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Characterization of protease from *bacillus* sp. on medium containing $FeCl_3$ exposed to magnetic field 0.2 mt

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Abstract This purpose of this research is to determine the character of the protease enzymes from *Bacillus* sp. on media content of $FeCl_3$ exposed to 0.2 mT magnetic field. The data obtained were analyzed descriptively. The result showed that protease enzyme without Fe resulted in the highest activity at pH 8, temperature 30°C with the addition of activator Mn^{2+}, and V_{max} of 0.28 U/ml, and K_m of 4.60 U/ml. The protease enzyme on media without magnetic field exposure and containing Fe yielded the highest activity at pH 8, temperature 30°C with the addition of activator Mn^{2+}, and V_{max} of 0.33 U/ml, and K_m of 5.64 U/ml. The protease enzyme on medium with magnetic field exposure and use Fe as inductors have the highest activity at pH 9, the temperature of 55° C with the addition of activator Mn^{2+}, and V_{max} of 0.35 U/ml, and K_m 10.04 U/ml.

Keywords: determine, inductor

1. Introduction

The production of the enzyme with high activity can be achieved by optimizing of medium conditions such as pH, temperature, carbon and nitrogen sources, or by providing additional treatment such as exposure to a magnetic field of the medium [1]. Ion Fe is an essential nutrient for almost all microorganisms in growth because, of its role as a cofactor in some enzymes.

Iron or Fe is one of the ferromagnetic metal ions. Fe is an essential nutrient for optimal growth of a cell. Fe acts as a cofactor for several enzymes is required in the biochemical processes of cell, respiratory and photosynthesis reaction [2].

Fe in the form of Fe^{2+} is more easily utilized by bacteria as Fe^{2+} is soluble in water. If the Fe in the form of Fe^{3+} the user is first converted to Fe^{2+} with the rest of the energy that has been used for growth [3]. Magnetic field exposure on bacterial medium containing Fe metal will cause the magnetization in the medium to motion moment - the moment of the dipole into the direction of the magnetic field. The change in direction dipole moment Fe suspected that would cause changes in enzyme activity is controlled by the bacteria.

Based on the research Selfiana et al [4] demonstrated that exposure to 0.2 mT magnetic field for 10 minutes on Fe metal ions affect the activity of *Bacillus* sp. to produce a protease enzyme. The addition of the metal ions Fe in salt form $FeCl_3$ as much as a 0.01% exposed to the magnetic field 0.2 mT for

10 minutes yields the highest enzyme activity 0.06 U/ml.

So far, the protease enzyme results of these studies have not been characterized. Characterization needs to be done to determine the optimum pH and temperature, the metal ions that can increase the activity of the enzyme, as well as chemical kinetics optimal protease enzyme so that to obtain high enzyme activity. Therefore, characterization studies protease from Bacillus sp. on media containing $FeCl_3$ was exposed to a magnetic field of 0.2 mT necessary.

2. Method

2.1. The Culture of Bacillus sp.

Rejuvenation culture is done by scraping the ose culture of *Bacillus* sp., *Bacillus* sp which is a collection of the laboratory of microbiology FMIPA UNILA. Then grown on a modified medium composition comprising Mendel's with skim; 0.5 g; Yeast extract 0.35 g; Water Tryptone 0.35 g; NaCl 0.2 g; KH_2PO_4 0.245 g; $MgSO_4.7H_2O$ 0.035 g; $(NH4)_2SO_4$ 0.175 g in 100 mL of distilled water and agar 1.5 g as the compactor. Later, it was tilt incubated at 37°C in an incubator for two days.

Proteolytic test on a modified solid media Mendel's

$FeCl_3$ which has been exposed by the magnetic field 0.2 mT for 10 minutes is used as an inductor in the growth media. The proteolytic test consisted of three treatments as follows:

a. **Treatment of media without inductors ($FeCl_3$).**
   As a control treatment, a modified solid media Mendel has not given exposure to a magnetic field and are not given the inductor.
b. **Media treatment with the inductor ($FeCl_3$).**
   Treatment Negative is treated using a modified solid media Mendel's and given inductor $FeCl_3$ neither the media nor the inductors are being exposed to a magnetic field.
c. **Media treatment with exposure to a magnetic field in the inductor ($FeCl_3$).**
   Positi treatment is treated using a modified solid media Mendel's and given inductor $FeCl_3$. $FeCl_3$ was exposed to 0.2 mT magnetic field for 10 minutes before use.

