Chitinase activity of *Pseudomonas stutzeri* PT5 in different fermentation condition

N Chalidah¹, I N Khotimah¹, A R Hakim¹, B A Meata¹, I D Puspita¹*, P S Nugraheni¹, Ustadi¹ and S Pudjiraharti²

¹Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora, Bulaksumur, Yogyakarta, Indonesia
²Research Unit for Clean Technology, Indonesian Institute of Sciences, Jl. Sangkuriang LIPI Complex Building 50, Bandung, Indonesia

*Email: indun_dp@ugm.ac.id

Abstract. This study aimed to determine the incubation condition of *Pseudomonas stutzeri* PT5 in producing chitin degrading enzyme in various pH and temperatures; to compare the production of chitin degrading enzyme in chitin medium supplemented with additional nitrogen, carbon and a mixture of nitrogen and carbon sources and to observe the production of chitin degrading enzyme in 250 mL-shake flasks and 2 L-fermentor. The parameters tested during production were chitinase activity (U·mL⁻¹) of culture supernatant and N-acetylglucosamine concentration (µg·mL⁻¹) in the medium. The results showed that *Pseudomonas stutzeri* PT5 was able to produce the highest chitinase activity at pH 6 and temperature of 37 °C (0.024 U·mL⁻¹). The addition of 0.1 % of ammonium phosphate and 0.1 % of maltose, increased the chitinase activity of *Pseudomonas stutzeri* PT5 by 3.24 and 8.08 folds, respectively, compared to the control. The addition of 0.1 % ammonium phosphate and 0.1 % maltose mixture to chitin medium resulted in the shorter time of chitinase production compared to the addition of sole nutrition. The production of chitinase using 2 L-fermentor shows that the highest chitinase activity produced by *Pseudomonas stutzeri* PT5 was reached at 1-day incubation (0.0283 U·mL⁻¹), which was shorter than in 250 mL-shake flasks.

1. Introduction

Chitin is the insoluble linear β-1,4 linked polymer of N-acetylglucosamine (NAG). After cellulose, chitin is the most abundant polysaccharide in nature and is primarily present in the exoskeletons of crustaceans such as crabs, shrimp, lobsters and also in various insects, worms, fungi and mushrooms [1]. Chitin is biocompatible, biodegradable and has antibacterial as well as wound-healing abilities. Furthermore, the hydrolyzed products of chitin such as oligomer, dimer and monomer have a very broad range of applications in different fields such as material science, microbiology, agriculture, waste water management, cosmetics, drug delivery system, food technology, biomedical, textile industry and much more. Chitin can be hydrolyzed to produce its oligomer, dimer and monomer fragments by a means of the chemical and biological process [2].

Generally, the chemical hydrolyzes of chitin are carried out by a strong acid such as hydrochloric acid (HCl). This procedure, however, produced low yield (below 65 %), costly and produced undesirable wastes which are one of environmental concern [3, 4]. Alternatively, the chitin hydrolysis can be performed bybiological processes, such as enzymatic reaction. Enzymatic hydrolysis of chitin can be performed by chitinase which is specifically hydrolyzed β-1,4 linkage of NAG present in chitin polymer. Chitinase is produced by a various organism from plantsto bacteria [5].
Chitinase production by bacteria gives more advantage compared to other organisms [6]. Therefore the exploration of chitinase-producing bacteria from various environments has given considerable attention from researchers [7, 8]. Chitinolytic bacteria can be isolated by cultivating the bacteria on the selective chitin agar media. The chitinolytic bacteria will produce the clear zone surrounding the bacterial colonies [9]. Pseudomonas is the most prominent bacterial genus in chitinase production. It is a Gram-negative bacteria, aerobic and can be easily found in soil, water, plant and animal [10]. Chitinase activity of Pseudomonas has been reported else where [11, 12, 13]. Previously, we isolated Pseudomonas stutzeri PT5 from shrimp pond sediment located at PT. Indokor Bangun Desa, Yogyakarta, showing achitinolytic index of 1.63 and chitinase activity of 0.0002 U·mL⁻¹ [14, 15].

