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Increasing Chitinase Activity of *Serratia marcescens* PT-6 through Optimization of Medium Composition

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Abstract

Chitin hydrolysate is one of the value added product derived from shrimp shell waste. Production of chitin hydrolysate using biological process offers an environmental friendly method compared to chemical process. *Serratia marcescens* PT-6, a gram negative chitinolytic bacterium isolated from shrimp pond sediment, shows good activity in hydrolyzing chitin. This study aimed to improve the chitinase activity of *S. marcescens* PT-6 culture by optimizing the component of chitin-containing medium (additional nitrogen source, additional carbon source, and colloidal chitin). The optimization of chitinase by *S. marcescens* PT-6 culture was done using one variable at a time method. The sequence of the research were to optimize 1) the type of additional carbon source (glucose, lactose, sucrose, and starch), 2) the type of additional nitrogen source (yeast extract, peptone, ammonium sulphate, and ammonium chloride), 3) the concentration of colloidal chitin (0.5; 1; 1.5; 2; and 2.5%), and 4) the concentration of the additional carbon and nitrogen source. The culture of *S. marcescens* PT-6 was incubated in colloidal chitin medium at 30 ^{o}C and chitinase activity from culture supernatant was analyzed. The results showed that starch gave the highest chitinase activity compare to other carbon source, meanwhile yeast extract was chosen as the best nitrogen source among others. The combination of 1.5% colloidal chitin with 0.5% starch and 0.1% yeast extract in medium increased the chitinase activity of *S. marcescens* PT-6 to 0.021 U/ml. These results indicated that an appropriate medium composition could increase the chitinase activity produced by *S. marcescens* PT-6 culture.

Keywords: chitinase, colloidal chitin, *S. marcescens* PT-6, starch, yeast extract

1. Introduction

Chitinase is an enzyme responsible to catalyze hydrolytic break down of glycosidic bonds in chitin into oligomer and monomer of N-Acetylglucosamine (NAG) (Liu et al., 2013). NAG was used as food supplement, medicine, and cosmetics (Chen, Shen, & Liu, 2010). Chitin is the value-added product derived from Crustacean shell waste. In Indonesia, approximately 40,000 ton/year of shrimp shell was produced as wastes based on National Statistic Board data in 2014 (LIPI, 2015). The utilization of shrimp shell waste helps seafood industries to decrease their operational cost for waste treatment.

One of the chitinase source is chitinolytic bacteria. *Serratia marcescens*, a member of Enterobacteriaceae, is known as high chitinolytic activity bacteria (Xia et al., 2011). In 2015, we isolated *S. marcescens* PT-6 from shrimp pond sediment with initial chitinase activity of 0.0002 U/mL (Triwijayani, Puspita, Murwantoko, & Ustadi, 2018). The optimal pH and temperature for chitinase production of *S. marcescens* PT-6 was determined and resulted in the increasing of chitinase activity into 0.002 U/mL (Sari, Isnaini, Puspita, Husni, and Ustadi, 2017), a 10 times higher than before the optimization. Chitinase is an inducible enzyme that is released by the bacteria in the presence of chitin in the medium. The hydrolyzed chitin provides nutrients, especially carbon and nitrogen, for bacterial growth (Monreal & Reese, 1968).

The addition of another carbon and nitrogen sources into chitin-containing medium has increased chitinase production of *S. marcescens*. Singh, Sharma, and Hoondal (2008) showed that media containing 0.5 % chitin and 0.5 % starch increased the chitinase activity of *S. marcescens* GG5 (0.3 U/mL) by 50 % compared to media containing only 0.5 % chitin (0.2 U/mL).

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Chakrabortty, Bhattacharya, and Das (2012) reported that organic nitrogen sources were better than inorganic source to increase the chitinase activity of *S. marcescens* marine isolate, and the addition of 0.1 % yeast extract showed the highest chitinase activity (9.56 U/mL) compared to other types of nitrogen sources. Furthermore, Arbakariya and Webb (1998) explained that C:N ratio in formulation medium is critical for higher enzyme production and biomass. The choice of nutrient source and concentration is an important consideration to provide the optimal condition for bacterial growth and enzyme production. This study was conducted to find out the best type of carbon and nitrogen sources, as well as the optimal composition in media for chitinase production by *S. marcescens* PT-6.

