Characteristics of mannanase enzyme in actinomycetes isolated from Taman Nasional Bukit Duabelas Jambi

S Evan¹, A Meryandini¹* and T C Sunarti²

¹Department of Biology, Faculty Mathematics and Natural Sciences, IPB University, IPB Darmaga Campus, Bogor 16680 Indonesia
²Department of Agroindustrial Technology, Faculty of Agricultural Technology, IPB University, IPB Darmaga Campus, Bogor 16680 Indonesia

*Corresponding author: ameryandini@yahoo.com

Abstract. Indonesia has about 14.03 million hectares of palm oil plantation. Palm kernel cake (PKC) is a byproduct of palm oil processing majority consist of β-mannan. Mannan is a component of hemicellulose which have an economic value. Mannan can be converted to the manooligosaccharides (MOS) using bacterial enzyme. This study aimed to produce MOS from PKC using actinomycetes from Taman Nasional Bukit Duabelas Jambi. The selection was begun with the production of the clear zone on medium containing PKC then enzyme activity was measured. Isolate HJ4-5B had the highest mannanolytic index up to 0.4. The enzyme activity of isolate HJ4-5B increased significantly up to 0.895 U/mL at 7th day of culture incubation. The highest mannanase activity of isolate HJ4-5B occurs at pH 6 and 70 °C with an incubation time of 7th day which reached 1.186 U/mL.

Keywords: Enzyme activity, mannanolytic, mannoooligosaccharides, palm kernel cake.

1. Introduction

The utilization of alternative raw materials has been done to overcome the problem of the high cost protein sources such as fish meal and soybean meal. Local feed raw materials can be used as alternative feed ingredients because they are easy to obtain and are usually in the form of industrial byproducts that have not been utilized optimally. One of the industrial byproducts that can be utilized is palm kernel cake.

Indonesia is the largest palm oil producer in the world with a total land area of 14.03 million ha with a production yield of 37.8 million tons per year [1]. Palm kernel cake (PKC) is a byproduct of the processing of palm oil produced about 45-46% of the palm kernel. As many as 20-40% of the composition of PKC is β-mannan [2]. Mannan is the second most abundant polysaccharide from after cellulose which is found in nature, in the form of glucomannan and galactomannan. Mannan can be converted to mannoooligosaccharides (MOS) using bacteria through an enzymatic process. The enzyme that plays a role in the β-mannan hydrolysis process in PKC is mannanase.

Mannanase is an enzyme that can hydrolyze mannan substrate into mannoooligosaccharides and a small number of mannoses, glucose, and galactose. Mannanase is needed in the feed, food, pulp and paper, pharmaceutical and pre-treatment biomass of lignocellulose in biofuel production [3]. Mannanase hydrolyzes the β-1.4 bond (polysaccharide molecule) between mannoses and mannoses.
Mannooligosaccharides (MOS) resulting from the degradation of PKC by mannanases can be used as prebiotics in animal feed mixes [4], which can stimulate the growth of microflora, and suppress the growth of pathogenic bacteria in the digestive tract [5]. Mannooligosaccharides and several other types of oligosaccharides function as functional food components as prebiotics [6]. Prebiotics are food that cannot be digested and can improve the health of its host by stimulating the growth and activity of one or several bacteria in the large intestine [7]. A food can be classified as a prebiotic if it is not hydrolyzed digestive tract, becomes a selective substrate for beneficial bacteria in the large intestine, can change the composition of the large intestinal microflora, and is beneficial to host health [8].

Some microorganisms that are known to produce mannanases are Bacillus circulans NT 6.7 [9], Streptomyces sp. BF 3.1 [10], and Streptomyces violascens BF 3.10 [11]. The utilization of mannanase enzymes is not optimal, due to the lack of culture of microorganisms capable of producing mannanase. Generally, mannanase enzymes are produced from microorganisms found in soil, compost, or animal rumen. Taman Nasional Bukit Duabelas Jambi (TNBD) is a lowland tropical rain forest rich in endemic biodiversity of flora and fauna. Previous researchers have succeeded in isolating actinomycetes from the soil from the Taman Nasional Bukit Duabelas Jambi but their ability to produce mannanase has not been examined. This study aims to determine the characteristics of the enzyme actinomycetes isolate mannanase from Taman Nasional Bukit Duabelas Jambi.