Chitinolytic bacteria requires an optimal environment condition to produce a maximum chitinase, hence, the optimization of environmental factor such as pH, temperature and nutrient concentration in the medium (carbon and nitrogen) are necessary. Nitrogen is one of the amino acid constituent, the small unit of protein, that plays an important role in the microbial functional system, metabolism for growth and enzyme production [16]. Meanwhile, carbon helps to increase the energy source for bacteria to grow and produces enzyme [17]. Chitinase production can also be increased by a fermentation process using a fermentor because the fermentation condition such as agitation, aeration, dissolved oxygen, turbidity and foam formation can be controlled [18]. This study was aimed to determine the incubation condition of Pseudomonas stutzeri PT5 in producing chitin degrading enzyme, including pH, temperatures, additional nutrients and type of fermentors.

2. Materials and Methods

2.1. Chitin and colloidal chitin preparation
Chitin preparation was comprised of deproteination, demineralization and depigmentation [19]. After depigmentation process, chitin was washed with water until the pH becomes neutral, dried using theoven at 60 °C for 24 h, mashed using a blender then strained in 20 mesh sized sieve to obtain chitin powder. Colloidal chitin preparation was carried on by the addition of HCl and 4 °C cold water [19].

2.2. Inoculum preparation
Refreshment of Pseudomonas stutzeri PT5 from the glycerol-stock culture was done in a chitin agar medium and used as a working culture. Refreshment was performed using a streak method and incubated at 30 °C for 3–5 days. The inoculum was prepared by taking a single colony from working culture and grown in 7 mL of chitin broth incubated for 3 days at 37 °C. Chitin agar medium was prepared by mixing a 2 % colloidal chitin; 2 % bacto-agar; 0.07 % K₂HPO₄; 0.03 % KH₂PO₄; 0.05 % MgSO₄; 0.0001 % ZnSO₄ and 0.0001 % MnCl₂ with aquadest and sterilized [20]. Chitin broth was prepared in the same way as chitin agar without the addition of agar.

2.3. Chitinase production in various condition of incubation and additional nutrient in medium
Chitinase production was performed by inoculating a 225 µL of inoculum to 45 mL chitin broth in 100 mL Erlenmeyer then incubated in water bath shaker for 5 days at 70 rpm. To observe the chitinase production at various temperatures, the pH medium was adjusted to six by the addition of 1 N HCl and temperature of incubation was varied into 30, 37 and 40 °C. Meanwhile, to observe the chitinase production in various pH (5, 6, 7 and 8), the temperature of incubation was maintained at 37 °C. The adjustment of pH was done by the addition of 1 N HCl or 1 N NaOH. Every 24 h, a culture filtrate was taken, centrifuged and analyzed to determine the chitinase activity. Incubation temperature and pH value of medium showing the highest chitinase activity was chosen as an optimum condition that was used afterward.

The chitinase production procedure described earlier was also performed to observe the production of chitinase in various source of 0.1 % (w/v) nitrogen (yeast extract, peptone, ammonium phosphate and ammonium oxalate) and 0.5 % (w/v) carbon (dextrose, maltose, glucose and starch) which was performed separately. Chitin medium without addition of other nitrogen and carbon source was used
as the control medium. After obtaining the optimum source of additional nitrogen and carbon source in the production medium, the experiment proceeded to observe the effect of concentration. The chitinase production procedure described earlier was performed for producing a chitinase in various concentration of the best nitrogen (0.05 %; 0.1 %; 0.15 % and 0.2 %) and carbon (0.5 %; 1 %; 1.5 % and 2 %) source.

2.4. Chitinase production in 250 mL-shake flasks and 2 L-fermentor
The combination of the best source of nitrogen and carbon with the optimum concentration obtained previously was used as a production medium. The production in 250 mL-shake flasks was performed by adding 0.5 mL of inoculum in 100 mL of production medium and incubated at 100 rpm. The condition of optimum temperature and pH obtained from the previous experiment were maintained. To scale-up the chitinase production, the process was performed in fermentor jar by inoculating a 60 mL of inoculum to 1 L production medium in a 2-L fermentor then incubated for 5 days at 200 rpm agitation speed, 1 vvm aeration. Every 24 h, a culture filtrate was taken, centrifuged and analyzed to determine the chitinase activity.