2. Material and Methods

2.1. Medium Preparation

Shrimp shell waste from a frozen shrimp industry located in Central Java, Indonesia was used in medium preparation. Chitin and colloidal chitin were produced from shrimp shell waste based on the method applied by Hargono and Sumantri (2008) and Arnold and Solomon (1986), respectively. Colloidal chitin was used as chitinase inducing substrate in medium. Colloidal chitin agar medium was made based on method by Hsu and Lockwood (1974) without $FeSO_4$ addition, and prepared using 0.03% (w/v) KH_2PO_4, 0.07% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4.5H_2O$, 0.0001% (w/v) $ZnSO_4$, 0.0001% (w/v) $MnCl_2$, 1.5% (w/v) colloidal chitin, and 2% (w/v) bacto agar (Oxoid), while colloidal chitin broth was prepared with similar composition without 2% bacto agar. Nutrient Broth (NB) (Oxoid) medium was used for the preparation of inoculum. The pH of all the medium used were adjusted into 7.

2.2. Bacteria Inoculum Preparation

S. marcesccens PT-6 used in the research was isolated from shrimp pond sediment owned by local company, PT. Indokor Bangun Desa, Yogyakarta, Indonesia (Triwijayani et al., 2018). A single colony of *S. marcescens* PT-6 incubated 24 h at 30 °C on colloidal chitin agar was put into 7 ml of NB (nutrient broth) and incubated for 24 hours at 30 °C. From NB medium, 0.6 mL culture was put into 30 mL NB and incubated for 8 hours (for logarithmic phase) in waterbath shaker at 30 °C and 100 rpm reciprocal agitation. Inoculum was ready to be used in the production medium.

2.3. Chitinase Production at Various Carbon and Nitrogen Sources

Approximately 0.225 mL inoculum was transferred into 45 mL colloidal chitin medium, as a control, colloidal chitin medium with the addition of 1 % (w/v) carbon sources (glucose, lactose, sucrose, or starch), and colloidal chitin medium with the addition of 0.1 % (w/v) nitrogen sources (yeast extract, polypeptone, ammonium chloride, or ammonium sulphate). Cultures were incubated at 30 °C and 100 rpm reciprocal agitation in waterbath for 3 days. Each treatment were done in triplicate. The chitinase activity and bacterial growth were measured at the end of incubation.

2.4. Chitinase Production at Various Concentration of Colloidal Chitin

Inoculum of 0.225 mL was transferred into 45 mL of colloidal chitin medium contained of additional carbon source (1.5 %) and nitrogen source (0.15 %) that were chosen as the best carbon and nitrogen sources from previous step. The concentration of colloidal chitin in the medium was varied into 0.5; 1; 1.5; 2; and 2.5 %. All treatments were done in triplicate and cultures were incubated for 3 days at 30 °C and 100 rpm reciprocal agitation in waterbath. The chitinase activity, N-acetylglucosamine concentration in culture broth and bacterial growth were measured at the end of incubation.

2.5. Chitinase Production at Various Ratio of Carbon and Nitrogen Sources

The best carbon and nitrogen source were determined for their optimal ratio for the chitinase activity produced by *S. marcescens* PT- 6. Approximately 0.5 mL of inoculum bacteria was put into 100 mL colloidal chitin broth at various concentrations of additional carbon source (0.5; 1.0; and 1.5 %, w/v) and nitrogen source (0.10; 0.15; and 0.2 %, w/v). Nine treatments combination were observed for their effects on chitinase activity. The concentration of colloidal chitin used in this experiment was prepared according to the optimal concentration of colloidal chitin obtained from the previous step. Culture were incubated in waterbath shaker for 5 days at 30 °C and 100 rpm reciprocal agitation. Once every 24 hours during incubation, bacteria growth and

chitinase activity were observed. All treatments were done in triplicate.