2. Materials And methods

2.1. Microorganism

Previous researchers have succeeded in isolating actinomycetes from the soil from the Taman Nasional Bukit Duabelas Jambi but their ability to produce mannanase has not been examined. Isolates used were HJ4-5B, OM2RH(e), and OM2RH(e)3b from collections of the Microbiology Laboratory, Department of Biology IPB University.

2.2. Rejuvenation of actinomycetes isolates

Rejuvenation of isolates was carried out on (locust bean gum) LBG medium. The isolate were rejuvenated in 100 mL 0.5% LBG liquid medium then incubated for 96 hours at room temperature with the agitation of 120 rpm. The isolates were grown on 0.5% LBG solid medium, incubated at room temperature for 96 hours. Rejuvenated isolates were transferred to 0.5% palm kernel cake solid medium. The isolate will be tested qualitatively and quantitatively for its enzyme activity.

2.3. Qualitative test of mannanase enzyme activity

The qualitative test of the enzyme activity of mannanase is carried out through the measurement of the mannanolytic index (MI). The qualitative test aims to determine the ability of isolates to degrade mannan. The test were carried out by the 0.1% congo red staining method [12]. One isolate colony was grown on 0.5% palm kernel cake solid medium, then incubated at room temperature for 72 hours. The congo red dye was poured over the medium and incubated for 30 minutes at room temperature. The remaining congo red dye was washed with 2 M NaCl solution three times until the clear zone was seen. The ability to degrade mannan can be determined by measuring the diameter of the clear zone around the isolate colony. The isolate with the largest clear zone was chosen as a potential isolate for further testing.

2.4. Crude enzyme production

Isolates which have the largest clear zone were re-grown on 0.5% palm kernel cake media and incubated for 96 hours. Pre-culture was made by means of a corkborer isolate inoculated in an erlenmeyer containing 125 mL of 0.5% palm kernel cake media. Pre-culture was shaker at 120 rpm at room temperature for 10 days. Harvesting is carried out every day, then a sample of 5 mL is centrifuged at a speed of 6000 rpm for 15 minutes, and the resulting supernatant is an enzyme of crude extract of mannanase to be tested for its activity.
2.5. Crude enzyme mannanase assay
The testing of mannanase enzyme activity is based on the formation of reducing sugars from the hydrolysis of mannan. The reducing sugar formed is measured by the dinitrosalicylic acid (DNS) method [13]. Measurement of the amount of reduced sugar using a 0.5% LBG substrate (in 50 mM phosphate buffer pH 6). The sample treatment was made in as much as 250 µL of the substrate reacted with 250 µL of crude extract enzyme, incubated for 15 minutes at room temperature. The reaction was stopped by adding 750 µL of DNS reagent. Control was made by means of 250 µL substrate added with a 750 µL DNS reagent, incubated for 15 minutes at room temperature, and added 250 µL of crude extract enzymes. Blanks were made by means of 500 µL distilled water reacted with a 750 µL DNS reagent. Samples, controls, and blanks were heated in a water bath for 15 minutes and cooled. Reducing sugar produced from samples, controls, and blanks was measured for absorbance at a wavelength of 540 nm. One unit of enzyme activity of mannanase is defined as the amount of enzyme produced to reduce 1 µmol mannone every minute based on the test conditions. Making a standard curve is done by reacting various mannone concentrations of 0-1mg / mL with an interval of 0.2.