2.5. Chitinase activity assay
Chitinase activity assay was performed according to [21]. The culture filtrate was centrifuged (6,000 rpm for 10 min at 4 °C). A total of 0.5 mL supernatant was added with 1 mL of 1.3 % of colloidal chitin (pH 7.4), incubated in water bath shaker at 37 °C for 30 min, boiled for 3 min and centrifugated at 10,000 rpm for 5 min. A total of N-acetylglucosamine (NAG) concentration was determined by taking 0.25 mL supernatant then added with 0.05 mL potassium tetraborate, boiled for 3 min and cooled in water [22]. The volume of 1.25 mL DMAB reagent was added then incubated in water bath shaker at 37 °C for 30 min then measured by a spectrophotometer colorimetric method, using a wavelength of 584 nm. The absorbance was compared with the standard curve of NAG. One unit (U) of the chitinase activity was defined as the amount of enzyme required to release 1 mmol of NAG from chitin per min.

2.6. NAG concentration assay
NAG concentration in the medium was determined by taking 1 mL of culture filtrate from the fermentation medium, then centrifugated (6,000 rpm at 4 °C for 10 min). A total of NAG concentration in the medium was determined according to the procedure described earlier [22].

3. Results and Discussion

3.1. Chitinase activity produced by Pseudomonas stutzeri PT5 in various pH medium and incubation temperature
The value of pH in the medium affects the environment condition for microorganisms to grow and produce the enzymes. Each species has a certain range of pH and optimum pH for growth. Microorganism needs to maintain a neutral cytoplasmic pH and for this, the plasma membrane may be relatively impermeable to protons. Drastic changes in cytoplasmic pH can harm microorganisms by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Prokaryotes will die if the internal pH drops below 5.0 to 5.5. External pH alterations also might alter the ionization of nutrient molecules and thus reduce their availability to the organism [23, 24]. Furthermore, the pH value of medium shows the total of H⁺ ion existed in the medium. Total ion affects enzyme structure that consisted of amino acids, particularly on the hydrogen bond. Enzymes activity closely related to its structure. Changes in enzyme structure will interfere enzyme-substrate interaction, causing the alteration of enzyme activity [16].

Chitinase activity of Pseudomonas stutzeri PT5 culture supernatant produced in various pH on day-4 fermentation is presented in figure 1. The highest chitinase activity of 0.0028 U·mL⁻¹ was reached at pH 5 followed by 0.0025 U·mL⁻¹ of pH 6. The condition of pH 5 and pH 6 giving a favorable
environment for *Pseudomonas stutzeri* PT5 to grow and produce the enzyme. We choose pH 6 for further experiment step because the activity was not significantly different from pH 5, moreover, the effort and resources required to prepare the pH medium of six are considered more efficient compared to pH 5. The result of enzyme activity was in accordance with the concentration of N-acetylglucosamine (NAG) in the medium, indicating that chitinase is secreted by the bacteria for hydrolyzing chitin in order to obtain carbon and nitrogen which is essential for cell growth [25]. The optimum pH obtained from this result was different from previous researchers [12], whose reported that the highest chitinase activity produced in chitin broth fermentation using *Pseudomonas stutzeri* YPL1 was obtained on the pH value of 7 with the maximum activity of 33.5 U·mL⁻¹, followed by pH 6, 8 and 5, respectively.

![Chitinase Activity and NAG Concentration](image)

**Figure 1.** Chitinase activity (U·mL⁻¹) produced by *Pseudomonas stutzeri* PT5 cultured in various pH of liquid chitin medium and the remaining concentration of N-acetylglucosamine (µg·mL⁻¹) in the medium.