Observations existence of a clear zone formed around colonies of bacteria do when the bacterial cultures were incubated for 18 hours at a temperature of 37°C. Colonies of bacteria and a clear zone formed around colonies of bacteria measured in diameter and further defined Proteolytic Index (PI):

$$PI = \frac{\text{the diameter of clear zone} - \text{the diameter of colony}}{\text{the diameter of the colony}} \quad (1)$$

2.2. Enzyme Production

Enzyme production is done by the respective treatment in the media without the addition of agar. Protease enzyme production is done by inoculating 5 ml starter *Bacillus* sp. in 45 ml liquid media Mendel's modified in Erlenmeyer 250 ml with the same treatment as the proteolytic test.

All treatments shake culture was incubated in an incubator at 120 rpm at a temperature of 40 ° C with a 24-hour long incubation time that is adjusted with previous studies. Extraction is done by centrifuging the protease enzyme of bacterial growth media at a speed of 10,000 rpm for 10 min at 4°C.

2.3 Protease Activity Test

0.1 ml protease sample of crude extract was added to the mixture using a 0.5 ml casein substrate as follows: A total of 0.5 ml of phosphate buffer pH 7 0:01 M was added and incubated at 39°C for 10 minutes. After wards plus 0.5 ml of 0.1 M TCA then incubated at 39°C for 10 minutes, and centrifuged at 10,000 rpm at 4°C for 10 minutes. Then 0.375 ml of the supernatant was taken and added with 1:25 ml of Na_2CO_3 0.4 M and 0:25 ml Folin reagent, incubated at 37°C for 20 minutes [5].

Observations were carried out by measuring the Optical Density (OD) at a wavelength of 578 nm. Blank value calculation is done in the same way, where the protease sample was replaced with distilled water. While the standard value calculation is done by replacing the protease samples with tyrosine 5 mM.

Protease activity was calculated in units of PU (protease units) per ml of the enzyme extract. One protease unit is defined as the amount of enzyme that can produce one μmol of tyrosine per minute on measurement conditions were measured using the following formula:

$$\text{PU} = \frac{Asp - Asbl}{Ast} \text{ X } \frac{1}{\text{T}} \qquad (2)$$

Description:

PU	:	Unit Activity Protease (Units/ml)
Asp	:	Value Absorbance sample
Ast	:	Value Absorbance standard
Abl	:	Value Absorbance Blank
T	:	Time

2.4 Determination of optimum pH

Effect of pH on the enzyme activity is tested by reacting a solution of crude extract enzyme and 0.25% incubated casein substrate at 39°C in a buffer 0.05M different series, namely: pH 4.5 (citrate buffer), pH 6.7 (phosphate buffer), pH 8.9 (tris-HCl buffer), pH 10,11,12 (glycine-NaOH buffer).

2.5. Determination of Optimum Temperature

The effect of temperature on the enzyme activity is tested by reacting a solution of crude extract enzyme at optimum pH with temperature variation test 25, 30, 35, 40, 45, 50 55, 60, 65, and 70°C.

2.6. Effect of Metal Ion on Protease Activity

Effect metal ions Ca^{2+} Mn^{2+} Cu^{2+} Mg^{2+} Fe^{3+} in the form of a salt of each - each CaCl2, $MnCl_2$, $CuCl_2$, $MgCl_2$, and $FeCl_3$ as an activator. As an inhibitor of the protease activity used is metal ion chelating compounds, ethylene diamine tetraacetic acid (EDTA) by reacting a solution of crude extract of enzymes, enzyme substrates used as casein 0.25% with one mM of the metal ion. The enzyme is incubated with the metal ions and inhibitors for 10 minutes at a temperature and pH optimum then tested the activity of the enzyme.