2.6. Bacteria Growth Measurement

Bacteria growth was measured by means of Optical Density (OD). For each treatment, absorbance of 1 ml bacteria in chitin broth medium was observed at 600 nm using spectrophotometer (Thermo Spectronic-Genesys 20).

2.7. Chitinase Activity Measurement

Cell-free supernatant was obtained by centrifugation of 1 mL culture medium at 10,000 rpm and 4 °C for 1 minute, then separated into two parts (500 μL each) in 1.5 mL microtube as sample and negative control. Negative control was heated to inactivate chitinase as a parameter to measure the initial concentration of N-acetylglucosamine (NAG). The value of negative control showed the NAG concentration in the culture medium. Both sample supernatant and negative control was then subjected to 1.3 % colloidal chitin (in 50 mM phoshate buffer pH 6) and incubated in shaker waterbath for 30 minutes at 37 °C and 100 rpm agitation. Colloidal chitin used in chitinase activity measurement was prepared from commercial chitin (99 % purity) (Sigma-Aldrich) using the same methods as mentioned previously. The mixtures were then boiled for 3 minutes to stop enzymatic reaction, cooled, and centrifuged at 10,000 rpm for 3 minutes (Wang & Chang, 1997). The concentration of NAG produced from the enzymatic reaction was measured using Reissig, Strominger, and Leloir (1955) method based on the absorbance detected on spectrophotometer (Thermo Spectronic-Genesys 20) UV-Vis at 584 nm. The absorbance of NAG standard (TCI) solution at concentrations of 5-50 μg/mL were also measured. The later was conducted to obtain standard curve equation as reference for sample absorbance. A unit of chitinase activity was defined as μmol NAG released per minute for 30 minutes incubation in enzymatic reaction condition mentioned.

2.8. Data Analysis

Complete randomized design in triplicate was applied during experiments. Obtained data were statistically analyzed using Analysis of Variance (ANOVA) 95 % confidence level. To determine optimum ratio of carbon and nitrogen source, regression analysis was applied. Regression equation was then used to calculate optimum period (x) if y'= 0 and chitinase activity (y) at x optimum, as well as coefficient of determination (R^2) to determine the best treatment.

3. Results and Discussion

Chitinase activity produced in *S. marcescens* PT-6 culture in various additional carbon sources is presented in Figure 1. The type of carbon source showed a significant effect on chitinase activity of *S. marcescens* PT-6 culture ($P<0.05$). The highest chitinase activity was shown by *S. marcescens* PT-6 culture in medium added with starch (0.004 U/mL). Meanwhile the addition of glucose, lactose, and sucrose showed negative effect on the growth and metabolic activity of *S. marcescens* PT-6.

Similar result was reported by Singh et al. (2008) that the addition of starch increased the chitinase activity of *S. marcescens* GG5 by 50 % compared to the chitin medium without starch addition. Meanwhile, Chakrabortty et al. (2012) reported that the addition of glucose and sucrose in the medium decreased the chitinase activity of *S. marcescens* culture compared to the control medium. Zarei et al. (2010) revealed that the addition of monosaccharides that were easily metabolized (glucose, lactose) inhibited chitinase formation by *S. marcescens* B4A. Holt et al. (1994) reported that growth of *S. marcescens* was inhibited in a medium containing urea, potassium chloride, and glucose. Presumably, the presence of simple sugar caused the rapid production of acidic compounds in the medium that resulted in the dramatically decrease of pH. The pH value of medium added with glucose, lactose, and sucrose ranged from 4.0-5.5 at day-3 of incubation, while starch and control were stable at 7. Low pH values inhibited bacterial growth and metabolic activity. It was indicated by the low OD value of *S. marcescens* PT-6 culture in the colloidal chitin medium added with glucose, lactose, and sucrose (Figure 1). Another explanation of the difference in chitinase activity produced in different type of additional carbon sources is due to the Carbon Catabolite Repression (CCR). CCR is a key regulatory system found in some microorganisms that have a preference in the utilization of certain carbon sources for the efficiency of energy usage (Vinuselvi, Kim, Lee, & Ghim, 2012). Starch was chosen as the best carbon source for the next optimization step.