Chitinase activity of *Pseudomonas stutzeri* PT5 culture supernatant produced in various temperature on day-4 fermentation is presented in figure 2. The highest chitinase activity of 0.0024 U·mL⁻¹ was reached at 37 °C incubation. The similar result was reported by previous researchers [26], that obtained the highest chitinase activity from *Pseudomonas pseudomallei* after 18 h fermentation at 37 °C reaching the value of 0.385 U·mL⁻¹. The maximum chitinase activity of *Pseudomonas aeruginosa* K-187 was produced at 37 °C [27]. The optimum range of temperature for *Pseudomonas* sp. to grow is 37–42 °C [28]. Temperature affects microorganism’s growth by influencing the enzyme-catalyzed reactions. Beyond a certain point of higher temperature, slow growth takes place and damages the microorganisms by denaturing enzymes, transport carriers and other proteins. The plasma membrane is also disrupted as lipid bilayer simply melts and the damage is such an extent that it cannot be repaired. At very low temperature, membranes solidify and enzymes do not work rapidly. In summary, when organisms are above their optimum temperature, both the function and cell structure is affected [23, 24]. Moreover, temperature affects the reaction rate of the enzyme-substrate complex [16]. Figure 2 indicated that at a temperature of 37 °C, the reaction rate occurred faster giving the results of the highest concentration of NAG product in the medium. Chitinase production in various environment condition showed that optimum chitinase activity obtained at pH 6 and temperature 37 °C. The optimum condition of pH and temperature obtained in this stage were used to produce chitinase in the next step.
Figure 2. Chitinase activity (U·mL⁻¹) produced by *Pseudomonas stutzeri* PT5 cultured in various temperature of incubation and the remaining concentration of N-acetylglucosamine (µg·mL⁻¹) in the medium.

### 3.2. Chitinase production in various nutrient source

Additional of suitable nitrogen and carbon sources in the medium other than chitin, which is more complex in structure, supplies more ready-to-use nutrient which helps to increase the available energy for the microorganism to grow and produce the enzyme for degrading chitin. Figure 3 shows that ammonium phosphate produced the highest chitinase activity compare to other treatment, resulted to an activity of 0.031 U·mL⁻¹ on day-3 of fermentation, meanwhile, control treatment produced highest chitinase activity of 0.0073 U·mL⁻¹ on day-4 of fermentation. However, this value was lower than that reported previously [12]. They reported the superiority of ammonium phosphate compares to other inorganic nitrogen sources added to chitin medium for *Pseudomonas stutzeri* YPL-1 in producing chitinase after incubation of 84 h at 30 °C. Chitinase activity produced by *Pseudomonas stutzeri* YPL-1 in chitin medium with the addition of ammonium phosphate, ammonium nitrate, ammonium sulfate, ammonium chloride, sodium nitrate and potassium nitrate were 21.4; 13.4; 11.4; 7.2; 1.4 and 0.8 U·mL⁻¹, respectively. However, this value is lower than that of produced with the addition of organic nitrogen, namely peptone, tryptone and asparagine, with the activity of 32.8; 24 and 22.8 U·mL⁻¹, respectively. The increase in chitinase activity of *Pseudomonas stutzeri* PT5, when added with ammonium phosphate, was followed by the increase of NAG concentration in the medium. It indicates that ammonium phosphate promotes the production of chitinase by *Pseudomonas stutzeri* PT5 to degrade chitin into NAG that would be used further as nutrition for growth.
Figure 3. The highest chitinase activity (U.mL\(^{-1}\)) produced by *Pseudomonas stutzeri* PT5 during cultivation in liquid chitin medium added with 0.1 % of various nitrogen source phosphate and the remaining concentration of N-acetylglucosamine (µg\cdot mL\(^{-1}\)) in the medium.