2.7. Determination of K_m and V_{max}

Determination of K_m and V_{max} is done by testing the activity enzyme at its optimum temperature and pH variation casein different substrate concentrations of 0%, 0.5%, 1%, 1.5%, 2% and 2.5%. The results of the activity test then tabulated and made the curve relationship between concentration and specific activity of the enzyme casein. Once it is put in the Lineweaver-Burk linear equations. Km value and Vmax is obtained by the formula [6]:

$$\frac{1}{V_o} = \frac{k_m}{V_{maks}} \frac{1}{[S]} + \frac{1}{V_{maks}} \quad (3)$$

Specification:
V_o : initial velocity on the substrate concentration [S]
V_{max} : Maximum Velocity
K_m : Constanta of Michaelis-Menten enzyme for a particular substrate

3. Results and Discussions

3.1. Bacillus sp. protease production

Proteolytic activity *of Bacillus* sp. on solid media Mendel's modified evident from the formation of a clear zone around bacterial colonies growing. With the protease enzyme produced by *Bacillus* sp., casein in the *Bacillus* sp medi The hydrolyzed into smaller peptides and amino acids, their degradation process is characterized by the formation of a clear zone around the colony.

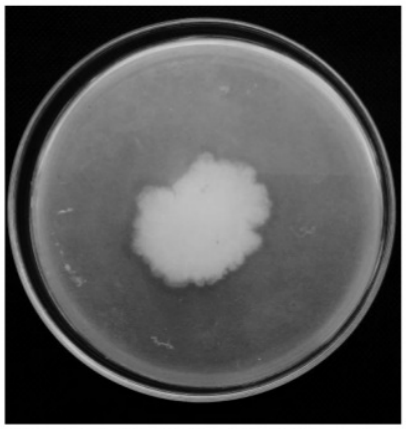

Figure 1. The test results of proteolytic media without $FeCl_3$ and no magnetic exposure. Isolate Bacillus sp. showed small the clear zone in protease medium

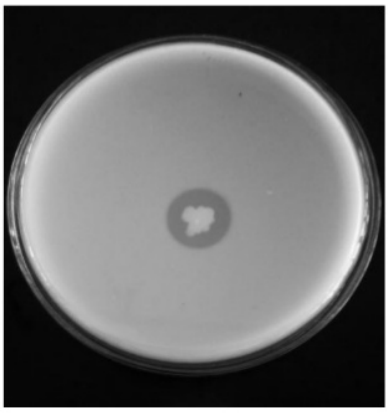

Figure 2. The test results on the media proteolytic treatment with Fe without exposure to magnetic fields. Isolate *Bacillus* sp. showed the clear zone in protease medium.

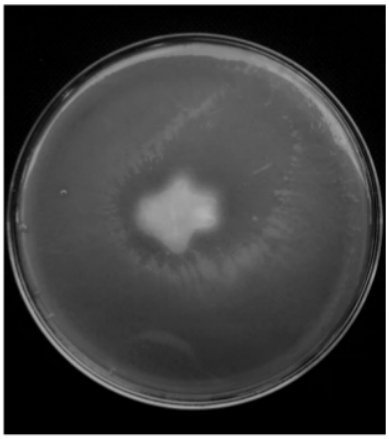

Figure 3. The test results on the media proteolytic treatment with Fe and exposure to magnetic fields. Isolate *Bacillus* sp. showed the clear zone in protease medium.

Proteolytic index value (PI) at the highest media *Bacillus* sp. after incubation for 10 hours of media treatment obtained by Fe outcome magnetic field exposure is equal to 5.62. The highest protease enzyme activity of 0.10 U/ml was obtained from the same media (Table 1).

Table 1. Comparison of proteolytic index and Protease Activity each treatment

Treatment media	Proteolytic Index (PI)	Protease Activity (U/ml)
Without Fe and no magnetic field exposure	0.54	0.08
without magnetic field exposure and containing Fe	2.65	0.07
with magnetic field exposure and containing Fe	5.62	0.10

The area that formed a clear zone indicates that the bacteria in a culture that has a high ability to change the substrate contained in a growth medium [7]. Proteolytic activity of bacteria is influenced by several factors given during treatment. The factors that influence proteolytic activity for each isolates are pH, substrate and enzyme concentration, temperature and the presence of activators and inhibitors. The high value of the proteolytic index on media treatment is given Fe results of magnetic field exposure is suspected because $FeCl_3$ which has been exposed by the magnetic field has induced a higher power and become more positive effect on the proteolytic activity of *Bacillus* sp.

Fe ions in salt $FeCl_3$ which is ferromagnetic have magnetic properties that the direction of the dipole moments of the movement direction of the external magnetic field. As a result of rectification movement can survive even if the external magnetic field has no [8].