2.6. Crude enzyme characterization
The crude extract enzymes obtained during the highest mannanase production were tested for their activity under various pH and temperature conditions. Determination of the optimum pH of mannanase production is done by testing the enzyme activity at pH 3-10 with a interval of 1 at room temperature (25 °C). The reagent composition contained 0.5% LBG in 0.5 mL buffer pH each and 0.5 mL crude extract enzyme incubated at room temperature for 15 minutes. Buffers used were 50 mM citrate buffer for pH 3-5, phosphate buffer 50 mM for pH 6-8, and 50 mM tris buffer pH 9-10. Determination of optimum temperature is done by testing the enzyme activity at a temperature level of 30-100 °C with an interval of 10 °C. The reagent composition contained 0.5% LBG in 0.5 mL buffer optimum pH and 0.5 mL crude extract of the enzyme incubated in the bath according to their respective temperatures for 30 minutes. Measurement of the activity of mannanase enzymes on various effects of pH and temperature is performed using the DNS method [13].

3. Results
3.1. Rejuvenation of actinomycetes isolates and qualitative test of mannanase enzyme activity
Three actinomycetes isolates were grown on locust bean gum (LBG) media (figure 1) as an initial step to induce the production of the enzyme mannanase. Galactomannan in LBG media can be used as a carbon source for actinomycetes metabolism and also enriched with minerals to induce mannanase [10]. The actinomycetes that have been grown on LBG media for four days are then grown on palm kernel cake media which is used as a carbon source to replace LBG.

Actinomycetes isolates that can degrade mannan can be seen from the value of the mannanolytic index of qualitative test results using a 0.1% red staining [12]. The observations showed that isolates that able to degrade mannan on 0.5% palm kernel cake solid media with the formation of clear zones were HJ4-5B, OM2RH(e).1, and OM2RH(e).3b with successive mannanolytic indices of 0.44, 0.31, and 0.21. HJ4-5B with the largest mannanolytic index (Figure 1) is the selected isolate and will be characterized by enzymes.

3.2. Mannanase enzyme production activity and time
Mannanase enzyme activity curves were produced from four experimental replications (figure 2). HJ4-5B isolate has mannanase enzyme activity, which continues to increase from the first day to the seventh day and decreases on the eighth day. The highest activity was found on the seventh day of 0.895 U/mL (figure 2).

3.3. Determination of pH and optimum temperature for mannanase production
The influence of pH and temperature on the activity of the enzyme mannanase can be seen in Figure 3. The enzyme mannanase can work optimally at pH 6, with the highest activity of 0.811 U/mL, but still has a high activity at pH 7. The mannanase enzyme is still relatively high at alkaline pH activity/base and higher value than acidic pH. The highest mannanase activity of isolate HJ4-5B occurs at pH 6 and 70 °C with an incubation time of 7th day which reached 1.186 U/mL (Figure 4).

![Figure 1](image1.png)

**Figure 1.** Growth of HJ4-5B isolates. (A) Results of rejuvenation of HJ4-5B isolates on 0.5% LBG solid media with 96 hours incubation, (B) Clear zone of HJ4-5B isolates grown on 0.5% palm kernel cake solid media with 96 hours incubation

![Figure 2](image2.png)

**Figure 2.** HJ4-5B mananase enzyme activity in 0.5% palm kernel cake substrate for 10 days, pH 6 (error bar = 5%)

4. **Discussion**

4.1. **Rejuvenation of isolates and mannanase enzyme activity selection**

Rejuvenation of isolates is done to fertilize and regrow isolates that have been previously isolated. Three actinomycetes isolates were grown on locust bean gum (LBG) media as an initial step to induce the production of the enzyme mannanase. LBG can induce the formation of mannanase complexes that can hydrolyze the galactomannan backbone chain and its side chains [4]. LBG is a (*Ceratonia siliqua*). LBG contains galactomannan which can be used as a carbon source for actinomycetes metabolism, in addition it is enriched with minerals to induce the enzyme mannanase [14]. Mannan is the main polysaccharide contained in legume plant seeds with the main element in the form of galactomannan [15]. Galactomannan is the optimum carbon source for the production of *Streptomyces galbus* NR
production [16]. The actinomycetes that have been grown on LBG media are then grown on palm kernel cake media. Palm kernel cake is used as a carbon source to replace LBG.