The highest chitinase activity produced by the addition of various carbon sources in the medium for all treatment was obtained at day-4 fermentations. Figure 4 shows that maltose addition to chitin medium produced the highest chitinase activity of 0.0173 U\cdot mL\(^{-1}\) among the other carbon source. This result indicates that *Pseudomonas stutzeri* PT5 preferred to use maltose compared to other carbon sources that might relate to the effectivity of energy production by this carbon source. Other carbon sources may be acted as an anti-induction or inhibiting enzyme production. The preference of bacteria against certain sugar compound is controlled by the Carbon Catabolite Repression (CCR) mechanism [29]. The preeminence of maltose in inducing chitinase production in *Pseudomonas stutzeri* YPL-1 was also reported previously [12]. After 84 h incubation at 30 °C, chitinase activity produced by *Pseudomonas stutzeri* YPL-1 in basal medium containing chitin, maltose, glucose, N-acetylglucosamine and starch were 31.2; 25; 24.2; 17.8 and 16.2, respectively.

Figure 4. The highest chitinase activity produced by *Pseudomonas stutzeri* PT5 during cultivation in liquid chitin medium added with 0.5 % of various carbon source and the remaining concentration of N-acetylglucosamine (µg\cdot mL\(^{-1}\)) in the medium.
3.3. Chitinase production in various concentration of nutrient source

Chitinase activity of *Pseudomonas stutzeri* PT5 in various concentration of ammonium phosphate as additional nitrogen sources in the medium are presented in figure 5. The 0.05 % and 0.1 % ammonium phosphate showed the highest chitinase activity of 0.030 U·mL⁻¹ and 0.024 U·mL⁻¹, respectively, in day-4 of fermentation. Meanwhile, the low chitinase activity was found in the medium added with 0.15 % and 0.20 % ammonium phosphate. The presence of abundant nitrogen amount in the medium caused the synthetic process of fermentation products was ineffective. A large number of total nitrogen from additional nitrogen source or from hydrolyzed chitin products in medium (NAG) will accelerate the growth of bacteria resulting in an increase in cell biomass. However, as the biomass increases, total nutrient available in the medium reduced, as the result of the competition to obtain the nutrients. The competition caused the imbalance energy released with the intake of nutrients and eventually, metabolism activity of microorganisms decreased [30]. Meanwhile, in a low concentration of ammonium phosphate of 0.05 % and 0.1 %, microorganisms are able to utilize the nitrogen effectively resulting in an optimum growth. The optimal growth of microorganisms showed a balance between biomass cell and the availability of a nutrient in the fermentation medium.

![Graph](image)

**Figure 5.** The highest chitinase activity (U·mL⁻¹) produced by *Pseudomonas stutzeri* PT5 during cultivation in liquid chitin medium added with a various concentration of ammonium phosphate and the remaining concentration of N-acetylglucosamine (µg·mL⁻¹) in the medium.

Chitinase activity of *Pseudomonas stutzeri* PT5 in various concentration of maltose as additional carbon sources in the medium are presented in figure 6. The highest chitinase activity produced by maltose addition in the medium for entire concentration was obtained at day-4 fermentations. The addition of 0.1 % maltose produced the highest chitinase activity 0.030 U·mL⁻¹. Maltose is a suitable carbon source for *Pseudomonas stutzeri* growth [31]. However, the reduction of chitinase activity was observed with the higher concentration of maltose. The role of maltose as a carbon source might relate to the ability of this sugar molecule to be transferred inside the cell for metabolism and growth. High concentration of maltose in the medium might cause the inhibition of chitinase gene expression. The relationship between sugar concentration and the mechanism of gene activation/shutting is controlled by the Carbon Catabolite Repression (CCR) mechanism [30].
Figure 6. The highest chitinase activity (U·mL⁻¹) produced by *Pseudomonas stutzeri* PT5 during cultivation in liquid chitin medium added with a various concentration of maltose and the remaining concentration of N-acetylglucosamine (µg·mL⁻¹) in the medium.