If $FeCl_3$ which has been exposed by the magnetic field is added to the bacterial growth media that is stored on the magnetic properties of media expected to affect the growth of bacteria grown on these

media. As reported by Sudarti et al [9] that the magnetic field can directly influence the metabolic activity of cells one to produce the enzyme. Farisna and Zulaikha [10] explains that *Bacillus* sp. able to grow on solid medium containing Fe as Fe metal ions required for the metabolism of bacterial cells even though in small amounts.

3.2. Determination of optimum pH

The optimum pH test results protease enzyme growth *of Bacillus* media prove that the treatment medium without Fe, pH optimum pH 8 to achieve the protease activity of 0.11 U/ml. While the optimum pH of protease enzyme in the treatment medium by Fe without exposure to magnetic fields reaches pH 8 with protease activity of 0.15 U/ml. As well as the optimum pH of the protease enzyme in the treatment by the medium Fe magnetic field exposure results reaches a pH of 9 with protease activity of 0.11 U/ml (Figure 4).

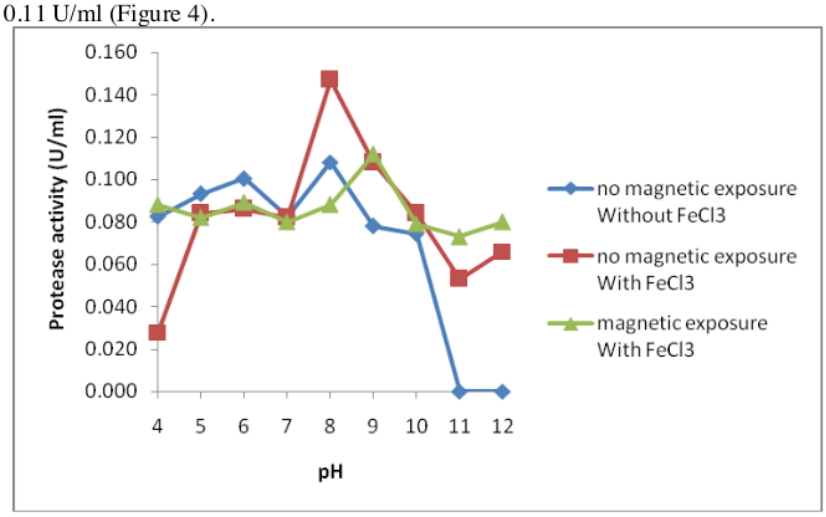

Figure 4. The activity of protease in difference pH

Bacillus sp isolates that it was inoculated in the three media treatments resulted in active proteases in the range of pH 8 and 9. Several studies have shown that the optimum activity of proteolytic enzymes varies from pH 8-10 [11]. Isolates of *Bacillus* sp. isolated from the digestive tract of chicken, protein degradation can grow at pH 7-9 [12]. Protease *.licheniformis B-05* LHSB also has an optimum pH 9. Some protease from genus *Bacillus* other has an optimum pH range 8-10 [13].

The optimum pH difference in the three treatment medium is thought to occur because of the influence of pH on the enzyme active site. The research result Ikehara et al [14], suggests that exposure to magnetic fields ELF has the effect of a reversible bond NH and CN of a peptide bond, and changing the secondary structure of the beta cells and alpha-helices in proteins, so easily have been the attachment of H^+ and OH^- on the side active enzymes.

At pH 4-7 protease enzyme activity tend to be smaller than the optimum pH for the number of H^+ ions that interfere with the enzyme active site, while at pH 10-12 decreased protease enzyme activity for enzyme functional groups disturbed by the many OH- that causes the enzyme denatured.

When $FeCl_3$ which has been exposed by the magnetic field is added in bacterial growth media will cause a difference in the number of H^+ and OH^- ions in the enzyme active site. Thus, there is a difference in the pH of the enzyme treated with magnetic field exposure are not given exposure [9].

3.3 Determination of optimum Temperature

Each enzyme has the maximum activity at a certain temperature; the enzyme activity will increase with increasing temperatures up to optimum temperature is reached. The optimum temperature of the protease enzyme in the treatment medium without Fe reaches a temperature of 30 ^{0}C with protease activity of 0.12 U/ml. Meanwhile, the optimum temperature of the protease enzyme treatment media by Fe without exposure to the magnetic field reaches a temperature of 30 ^{0}C with protease activity of 0.44 U/ml. As well, the optimum temperature of the protease enzyme treatment given media exposure results Fe magnetic field reaches a temperature of 55^{0}C with protease activity of 0.13 U/ml (Figure 5).