The effect of different type of nitrogen source on chitinase activity produced by *S. marcescens* PT-6 is shown in Figure 2. The type of nitrogen source showed a significant effect on chitinase activity ($P<0.05$). Chitinase activity of all treatments were higher compared to control. It is expected that the additional nitrogen sources provide more nitrogen

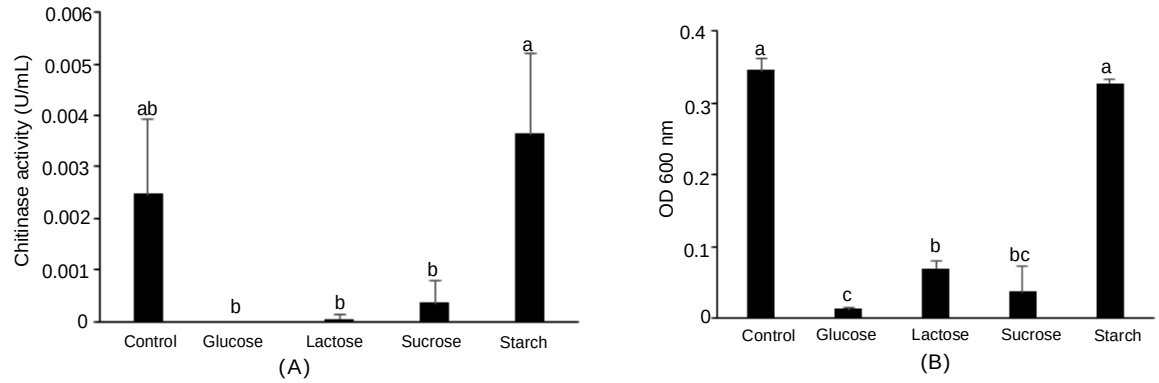

Figure 1. Chitinase activity (A) and optical density (B) of *S. marcescens* PT-6 culture in colloidal chitin broth added with various carbon sources at 1%. Incubation condition: Inoculum 0.5%, colloidal chitin 2%, pH 7, 30°C, day-3.

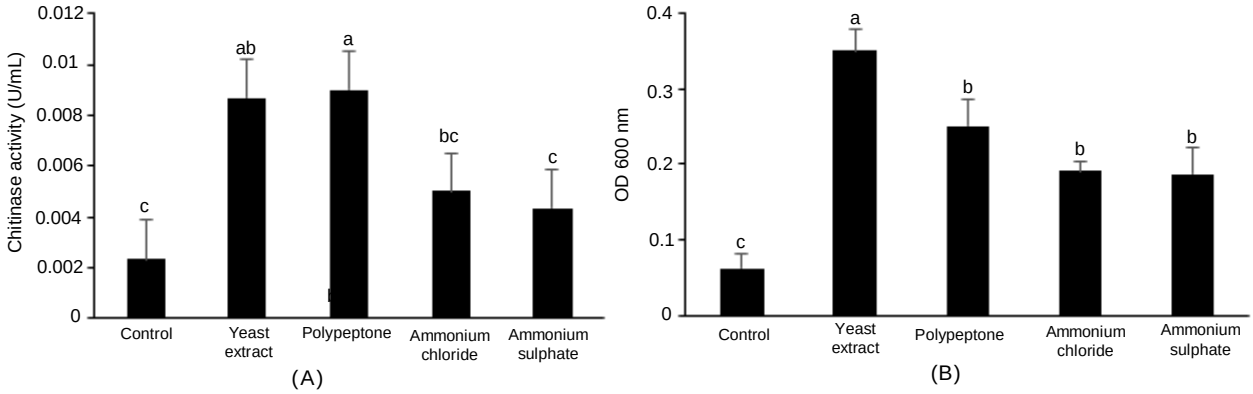

Figure 2. Chitinase activity (A) and optical density (B) of *S. marcescens* PT-6 culture in colloidal chitin broth added with various nitrogen sources at 0.1%. Incubation condition: Inoculum 0.5%, colloidal chitin 2%, pH 7, 30°C, day-3.

