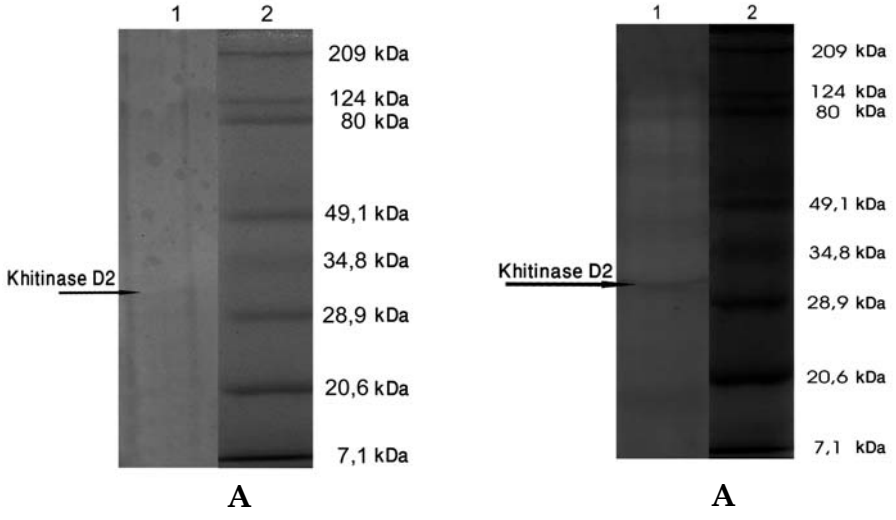


Figure 4. SDS-PAGE analysis of chitinase *Bacillus* sp. D2 (a= Stained with 0.25% (w/v) Comas Brilliant Blue R-250; b= Stained with 0.1% (w/v) Silver nitrate)

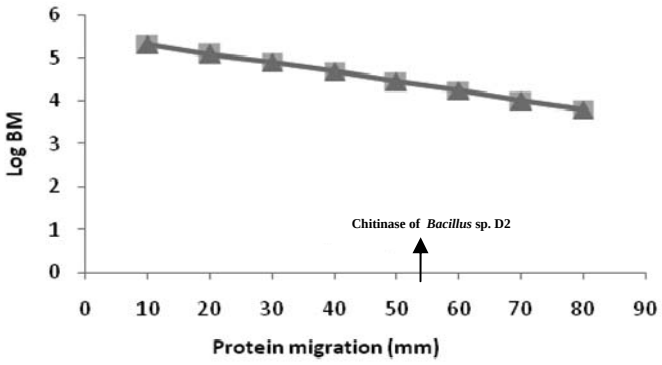


Figure 5. Regression equation of estimation of molecular weight

Characterization of the chitinase molecular weight of chitinase

The molecular weight of chitinase produced by *Bacillus* sp. D2 which performed through on 12.5% SDS-PAGE (Figure 4). The determination of molecular weight using relative mobility calibration curve of standard polypeptides was found out that the chitinase of *Bacillus* sp. D2 was about 30 kDa.

Figure 5 showed that the molecular weight estimation was determined using regression equation Y=5.184-0.0158X (with r = -0.98).

The character of chitinase protein produced by *Bacillus* sp. D2 could be performed on 12.5 % SDS-PAGE (Figure 5). The single band as the result of DEAE-Cellulose filtration was chitinase and it was also approved by the increasing of chitinase activity and enzyme purity. According to Wang *et al*. (1997), molecular weight of bacterial chitinase range from 20 to 110 kDa, but different step of fractionation could give different result on estimation. Sheng *et al.* (2002), reported that purification of *Bacillus brevis* G1 chitinase using 50% ammonium sulphate followed with ion exchange and gel filtration resulted in the chitinase molecular weight about 85 kDa. The purity of its enzyme was 19.57 times compared to its crude enzyme. Toharisman *et al.*(2005) reported that, purification of chitinase from *Bacillus licheniformis* Mb-2 using sequential chromatography (gel filtration, ion exchange and affinity adsorption) resulted in 15.33 times for its purification factor and a single band with the molecular weight 67 kDa. Dahiya, *et al*. (2005) reported that *Enterobacter* sp. NRG4 showed excreting chitinase into the culture supernatant when cultivated in medium containing colloidal chitin and had MW 60 kDa.