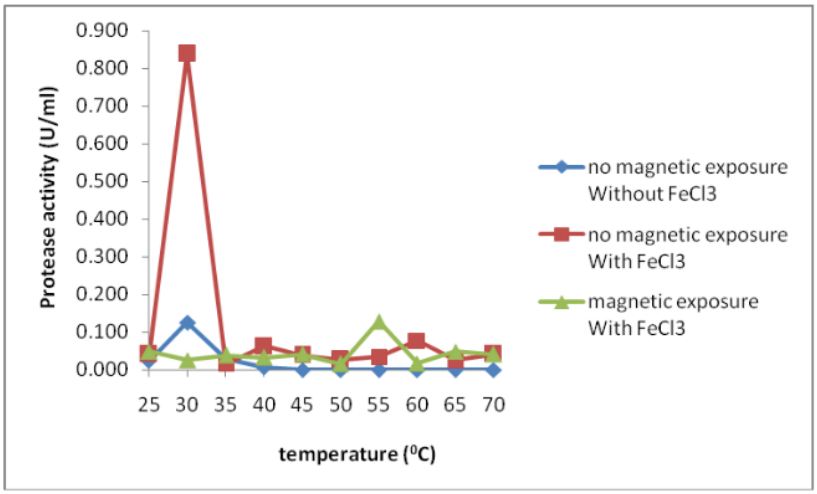

Figure 5. The activity of protease in difference temperature

Further increase in temperature will cause decreased enzyme activity. At temperatures lower than the optimum temperature, the enzyme activity is also low, due to lower activation energies available. The energy needed to create the conditions of an active complex level, both of the molecules of enzyme or substrate molecules.

Protease enzyme was produced on all three medium treatment has the optimum temperature 30 and 55^{o}C. This is confirmed by the results of an intensive search Fitriani [15], where he reported isolates of protease *Bacillus* KUB B19 CC BPPT actively working at a temperature of 30 to 65°C with the optimum temperature at 60°C. Based on the optimum temperature of the protease is classified as thermophilic protease active means to work at high temperatures. Thermophilic proteins that are different characteristics in its structure compared to mesophilic proteins. Thermophilic proteins have more salt bridge structure coupled with the presence of the main chain hydrogen bonds are plentiful when compared to mesophilic proteins. Also, this protein has an amino acid residue more hydrophobic than mesophilic proteins. This will lead to increased activity of enzyme proteins at high temperatures [16]

The difference between the values of the activity of Fe media with media treatment without treatment by Fe without exposure to a magnetic field is thought to occur due to exposure to a magnetic field causes a change of Ca ion movement^{2+} and the increasing rate of movement of the ion. Such conditions lead to changes in transport in the cell membrane that affect cellular metabolism activity. It can have an impact on the process of cell growth. Increased bacterial growth will result in differences in enzyme activity value [17].

The temperature difference optimum media treatment by Fe the result of exposure to magnetic fields with the protease enzyme in media other treatments, allegedly caused by treatment $FeCl_3$ which is exposed to a magnetic field causes motion moments of the dipole direction of the magnetic field from the outside so that the influence of electromagnetic waves that pass through it. While the results of research Ikehara et al .[14], suggests that exposure to ELF magnetic fields have reversible effects on the NH and CN bond of peptide bonds, and changing the secondary structure of the beta cells and alpha-helical proteins. As a result is the release of bonds of constituent proteins and changes in the molecular structure.

3.4. Effect of Metal Ion and Specific Inhibitor

Addition of Mn^{2+} ions to a final concentration of 5 mM can raise the activity of the protease enzyme in the treatment medium without Fe into 0.27 U/ml. The addition of Mn^{2+} ions on the addition of Mn^{2+} ion concentration of 1 mM protease enzyme activity in the media treatment by Fe without exposure to a magnetic field increasing to 0.18 U/ml. But with a final concentration of 5 mM was not able to raise the activity of the protease enzyme in media exposure treatment without magnetic field and added inductors Fe, The same thing happened on the media treatment of the protease enzyme by Fe result of exposure to magnetic fields. The addition of Mn^{2+} ions on the addition of Mn^{2+} ion concentration of 1 mM protease enzyme activity increased to positive treatment 0.30 U/ml. But, with a final concentration of 5 mM was not able to raise the activity of the protease enzyme in the treatment with magnetic field exposure and added inductor Fe (Figures 6 and 7).