![Figure 3. Characterization of crude mannanase HJ4-5B activity in various pH at room temperature (error bar = 5%)](image3)

Figure 3. Characterization of crude mannanase HJ4-5B activity in various pH at room temperature (error bar = 5%)

![Figure 4. Characterization of crude mannanase HJ4-5B activity in various temperature at phosphate buffer pH 6 (error bar = 5%)](image4)

Figure 4. Characterization of crude mannanase HJ4-5B activity in various temperature at phosphate buffer pH 6 (error bar = 5%)

The isolates tested were obtained from Taman Nasional Bukit Duabelas Jambi, which was successfully isolated from the soil. Qualitative test of mannanase enzyme activity includes three main isolates are HJ4-5B, OM2RH(e).1, and OM2RH(e).3b. Mannanolytic isolate selection was carried out to obtain isolates that have the highest ability to produce mannanase. The mannanolytic index (MI) was used to determine the ability of isolates to degrade mannan (figure 1). The qualitative test results showed that the HJ4-5B actinomycetes isolate was the isolate that had the largest mannanolytic index, so that the HJ4-5B isolate was selected as a potential isolate to be tested further. The clear zone produced around the colony and the large mannanolytic index value indicates that the three isolates can degrade the palm kernel cake substrate into manooligosaccharides (MOS) and can produce the enzyme mannanase. Congo red coloring in the media can clarify the clear zone formed so that the mannanolytic index can be calculated.

Congo red dye is a specific polysaccharide dye that can bind the β-1,4-D-mannopiranosil bonds in mannan. When mannan has been hydrolyzed into MOS, the red color of congo red does not completely bind the oligosaccharides. This is caused by the β-1,4-D-mannopiranosil bond in oligosaccharides only in small amount. The congo red dye used was 0.1% [12]. The congo red was washed with NaCl, so that the clear zone will be seen more clearly [17].
4.2. **Daily curves of mannanase enzyme activity**

The actinomycetes isolate was grown on liquid media containing palm kernel cake as a carbon source to induce mannanase production. The growth of actinomycetes in the liquid media of the palm kernel cake can be seen through the change in the color of the liquid media from dark brown rather black to light brown rather yellowish. This can indicate that actinomycetes can utilize palm kernel cake as a source of carbon for its growth. Determination of the highest enzyme production time can be seen from the highest mannanase activity. One unit of mannanase activity is defined as the number of enzymes that can produce 1 μmol mannose in 1 minute.

The growth of a bacterium is influenced by aeration and the number of nutrients. The low water solubility of oxygen and slow replacement of oxygen through diffusion are inhibiting factors for aerobic bacteria [18]. The highest mannanase enzyme activity was produced on the seventh day (figure 2). Mannanase enzyme activity decreases on the eighth, ninth, and tenth days. The reduced product in the form of mannose or MOS will increase mannanase activity because this product is used by bacteria as a source of carbon and nutrients for its growth [4]. The results of hydrolysis such as mannoooligosaccharides (MOS) can suppress the synthesis of enzymes used to decompose polysaccharides, so that enzyme activity is decreased [19].

Enzyme activity is influenced by several factors, including the concentration of enzymes, substrates, products, inhibitor or activator compounds, pH, temperature, time, agitation, and aeration [20]. The activity curve of the HJ4-5B actinomycetes isolates enzyme has a different incubation time with other microbes to produce the enzyme mannanase. The results of the research [11] explained that the enzyme mannanase produced from *Streptomyces violascens* BF 3.10 had the highest activity of 16.38 U/mL at pH 6 and a temperature of 70°C. The optimum production time for *Streptomyces violascens* BF 3.10 mannanase at 72 hours, *Nonomuraea* sp. ID06-379 has the optimum mannanase production time on the fifth day with the highest enzyme activity of 4.40 U/mL [21], *Klebsiella oxytoca* KUB-CW2-3 has the optimum mannanase production time on the 24 hours with the highest enzyme activity of 0.52 U/mL [20], and *Fusarium oxysporum* had an optimum mannanase production time on the fifth day with enzyme activity of 3.4 U/mL [17].