availability and resulted in the enhancement of chitinase production by bacteria. According to Karthik, Akanksha, Binod, and Pandey (2014), the production of extracellular chitinase enzymes is influenced by the composition of the medium, one of which is the availability of nitrogen. Moreover, Figure 2 shows that the chitinase activity produced by the cultures added with organic nitrogen source (yeast extract and peptone) tends to be higher than inorganic sources (ammonium sulfate and ammonium chloride). In addition to the nitrogen component, organic nitrogen sources provide amino acids, vitamins, and growth factors that support bacterial growth (Kalil, Alshiyab, & Yusoff, 2008). It was indicated from the turbidity of the culture in the medium added with yeast extract and polypeptone were higher than ammonium chloride and ammonium sulphate (Figure 2).

The addition of yeast extract and polypeptone resulted in the similar chitinase activity, but the bacterial density was higher in the culture added with yeast extract (Figure 2). Chakrabortty et al. (2012) reported that the highest chitinase activity from *S. marcescens* culture was also obtained from medium added with yeast extract (9.56 U/mL) compared to other organic and inorganic nitrogen sources. Yeast extract was able to support bacterial growth due to the presence of growth factors (Nawani & Kapadnis, 2005). Yeast extract was chosen as the best nitrogen source for the next optimization step, as it also served as the best nitrogen source to support bacterial growth.

The low chitinase activity of *S. marcescens* PT-6 cultured in the medium added with inorganic nitrogen was different from Xia et al. (2011) whose reported that *S. marcescens* XJ-01 showed the highest chitinase activity in the medium added with ammonium chloride compared to other organic and inorganic N sources. It indicates that the same bacterial species does not necessarily required the same nitrogenous compound to produce the highest chitinase activity. According to Wang, Yan, and Cao (2014), the ability of the *S. marcescens* to produce chitinase depends on the strain used. This suggests that the achievement of chitinase activity in different types of nitrogen sources is caused by different strains of bacteria, therefore the choice of nitrogen source is critical before the optimization of chitinase production.

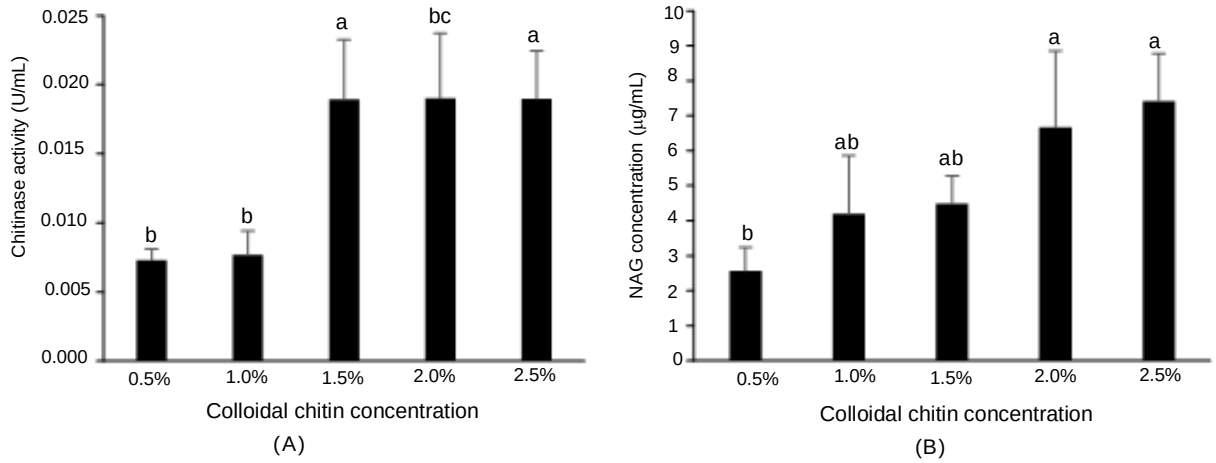

Figure 3. Chitinase activity (A) and N-acetylglucosamine concentration in medium (B) of *S. marcescens* PT-6 culture in colloidal chitin broth prepared by various concentration of colloidal chitin added with 1.5% starch and 0.15% yeast extract. Incubation condition: Inoculum 0.5%, pH 7, 30°C, day-3