This report might be the first result of *Bacillus* sp. chitinase with MW 30 kDa which isolated from rhizosphere of potato plantation in Banjarnegara, Central Java, Indonesia and potentially to be applied for biological control of PCN in the field.

Optimum pH for Bacillus sp. D2 chitinase activity

Figure 6 showed that the most optimum pH value was 7 with maximum chitinase activity up to 17.3 U/ml. At the optimum pH, catalytic sites of enzyme which contain group that act as donor and acceptor for protons had more ionization degree to support the optimum condition. pH also has influence in substrate solubility. The extreme changes of pH resulting changes in enzyme structure and follows with decrease in affectivity and efficiency of enzyme activity (Farabee, 2001).

Chitinase of *Bacillus* sp. D2 had maximum activity at pH7. Wilson and Walker (2005) stated that enzyme activity was determined by the presence of proton donor and acceptor at the expected ionization degree, so that procured an optimum pH. Scopes (1994) reported that most of all enzyme has its maximum activity at pH 5,5-7,5, furthermore, in more acidic (pH 4-5) or alkaline (pH 8-10) environment, the enzyme

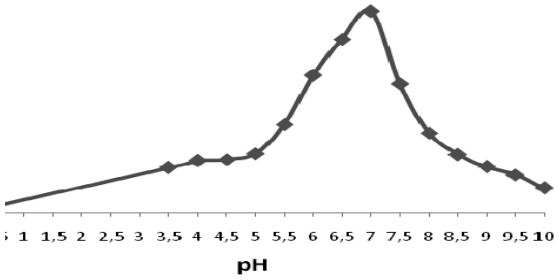


Figure 6. Effect of buffers pH on chitinase of *Bacillus* sp. D2

activity would decrease or become not active. The optimum pH for enzyme activity was not must equal to the environmental pH.

Optimum temperature for Bacillus sp. *D2 chitinase activity*

Almost all bacterial chitinases achieved its maximum activity at the wide range of temperature whereas many of them achieved its maximum activity at the optimum temperature 30^0C-37^0C (Frandberg *et al.*, 1994). The chitinase of *Bacillus* sp. D2 had no activity at 0^0C. Maximum activity chitinase of *Bacillus* sp. D2 was achieved at temperature 30^0C with activity up to 11.57 U/ml, then decreased slowly in 37^0C = 10.52 U/ml (Figure 7).

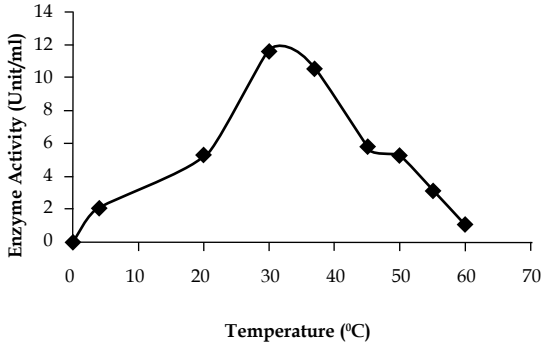


Figure 7. Effect of temperature on chitinase of *Bacillus* sp. D2

The chitinase activity of *Bacillus* sp. D2 increased at temperature range 20^0C–37^0C with maximum activity at 30^0C. This result also indicated that the speed of reaction increased with the increasing of temperature. The maximum activity of chitinase at optimum temperature (30^0C) was a common features of chitinase and this common features also depends on the habitat of the organism (Wen *et al.*, 2002). The chitinase activity of *Bacillus sp*. D2 was decreased at temperature above 40^0C. Scopes (1994) reported that treatment of temperature to the reaction mixture was higher than the optimum temperature so the enzyme will lose its catalytic activity.