The principle of testing the activity of mannanase enzymes is the reaction between the enzyme and the substrate to produce a product in the form of mannose or MOS. Mannose products in the form of reducing sugars will react with dinitrosalicylic acid (DNS) reagents. Dinitrosalicylic reagent consisting of 3,5-dinitrosalicylic acid which is yellow will be reduced to 2-amino-5-salicylic acid. The nitro group that occurs in the reduction reaction is due to the presence of reducing sugars resulting from the hydrolysis of the substrate by mannanase [13]. DNS reagents also function to stop the reaction and color the solution so that its absorbance can be measured using a spectrophotometer. The color formed depends on the amount of reducing sugar produced. Warming up during the enzyme activity test aims to maximize the cessation of the reaction between the enzyme and the substrate. The use of controls in the enzyme activity test aims to determine that the results obtained are the result of a reaction between the enzyme and the substrate during incubation.

4.3. **Determination of pH and optimum temperature for mannanase enzyme production**

Enzymes are protein compounds that function as catalysts for chemical reactions in biological systems. Enzymes have an enormous catalytic ability. Enzymes can increase the reaction rate from $10^6$ to $10^{20}$ times compared to spontaneously reaction. In addition to the catalytic power reaching extraordinary values, enzymes have specificity to the substrate of the reaction they catalyze [22]. The enzyme must bind the exact substrate and position at the active catalyst amino acid substrate and at the active site of the enzyme to catalyze a specific reaction. Enzyme substrate complexes show reactive groups and specific bond positions on the substrate, which will produce a reduction in activation energy and convert the substrate into a product.

Environmental factors can affect the activity of enzymes such as external factors and internal factors. External (environmental) factors include pH, temperature, substrate concentration, and
enzyme inhibitors [23]. Internal factors can include the availability of macro and micronutrients, vitamins, or media [22]. Determination of optimum pH and temperature is important for the growth of microorganisms and their metabolite production. The availability of good supporting factors can increase enzyme activity. High enzyme activity shows that the conformation that forms between enzymes and substrates is also high, so the products it produces are high.

Enzymes have a specific pH range for each enzyme. Changes in active site configuration and loss of catalyst properties occur in the enzyme. The change in pH affects the ionization of the R-amino acid group charge at the active site [22]. The pH level will affect the conformation of the enzyme. The conformation of an enzyme is related to the ability of an enzyme to bind or react with a substrate. If the amount of substrate reacts with enzymes is high then the product will produce more. Besides, changes in pH can also cause enzyme denaturation and result in a loss of enzyme activity [24]. The balance between denaturation of the enzyme at high or low pH and the effect of the enzyme charge, the substrate, or both reflect the relationship of enzyme activity with the concentration of hydrogen ions [23]. pH can stop the enzyme activity by denaturing the three-dimensional structure of the enzyme by breaking the ionic and hydrogen bonds from the enzyme. Increasing the concentration of the substrate will increase the activity of the enzyme until it reaches its saturation point [23].

The results of the characteristics of the enzyme mannanase HJ4-5B actinomycetes isolate showed that this enzyme works optimally at pH 6, but still produces enzymes which are quite high at alkaline pH (figure 3) and works optimally at 70°C pH 6 (Figure 4), including the thermophilic enzyme. The HJ4-5B actinomycetes isolate produces its maximum activity at a pH that is near neutral that is pH 6 of 0.812 U/mL (Figure 3). Similar research results have been reported including the optimum Bacillus circulans NT 6.7 at pH 6 [9], Paenibacillus sp. Optimum DZ23 at pH 6 [25], Aspergillus niger and Trichoderma spp. optimum at pH 5.5 [26], Iodamarina zobelii KMM231 optimum at alkaline pH (pH 9) with an activity of 0.476 ± 0.009 U/mL [27], Bacillus sp. MB 71 optimum at pH 9 with an activity of 0.528 ± 0.057 U/mL [27], Klebsiella oxyctoa KUB-CW2-3 optimum at pH 4 [20], and Fusarium oxysporum at pH 4 with optimum enzyme activity of 18 U/mL [17].