The concentration of colloidal chitin showed a significant effect on chitinase activity produced in *S. marcescens* PT-6 culture ($P<0.05$). Figure 3 showed that chitinase activity in medium with colloidal chitin of 1.5; 2; and 2.5 % were higher than 0.5 and 1 %. Pandey, Webb, Soccol, and Larroche (2006) stated that colloidal chitin of 1-1.5 % was able to increase chitinase activity. According to Monreal and Reese (1968), chitinase is an inducible enzyme which is released by the microorganisms upon the presence of the related substrate in the environment. Smith and Grula (1983) reported that solubilized components such as D-glucosamine, N-acetylglucosamine, chitobiose resulted from sterilization of chitin were good inducers for chitinase synthesize in *Beauveria bassiana*. Moreover, Green, Healy, and Healy (2005) reported that the type of chitinous substrate affects the induction of chitinase in *S. marcescens* QMB1466, and colloidal chitin was the most effective inducer on the dry weight basis. The initial concentration of NAG in medium ranged from 1.42-1.78 µg/mL. This small amount of NAG in the medium possibly induced the synthesis of *S. marcescens* PT-6 chitinase. Figure 3 shows that the increased in the concentration of inducers component (colloidal chitin), increased the chitinase activity of *S. marcescens* PT-6 culture. We also found that the increased in chitinase activity was in concurrent with the increased of NAG concentration in culture medium. Meanwhile, the OD (600 nm) of culture from all treatments did not show significant different. The stable chitinase activity in *S. marcescens* PT-6 culture was observed at the colloidal chitin concentration of above 1.5 %. Therefore, colloidal chitin of 1.5 % was used in the next optimization step.

There was generally increasing growth during incubation day-0 to 5 when *S. marcescens* PT-6 were cultured in various concentration of starch and yeast extract ratio (Figure 4). In the beginning of exponential phase (day-1), chitin broth medium color changed to red. Khanafari, Assadi, and Fakhr (2006) mentioned that the phenomena occurred due to the expression of red pigment gene by high number of *S. marcescens* during exponential phase which live in a suitable condition. The highest bacteria growth was obtained by the addition of starch to yeast extract ratio (%) of 0.5 : 0.15 followed by 1.0 : 0.15 and 1.5 : 0.15 (Figure 4). Madigan, Martinko, Dunlap, and Clark (2012) explained that carbon plays the role in cell material formation during cell division, whereas nitrogen is important in protein synthesis (enzyme). Results indicated tendency that higher yeast extract ratio induced higher bacteria growth. Shaikh (2016) mentioned that yeast extract is the source of amino acid, vitamin, coenzyme, and growth factor for *S. marcescens*. Kalil et al. (2008) also noted that the substance is rich of nitrogen and growth factor, thus often used in organism growth media.

In general, the variations of starch concentration did not show any positive effect on *S. marcescens* PT-6 growth. It was evidenced by the decreased in culture's optical density with the greater concentration of starch. Probably the added concentration of carbon source was higher than that was required by the cells. In some extent, the higher concentration of carbon can even inhibit growth, as microorganisms have a regulatory system in utilizing carbon sources, commonly called carbon catabolite repression (CCR). CCR helps microorganisms balance the metabolic capacity and the ability to absorb the maximum amount of sugar. Microorganisms have the ability to absorb nutrients to survive in a fluctuating environment, therefore optimal absorption and assimilation of nutrients is needed (Vinuselvi et al., 2012).