Determination of V_{max} and K_m value

The increasing of substrate until achieved saturation as well as will not increase the velocities of enzymatic reaction at each substrate concentration. Michaelis Menten's constant were determined using the optimum reaction condition in experiment designs to calculate the velocities of enzymatic reaction at each substrate concentration (Farabee, 2001).

The determination of V_{max} and K_m value was based on the optimum pH and temperature condition that have been procured. Michaelis Menten's (K_m) constant value analysis and maximum velocity (V_{max}) can be seen at the following Table 3.

Table 3. K_m value and V_{max} for chitinase of *Bacillus* sp. D2

No	[S]	1/[S]	Vi	1/Vi	Regression equation
1	0,25	4,00	0,0004412	2266,545	Y =
2	0,5	2,00	0,0005573	1794,365	917,94+455,14
3	0,75	1,33	0,0007907	1264,6651	r = 0,99
4	1	1	0,002129	469,704	Km= 2 mg/ml
					$\mathbf{V}_{max=2,2}$ µg/h

[S] = *substrates saturation*
Vi = *catalytic velocity*

Based on the calculation, there was quantitative relationship between the velocity i (Vi) with substrate saturation [S]. Michelis Menten's (K_m) constant was procured about 2 mg/ml and maximum velocity (V_{max}) as much as 2,2 µg/h with regression equation Y= 917,94+455,14 and had correlation value about r = 0,99, the lowest of K_m value, has the highest affinity of enzymatic reaction. Chitinase from *Bacillus* sp WY22 had a molecular

weight about 35 kDa, with K_m value 3 mg/ml towards colloidal chitin (Woo and Park, 2003). Compared to chitinase of *Bacillus* sp WY22, the K_m value of *Bacillus* sp. D2 (2 mg/ml) was lowest. Yong, *et al*. (2005) showed that Km and Vmax values of novel chitinase of bacterium C4 apparent on colloidal chitin were 6.95 mg/ml and 10.53 U/min/mg, respectively.

The results indicated that *Bacillus* sp. D2 had highest affinity of enzymatic reaction than chitinase of *Bacillus* sp WY22. Margino (2010) reported that K_m value for chitinase *Streptomyces* sp. IK was 2,92 mg/ml. The lowest K_m value for chitinase of *Bacillus* sp. D2 will also contributes to the future commercial production of chitinase.

The result of qualitative relation determination between the velocities of reaction (V) with substrate saturation [S], was drawn in linear form and based on Lineweaver-Burk equation (Figure 8).

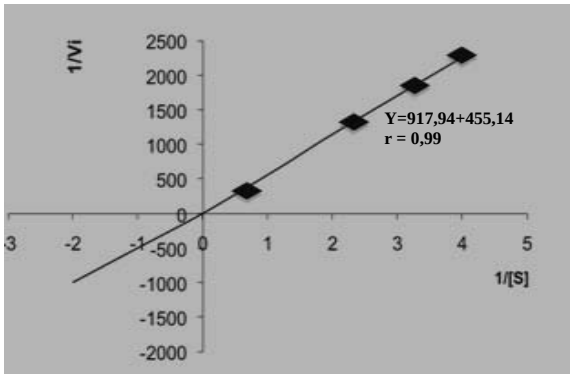


Figure 8. Lineweaver-Burk equation for chitinase of *Bacillus* sp. D2

In conclusion, purification of chitinase *Bacillus* sp. D2 using ion exchange chromatography (DEAE-cellulose) had specific activity of 2691,052 U/mg. The degree of purity 7,94 times than crude enzyme and its yield 4,87% . Characterization of chitinase of *Bacillus* sp. D2 using SDS-PAGE 12,5% resulted in molecular weight about 30 kDa. The apparent K_m and V_{max} of chitinase towards colloidal chitin were 2 mg/ml and 2,2 µg/h, respectively and the optimum condition was at pH 6 and temperature of 30°C.

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