Mannanase produced by the HJ4-5B actinomycetes isolate gives a different response to changes in pH. Initial conditions of incubation at acidic pH showed a slight activity of the enzyme mannanase (Figure 3). Mannanase produced by HJ4-5B can work at optimum pH 6, but still has a high activity at pH 7 and alkaline pH. Streptomyces sp. CS428 can produce mannanase in a board range of pH 5-12.5 and may be most alkaline tolerant so far reported from Streptomyces. The substrate specificity of enzyme showed its highest specificity towards galactomannan, which was further hydrolyzed to produce mannose, mannobiose, mannotriose, and a series of manooligosaccharides. Manooligosaccharides can be further converted to ethanol production [28].

There are three types of main chain degrading enzymes and two side chain degrading enzymes attached to the backbone of mannan. The main chain degrading enzymes are β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.21), and β-glucosidase (3.2.1.25). Side chain degrading enzymes are acetyl mannan esterase (EC 3.1.1.6) and α-galactosidase (EC 3.21.22) (Moreira and Filho 2008). β-mannanase is an endoenzyme that cuts the β-1,4 mannosidic linkages in the mannan or galactomannan backbone randomly producing mannose or manooligosaccharides [29]. Manooligosaccharides are then broken down into human monomers by β-mannosidase. β-mannosidase is an exoenzyme that cuts the β 1-4 bonds in the human backbone or manooligosaccharides from the non-reducing end to produce mannose. α-galactosidase is an enzyme that cuts the α-D galactose chain in the backbone of mannan [29].

Besides pH, temperature also influences enzyme activity. Temperature works in a specific temperature range for each organism. Temperature directly affects the growth rate of microorganisms, the speed of enzyme synthesis, and the speed of enzyme inactivation [24]. The reaction rate increases with increasing temperature followed by fast molecular movements such as enzyme-catalyzed reactions which will peak at optimum temperatures then decrease. The secondary and tertiary structure of protein molecules has weak molecular bonds such as hydrogen bonds. Excessive heat will cause
denaturation, and three-dimensional folding structures will open [17]. The reaction speed of almost all enzymes doubled with every 10°C rise in temperature [30]. The optimum temperature of the HJ4-5B actinomycetes isolates mannanase occurred at 70°C pH (figure 4). Similar research results have been reported including β-mannanase from Fusarium oxysporum thermophilic actinomycetes optimum at 70°C with enzyme activity of 22 U/mL [17], A. aculeatus which has an optimum temperature at 70°C [31], Thermomonospora fusca optimum at 80°C alkaline pH [32], and Bacillus agaradhaerens strain HMTS15 optimum at 75°C pH 10 [33]. Based on the activity of the enzyme produced at the optimum temperature of 70°C, the enzyme is classified as a thermophilic enzyme. The response of enzyme activity to changes in temperature rises slowly following the rise in temperature, but after reaching its optimum temperature the activity returns down. Enzyme activity slowly decreases with increasing temperature. Enzyme inactivation by temperature occurs due to the weakening of intermolecular forces, thereby affecting the stability of the three-dimensional structure of the enzyme and its decreased catalytic ability [34].

5. Conclusion
HJ4-5B isolates can produce enzymes mannanase with the highest mannanase activity obtained on the 7th day of 0.895 U/mL. The resulting mannanase works at an optimum temperature of 70°C and an optimum pH of 6. HJ4-5B isolates can produce thermophilic mannanases.

Acknowledgments
This research was funded by CRC990 ABS Fund 2018 Project B02 for Anja Meryandini.