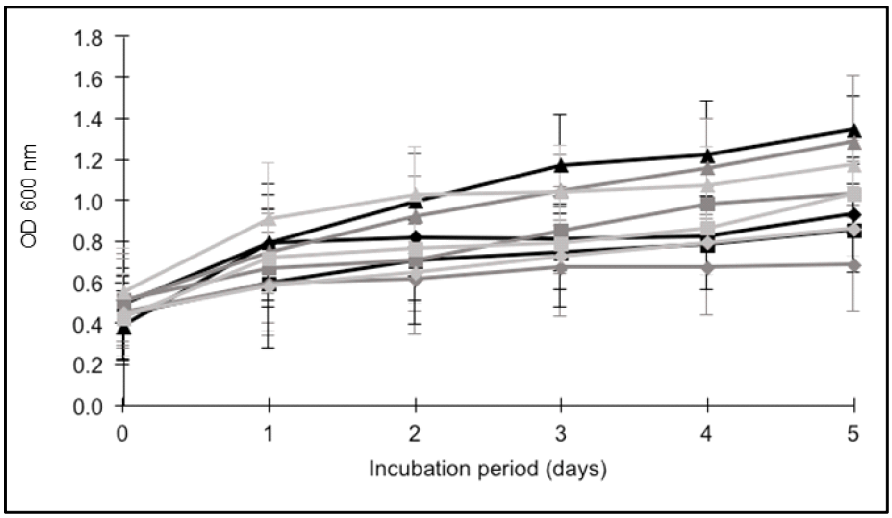

Figure 4. *S. marcescens* PT-6 growth during 5 days incubation at various starch : yeast extract ratio (%) (starch 0.5% :▬; 1.0% :▬; 1.5% :▬; yeast extract : 0.05% :◇; 0.10% :□; 0.15% :△)

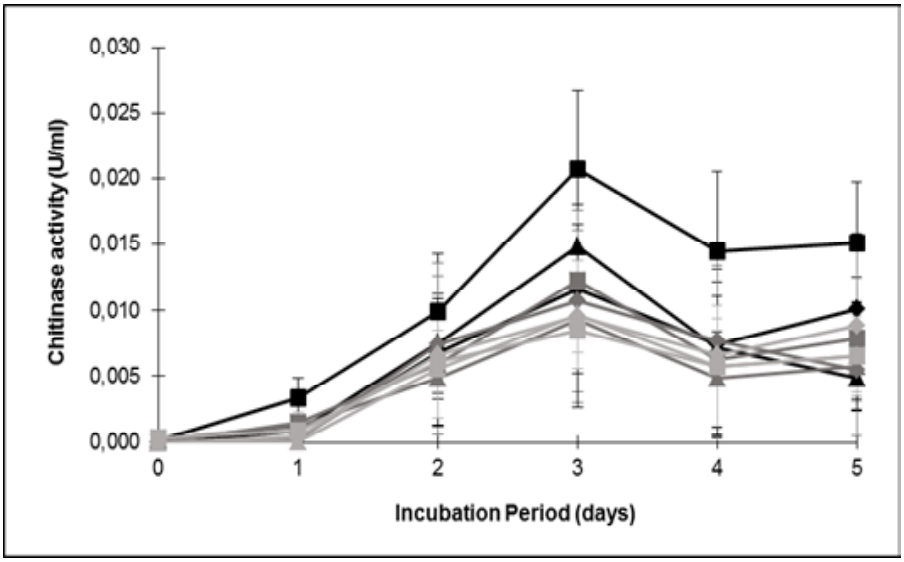

Figure 5. Chitinase activity produced by *S. marcescens* PT-6 during 5 days incubation at various starch : yeast extract ratio (%) (starch 0.5% :▬; 1.0% :▬; 1.5% :▬; yeast extract : 0.05% :◇; 0.10% :□; 0.15% :△)

Chitinase activity tends to increase during day-1 to 3 fermentation according to quadratic equation (Figure 5). There was no significant effect among various starch and yeast extract concentration ratio as C and N source (P>0.05) on chitinase activity of *S. marcescens* PT-6. However, there was a clear trend that the highest chitinase activity of 0.0207±0.006 U/mL was obtained by the addition of starch to: yeast extract ratio (%) of 0.5:0.10 during day-3 (Fig. 5). Results indicated the tendency of decreased in chitinase activity with higher starch to yeast extract ratio, marked by chitinase activity below 0.01 U/mL, obtained by all treatments of 1.5 % starch. Zuhri, Agustien, and Rilda (2013) mentioned that high concentration of carbon source in medium might play another role as catabolic repressor hence lower enzyme production. Schmidt and Schaechter (2012) stated that bacterial cells require nutrients in the form of carbon and nitrogen with a ratio of 4-5:1. The best treatment of starch to yeast extract ratio (%) of 0.5:0.1 were closely matched the nutritional requirements of bacterial cells.