The addition of EDTA inhibitor compounds with a final concentration of 5 mM greatly decreases the activity of the protease enzyme in the three media such treatment, until there is no activity at all three enzymes (Figure 6 and 7).

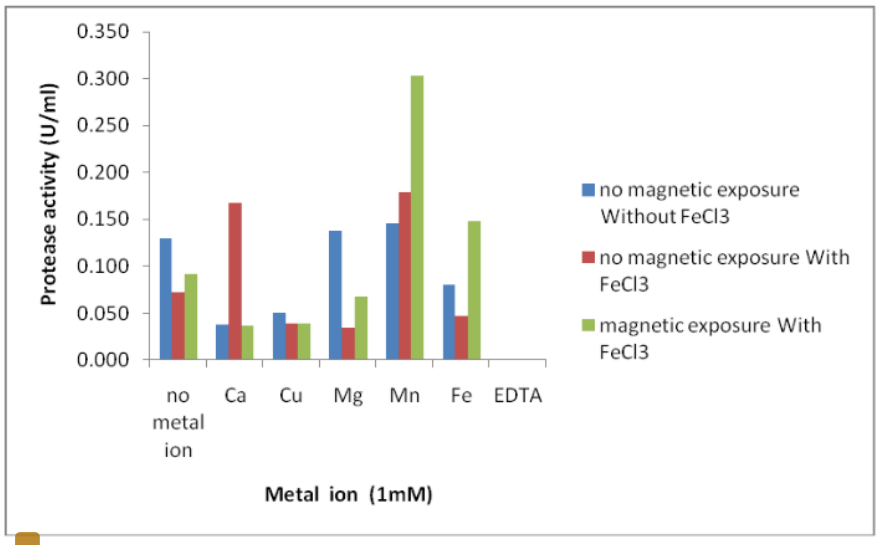

Figure 6. Effect of metal ions and specific inhibitor of the protease enzyme activity in a concentration of 1 mM

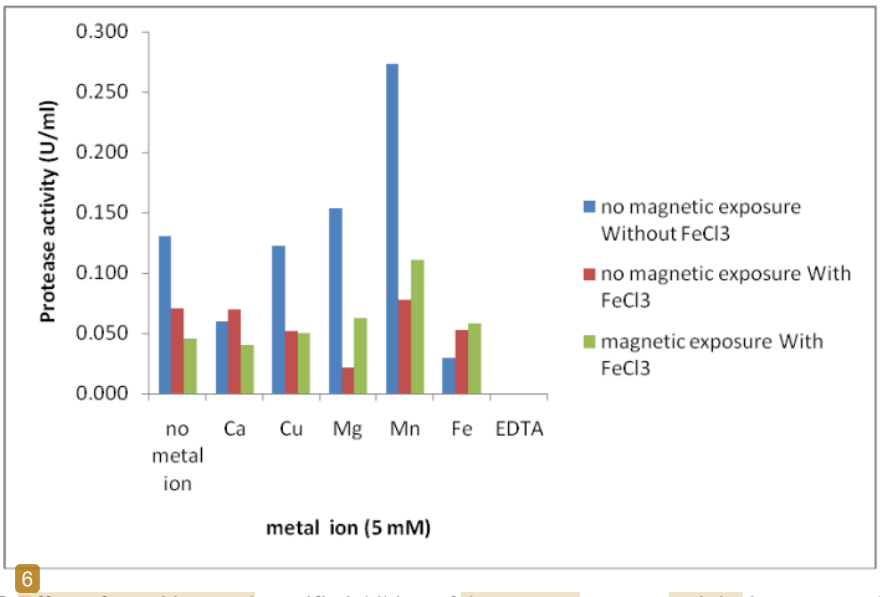

Figure 7. Effect of metal ions and specific inhibitor of the protease enzyme activity in concentrations of 5 mM