3.4. Chitinase production in a combined nutrient source using shake flask and laboratory bench top jar fermentor

The combination of nutrient sources of nitrogen and carbon was added to the fermentation medium to produce a maximum chitinase by *Pseudomonas stutzeri* PT5. The combination of 0.1 % ammonium phosphate and 0.1 % maltose were chosen, as in this concentration (when solely added to chitin medium), the chitinase activity and NAG concentration in the medium were high. Chitinase activity in a combined ammonium phosphate and maltose as additional nutrient sources in the medium are presented in figure 7. The result shows that chitinase activity starts to increase in a day-2 of fermentation with the activity of 0.0167 U·mL⁻¹ and reached the highest chitinase activity of 0.021 U·mL⁻¹ on day-3 fermentations. Combined nitrogen and carbon source as an additional nutrient in chitin medium showed the lower chitinase activity compared to single additional of nutrient sources (0.030 U·mL⁻¹ both for the single addition of 0.1 % ammonium phosphate or 0.1 % maltose), but shorter fermentation time of fermentation was observed. In a single addition of nutrient source, the highest activity was reached at day-4 fermentation, meanwhile, in a combined addition of nutrient source, the highest activity was reached at day-3 fermentations. Allegedly, a high concentration of substrate will become an inhibitor of the enzyme to hydrolyze the substrate. The appropriate ratio of combined nitrogen and carbon source required in chitin medium must be observed further in order to provide a nutrient balance for bacteria to grow (increase cell biomass) and to synthesize enzyme.

Microbial enzyme production in a large amount is usually prepared using a fermentor in which the factors that support microbial growth is controllable [32]. The time for *Pseudomonas stutzeri* PT5 produced the highest chitinase activity in a laboratory fermentor was shorter compared to shake flask Erlenmeyer (figure 7a). The highest chitinase activity observed in the culture supernatant of *Pseudomonas stutzeri* PT5 cultured in the laboratory fermentor (0.028 U·mL⁻¹) was reached at the first day of incubation, meanwhile, the highest chitinase activity in the shake flask erlenmeyer (0.021 U·mL⁻¹) was observed after 3 days of incubation. The shorter time of fermentation was reached using a fermentor because other factors that supported the growth of *Pseudomonas stutzeri* PT5 was controlled, such as agitation and aeration. An optimum condition for microbial growth caused the chitin hydrolyzing process occurred faster. This condition implied to the faster rate of NAG
consumption by the bacteria which was observed by the lower NAG concentration in the medium compared to shake flask fermentation (figure 7b). The production of chitinase by Lecanicillium muscarium CCFEE 5003 increase 23 % in Bench-top CSTR Bioreactor 2 L compared to the conventional method using shake flask Erlenmeyer [33]. However, the difference in chitinase activity obtained using both fermentation vessels was not observed in this experiment, indicating that the fermentation condition using fermentor must be optimized further.

(a)

(b)

Figure 7. (a) Chitinase activity (U·mL⁻¹) produced by Pseudomonas stutzeri PT5 cultured in chitin medium added with 0.1 % ammonium phosphate and 0.1 % maltose using 250 mL-shake flask erlenmeyer (○) and 2 L-laboratory fermentor (●); (b) the remaining NAG (µg·mL⁻¹) concentration in the medium of shake flask (○) and fermentor (●).

4. Conclusion
This paper shows that the production of chitinase by Pseudomonas stutzeri PT5 was able to be optimized by the addition of suitable nutrient of nitrogen and carbon other than chitin in the medium. The combination of additional nitrogen and carbon other than chitin into medium resulted in the shorter time of chitinase production. Moreover, the production of chitinase using 2 L-fermentor was able to shorten the incubation time in order to reach the highest chitinase activity compared to 250 mL-shake flasks. It can be concluded that the production of Pseudomonas stutzeri PT 5 was affected by the condition of fermentation.

References
[1] Muzzarely R A A 1977 Chitin (Oxford: Pergamon Press)
[2] Rinaudo M 2006 Polymer Science 31(7) 603–632
[3] Ju-Hee K, Woo Jin J, Gyu-hyun J, Joon-Seob A, Kil-Yong K and Ro-Dong P 2005 Biotechnology Letters 27 7–11
[4] Sashiwa H, Fujishima S, Yamano N, Kawasaki N, Nakayama A, Muraki E, Hiraga K, Oda K and Aiba S 2002 Carbohydrate Research 337 761–763
[5] Haliza and Suhartono M T 2012 Bulletin Teknologi Pascapanen Pertanian 8(1) 1–14
[6] Bhattacharya A, Nagpure and Gupta R K 2007 Critical Reviews in Biotechnology 27(1) 21–28
[7] Brzezinska M S, Jankiewicz U, Burkowska A and Walczak M 2014 Curr. Microbiol. 68 71–81
[8] Kielak A M, Cretoiu M S, Semenov A V, Sorensen S J and van Elsas J D 2013 Applied and
Environmental Microbiology 79(1) 263–272