Influence of each starch and yeast extract ratio on chitinase activity of *S. marcescens* PT-6 was

Table 1. Regression equation of *S. marcescens* PT-6 chitinase activity at various starch and yeast extract ratio

Treatment ratio (%)		Quadratic equation	Coefficient of determination	Optimum	Chitinase activity[b]
Starch	Yeast extract		(R^2)	period [a] (days)	(U/ml)
0.5	0.05	$y= -0.0005x^2 + 0.0049x - 0.0013$	0.7987	4.9	0.0107
0.5	0.10	$y = -0.0012x^2 + 0.0092x - 0.0018$	0.8378	3.8	0.0158
0.5	0.15	$y = -0.0013x^2 + 0.008x - 0.0021$	0.6885	3.1	0.0102
1.0	0.05	$y = -0.0009x^2 + 0.0063x - 0.0017$	0.7931	3.5	0.0093
1.0	0.10	$y = -0.0007x^2 + 0.0054x - 0.0011$	0.7245	3.9	0.0093
1.0	0.15	$y = -0.0006x^2 + 0.0042x - 0.0007$	0.7318	3.5	0.0065
1.5	0.05	$y = -0.0005x^2 + 0.0043x - 0.001$	0.8211	4.3	0.0082
1.5	0.10	$y = -0.0005x^2 + 0.0041x - 0.0006$	0.8122	1	0.0078
1.5	0.15	$y = -0.0006x^2 + 0.0046x - 0.0013$	0.7553	3.8	0.0075

Note : a: period (x) obtained at y'= 0 ; b: chitinase activity (y) at x optimum;

measured by coefficient of determination (R^2) (Table 1), with the highest of $R^2 = 0.8378$ obtained by starch to yeast extract ratio (%) of 0.5:0.10, the most optimum ratio to increase *S. marcescens* PT-6 chitinase activity. Meanwhile, optimum period of chitinase production in fermentation medium can be predicted through quadratic equation when y'= 0, then the highest chitinase activity can be determined from optimum period. Based on quadratic equation, optimum period was predicted to be obtained during day-3.1 to 4.9, with optimum chitinase activity predicted using quadratic equation of 0.0158 U/mL (day-3.8).

In all treatment, the highest chitinase activity was observed at the third day of incubation (Figure 5) when bacteria were in their late logarithmic phase or early stationary phase (Figure 4). Ruiz et al. (2010) stated that the late log phase in bacterial growth is characterized by the formation of secondary metabolites in the culture. The production of secondary metabolites is the response of the cells to the environmental conditions, one of which is due to the decrease in nutrient availability. Bacteria produce chitinase to degrade chitin to obtain NAG which functions as a source of nitrogen and carbon for the living process of bacteria (Brzezinska & Donderski, 2001). However, when NAG accumulates in the medium, it inhibits the activity of bacterial chitinase through the formation of catabolic inhibitors during the synthesis of chitinase (Donderski & Trzebiatowska, 1999). This might explain the decreased in chitinase activity after the third day of incubation, meanwhile the accumulated NAG in the medium was continually used by the bacteria to maintain growth.

4. Conclusion

This experiment showed that the additional of carbon and nitrogen sources other than chitin improved the chitinase activity of *S. marcescens* PT-6. Starch and yeast extract were the best source to enhance the chitinase activity of *S. marcescens* PT-6. The highest chitinase activity was achieved in the medium added with 1.5 % colloidal chitin, 0.5 % starch, and 0.1 % yeast extract. This optimization of medium composition with the addition of starch and yeast extract increased the chitinase activity of *S. marcescens* PT-6 from 0.002 U/ml (before starch and yeast extract addition) to 0.021 U/ml. This study revealed that medium optimization effectively improved chitinase production of *S. marcescens* PT-6 with a 10-fold increased in activity. Improvement of chitinase production through genetic approach using recombinant chitinase of *S.marcescens* PT-6 is currently underway. Furtherwork on scale-up production is required to make the application of this enzyme feasible in industrial scale.

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