References
[1] [BPS] Statistics Indonesia 2018 (https://www.bps.go.id)
[2] Yopi, Purnawan A, Thontowi A, Hermansyah H and Wijanarko A 2006 J. Teknologi. 4 312
[3] Sigres D P and Sutrisno A 2015 J. Pangan. Agroindust. 3(3) 899
[4] Meryandini A, Ambarawati D and Rachmania N 2008 451-3 JIPI 13(1) 1
[5] Titapoka S, Keawsompong S, Haltrich D and Nitisinprasert S 2008 World. J. Microbial. Biotechnol. 24 1425
[6] Qiang X, YongLie C and QianBing W 2009 Carbohydr. Polym. 77 35
[7] Gibson G R, Roberfroid M B, Van Loo J, Rastall R A and Probert H M 2004 Nutr. Res. Rev. 17 259
[8] Gibson G R and Roberfroid M B 1995 J. Nutr. 125 1401
[9] Pangsi P, Piwpankaew Y, Ingkakul A, Sunee N and Keawsompong S 2015 SpringerPlus. 4 771
[10] Ariandi, Yopi and Meryandini A 2015 Hayati. J. Biosci. 22 79
[11] Safitri A H, Yopi and Meryandini A 2014 Media. Petern. 37 190
[12] Meryandini A, Anggreandari R and Rachmania N 2008 Biota. 13 82
[13] Miller GR 1959 Anal. Chem. 31(3) 426
[14] Hakobyan L, Gabrielyan L and Trechouian A 2012 Int. J. Hydrogen. Energy. 8 6519
[15] Dey P, Maiti S and Biswanath S A 2012 J. Curr. Pharm. Res. 4 7
[16] Kansoh A L and Nagieb Z A 2004 Antonie. van. Leeuwenhoek. 85 103
[17] Sumardi 2007 J. Sains. MIPA. 13 43
[18] Madigan M T, Martinko J M and Parker J 2006 Brock Biology of Microorganisms 10thEd (New Jersey: Prentice-Hall, Inc)
[19] Williams and Wilkins 1996 Basic Medical Biochemistry: A Clinical Approach Ed ke-1 (Jakarta: EGC)
[20] Chantorn T S, Nawapan P, Suttipan K, Arunuee I, Dietmar H and Sunee N 2013 ScienceAsia. 39 236
[21] Ratnakomala S, Yopi, Prasetya B, Suhartono M T and Meryandini A 2015 Malay. J. Microbiol. 11 284
[22] Sunatmo T I 2009 *Eksperimen Mikrobiologi dalam Laboratorium* (Jakarta: Percetakan Ardy Agency)
[23] Murray R K, Bender D A, Botham K M, Kennelly P J, Rodwell V W and Weil P A 2014 *Harper’s Illustrated Biochemistry* Ed ke-29 (Jakarta: EGC)
[24] Richana N, Lestari P, Thontowi A and Rosmimik 2000 *J. Microbiol. Ind.* 5 54
[25] Chandra M R S, Lee Y S, Park I H, Zhou Y, Kim K K and Choi Y L 2011 *J. Korean. Soc. Appl. Biol. Chem.* 54 325
[26] Adesina F C, Oluboyede O A and Onilude A A 2013 *Afr. J. Microbiol. Res.* 7 282
[27] Tasia W, Zuraida R and Yopi 2016 *JPB. Kelautan. Perikanan.* 11 101
[28] Pradeep G C, Seung S C, Choi Y H, Qchoi Y S, Jun-Pil Jee, Seong C N and Yoo J C 2016 *World. J. Microbiol. Biotechnol.* 32 84
[29] Moreira L R S and Filho E X F 2008 *Appl. Microbiol. Biotechnol.* 79 165
[30] Murray R K, Granner D K and Rodwell V W 2009 *Biokimia. Harper* (Jakarta: EGC)
[31] Pham T A, Berrin J G, Record E, To K A and Sigoillot J C 2010 *J. Biotechnol.* 148 163
[32] Hilge M, Gloor S M, Rypniewski W, Sauer O, Heightman T D, Zimmermann W, Winterhalter K and Piontek K 1998 *Structure.* 6 1433
[33] Liao T, Zhai L, Gao C, Xueand Y and Ma Y 2011 *Wei. sheng. wu. xue. bao.* 51 1520
[34] Bommarius A S and Broering J M 2005 *Biocatal. Biotransform.* 23 125