[9] Muzarrelli R A A 1999 Native, Industrial and Fossil Chitins (Basel: Springer Basel AG)

[10] Siegrist J 2010 Pseudomonas: A Communicative Bacteria (St Louis: Sigma-Aldrich)

[11] Velusamy P, Ko H and Kim K 2011 Agriculture, Food and Analytical Bacteriology 1 15–23

[12] Ho-Seong L and Sang-Dal K 1994 J. of Microbiology and Biotechnology 4(2) 134–140

[13] Wang S L and Chang W T 1997 Applied and Environmental Microbiology 63(2) 380–386

[14] Kholifah A 2015 Isolasi dan Identifikasi Bakteri-bakteri Kitinolitik dari Sedimen Tambak Udang (Yogyakarta: Fakultas Pertanian Universitas Gadjah Mada)

[15] Triwijayani A U 2016 Identifikasi Bakteri Kitinolitik dari Sedimen Tambak Udang dan Karakterisasi Gen Kitinasenya (Yogyakarta: Fakultas Pertanian Universitas Gadjah Mada)

[16] Lehninger A 1982 Dasar-Dasar Kimia Jilid I (Jakarta: Penerbit Erlangga)

[17] Pandey A 1999 Types of Fermentations and Factors Affecting It Biotechnology: Food Fermentation (New Delhi: Educational Publisher) pp 383–426

[18] Satiawihardja B 1983 Buletin Pusbantepa (Bogor: FTDC Institut Pertanian Bogor) pp 62–70

[19] Hargono H, Abdullah A and Sumantri I 2008 J. Reaktor 12(1)53–57

[20] Hsu and Lockwood J 1975 Applied Microbiology 29(3) 422–426

[21] Wang S L, Lin B S, Liang T W, Wang C L, Wu P C and Liu J R 2010 J. of Microbiology and Biotechnology 20(6) 1001–1005

[22] Reissig J L, Strominger J L and Leloir L F 1955 J. of Biological Chemistry 217 959–966

[23] Pommerville J C 2014 Fundamentals of Microbiology 10th Edition (Glendale Community College)

[24] Tortora G J, Funke B R and Case C L 2010 Microbiology an Introduction 10th Edition (San Francisco: CA Pearson Benjamin Cummings)

[25] Reinhold B and Fritz T 2002 FEMS Microbiology Letters 209(2) 141–148

[26] Purkan, Azizah B, Baktir A and Sumarsih S 2014 Molekul 9(2) 128–135

[27] Wang S L and Chang W T 1997 Applied and Environmental Microbiology 63(2) 380–386

[28] Brooks M 2012 Changes in Cell Structure, Morphology and Activity of Streptomyces Venezueleae During The Growth, Shocking and Jadomycin Production Stages (Canada: Microbiology Biochemical Technology Dalhousie University Canada)

[29] Parisutham V, Kim M K, Lee S K and Ghim C M 2012 Rewiring Carbon Catabolite Repression for Microbial Cell Factory (South Korea: Korean Society Biochemistry and Molecular Biology)

[30] Suresh P V 2012 World Journal of Microbiology and Biotechnology 28 2945–2962

[31] Lalucaj J, Antoni B, Rafael B, Elena G V and Noberto J P 2006 Microbiology and Molecular Biology Reviews 70(2) 510–547

[32] Mejia-Saulés J E, Waliszewski K N, Garcia M A and Cruz-Camarillo R 2006 Food Technology and Biotechnology 44(1) 95–100

[33] Fenice M, Barghini P, Selbmann L and Federici F 2012 Microbial Cell Factories 111–112