Test results show that a strong activator for the protease enzyme of isolates of *Bacillus* sp. the third was inoculated on media treatment is Mn^{2+} at a final concentration of 5 mM able to enhance the catalytic activity of the protease enzyme *of Bacillus* sp. The influence of the addition of metal ions from a few studies have been done, such as the activity measurement results with the addition of metal ions on the substrate casein, metal ion Mn^{2+} (5 mM) is a potent activator of protease *Bacillus epidermidis*, which can increase the activity of protease respectively 3 and 2 times folding of the protease control [18]. For *Bacillus* A1 strain Mn metal ion^{+2} as an activator can increase the enzyme activity according to the research it Adinarayana et al [19]. Inhibitors are compounds that can inhibit the enzyme activity. Extra activity inhibitor compound is one method to identify the enzyme. Differences in protease activity value on each concentration allegedly caused by the difference itself. Enzymes in treatment media which has the highest enzyme activity at a concentration of 1 mM decreased in concentration 5mM, allegedly can not grow optimum conditions of high metal concentrations, but the optimum conditions a low concentration.

Effect of protease inhibitors on the activity was shown in Figure 10, EDTA is a chelating metal (metalloprotease inhibitor) 5 mM slow down to up do not occur enzyme activity in it. The addition of 2 mM EDTA in protease of *Bacillus thermophilic* activity strain HS08 lowered relative to respectively 6% and 4% [20]. The results of analysis show that the protease activity of *Bacillus sp*. perfect inhibited by EDTA so that it can be concluded that these proteases are classified into metalloprotease protein [21]. Chantawannakul [22] reported a protease inhibitor that inhibits the fibrinolytic activity of strains *Bacillus* fermented soybeans 38 isolates from Thailand, namely 1.10 fenantrolin and identified as metalloprotease.

3.5. Protease Kinetics Chemistry

Determination of the value of K_m and V_{max} is useful to determine the enzyme kinetics that can be known how much bonding enzymes and enzyme substrate and how fast it can perform the activity. Determination of the value of V_{max} and K_m is done by measuring the initial velocity at various concentrations of protease enzyme substrate.

From the research that has been conducted shows that the increasing concentration of substrates will increase the reaction rate. However, at a certain concentration limits, no increases the reaction rate even if the substrate concentration is enlarged. This is because at a certain substrate concentration limit, all active parts have been set by the substrate or has been saturated with the substrate [23]. In this study, obtained the value of K_m and V_{max} of the enzyme protease in the treatment medium without Fe each - each amounted to 4.60 mM and 0.28 U/ml. It shows one ml of the enzyme can produce products as much as 0.28 units. As well, the value of K_m and V_{max} of the enzyme protease on media treatment by Fe without exposure to magnetic fields, respectively - each of 5.64 mM and 0.33 U/ml. Show one ml of the enzyme can produce products as much as 0.33 units. Meanwhile, the value of K_m and V_{max} of the enzyme protease media Fe treatment by exposure to magnetic fields result, each - each of 10.04 mM and 0.35 U/ml. This means that one ml of the enzyme can produce products as much as 0.35 units (Table 2).

Table 2. Comparison of Km and Vmax values between each treatments

Treatment media	Km (U/ml)	Vmaks (U/ml)
Without Fe and no magnetic field exposure	4.60	0.28
without magnetic field exposure and containing Fe	5.64	0.33
with magnetic field exposure and containing Fe	10.04	0.35

According to Kovacs et al [24], exposure to magnetic fields on a protein enzyme trypsin can produce different ranges in a redox reaction due to changes in the shape of the helix peptide bonds and hydrogen bonds, thus increasing the Km significantly. A value of K_m are high indicates a low affinity for the substrate and instead of.

In general, the crude extract of the enzyme will give a high value of K_m. It can be caused by the presence of contaminants other enzymes which also have an affinity for the substrate. The higher the value of K_m, the concentration required to achieve half of the maximum reaction rate is also higher. Maximum velocity (V_{max}) will be achieved when all of enzyme molecule has interacted with the substrate or enzyme has been saturated with the substrate, so that an increase in substrate concentration will not increase the rate of reaction [25].

4. Conclusion

From the discussion in this study, the character of the protease enzyme *of Bacillus* sp on media content of $FeCl_3$ in said the magnetic field of 0.2 mT. Where the characters are, among others: the optimum pH and temperature influence of metal ions and inhibitors, as well as the value of enzyme kinetics.

Acknowledgments

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