

Chitinase Activity of *Serratia marcescens* PT-6 Cultured in Bioreactor with Various Aeration Rate

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Abstract. Chitin is a biopolymer composed of N-acetylglucosamine (NAG) monomers. Bacterial chitinase helps in bioconversion process of chitin from shrimp shell waste into NAG. This study aimed to determine the effect of aeration on chitinase activity, NAG concentration in medium, bacterial growth, and pH medium; to determine the optimum aeration on chitinase production of *Serratia marcescens* PT-6. Chitinase was produced using a 1.5 L bioreactor at pH 7, 30°C, 200 rpm agitation speed, and variation of aeration rates (0.4, 0.6 and 0.8 vvm). Parameters examined included pH, bacterial growth (log CFU/ml), chitinase activity (U/ml), and NAG concentration in medium (µg/ml). Bacterial growth was measured by enumeration of bacterial colony in medium, while chitinase activity and NAG concentration were analyzed by colorimetric assay. Parameters was measured every 8 hours during 60 hours of incubation. Aeration rate showed a significant effect ($P \leq 0.05$) on chitinase activity and NAG concentration, but not significant ($P \geq 0.05$) on bacterial growth and pH medium. Optimum aeration rate on producing chitinase was 0.6 vvm with the highest chitinase activity of 0.0125 U/ml at 40 hours incubation. This study implies that optimization of bioreactor condition improved the production of chitinase by *S. marcescens* PT-6.

1 Introduction

Chitin is a biopolymer composed of N-acetylglucosamine (NAG) monomers and linked by β -1.4 glycosidic bonds [1]. Chitin is commonly found in crustacean shells, exoskeletons of insects, mollusks, and cell walls of fungi or algae [2]. Shrimp shells are wastes that cause environmental pollution and have low economic value [3]. Therefore, bioconversion process of shrimp shell waste into chitin or hydrolysis products is needed to provide value added and reduce environmental pollution [4]. Chitin can be hydrolyzed into oligomer, dimer and monomer fragments through chemical and biological process. Chitin hydrolysis products have the advantage of having an active free amino groups that can bind many chemical reactions [5]. The hydrolysis process can expand product applications, such as applications

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in the fields of microbiology, agriculture, waste water management, cosmetics, medicine, food technology, and the textile industry [6]. Chitinase, an enzyme that capable in degrading β -1,4-glycosidic bond of chitin, is used in the biological process of chitin hydrolysis.

Chitinase is produced by bacteria, fungi, plants, and animals [7]. One of the chitinolytic microorganisms is *Serratia marcescens* [8]. *S. marcescens* produces more than 1 type of chitinase enzyme [9]. *S. marcescens* chitinase showed specific characteristics and synergistic activities in degrading chitin [10]. Moreover, *S. marcescens* has been known as a good producer of chitinase with high chitinase activity, i.e. 35.2 U/ml [11], 61.01 U/ml [12], and 77.3 U/ml [13].

S. marcescens PT-6 is a bacteria collection of Laboratory of Fisheries Products Quality and Safety, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. Studies on the optimization of chitinase production by *S. marcescens* PT-6 has been carried out by previous studies [14, 15, 16]. Aeration rate is one of the factors affecting the fermentation process [17]. An optimum aeration rate of 0.04 vvm was reported improving chitinase activity of *S. marcescens* of 34 U/ml [18]. Aeration supplies oxygen for cell growth, helps to homogenize the broth, and removes waste during the fermentation process [19]. This research was a further study to optimize chitinase activity of *S. marcescens* PT-6 using the variation of aeration rates in 1.5 L bioreactor. This study aimed to determine the effect of aeration on chitinase activity, NAG concentration in medium, bacterial growth, and pH medium. This study also determine the optimum aeration rate in bioreactor for chitinase production by *S. marcescens* PT-6.

2 Materials and methods

2.1 Chitin and colloidal chitin preparation

Chitin preparation was comprised of deproteination, demineralization and depigmentation [20]. After depigmentation process, chitin was washed with water until the pH becomes neutral, dried using the oven 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 [21].

2.2 Inoculum preparation

Refreshment of *S. marcescens* PT-6 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 72 hours. The inoculum was prepared by taking a single colony from working culture and grown in 7 mL of chitin broth incubated for 8 hours at 30°C. Chitin agar medium was prepared by mixing a 2 % colloidal chitin; 2 % bacto-agar; 0.07 % K_2HPO_4 ; 0.03 % KH_2PO_4 ; 0.05 % $MgSO_4$; 0.0001 % $ZnSO_4$ and 0.0001 % $MnCl_2$ with aquadest and sterilized [22]. Chitin broth was prepared in the same way as chitin agar without the addition of agar.

2.3 Chitinase production at 150 rpm and 200 rpm agitation rate

Optimization of agitation rate was carried out to obtain optimum and accurate results in the main study. This preliminary study needed to be done because in the previous study, there was possibility of higher chitinase activity using an agitation rate of lower than 200 rpm [16]. Preliminary study was carried out with 200 rpm and 150 rpm agitation rate using a 1.5 L

bioreactor. The same condition of fermentation as previous study was used [16] except the capacity of the bioreactor from 2 L was changed into 1.5 L.

2.4 Determination of incubation time and sampling period

Determination of incubation time and sampling period were carried out based on previous research [16] that reported the trend of optimum chitinase activity achieved on day 2 (48 hours), and decreased afterward. Determination of incubation time and sampling period needed to be done to determine the time when optimum chitinase activity was reached and the efficiency of the fermentation process.

2.5 Chitinase production at various aeration rate

The fermentation process was carried out in 1.5 L bioreactor at 30 °C, pH 7, agitation speed of 200 rpm, and aeration rate variations of 0.4 vvm, 0.6 vvm and 0.8 vvm. Each treatment was done in duplicate. The design of aeration rate variations was based on the optimum aeration rate to produce chitinase by *S. marcescens* QMB1466 [12] and *S. marcescens* SR1 [13]. The interval of 0.2 vvm at each aeration rate was chosen to obtain a significant result.

2.6 Measurement of bacterial growth

Bacterial growth assay was performed according to Standar Nasional Indonesia (SNI) 01-2332.3-2006 [23]. Bacterial growth assay was carried out by taking 1 ml of fermented culture, then put it in 9 ml of sterile distilled water, serially diluted, and planted on Nutrient Agar (NA) media in duplicate. Then incubated at 30°C and calculated the number of bacteria.

2.7 Measurement of chitinase activity and NAG concentration in medium

Chitinase activity assay was performed according to previously reported method [24]. Briefly, the culture filtrate was centrifugated (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 released from the enzymatic reaction was determined by spectrophotometry [25]. 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. NAG concentration in the medium was determined by the same method.

2.8 Measurement of pH medium

A 5 ml of culture sample was taken and measured with a pH meter.

3 Results and discussions

3.1 Chitinase production at 150 rpm and 200 rpm agitation rate

Figure 1A shows the number of *Serratia marcescens* PT-6 in a 1.5.L bioreactor. The optimum result was 8.05 log cfu/ml achieved at 200 rpm on day-2 of incubation. In general, the number of bacteria at an agitation rate of 200 rpm was higher than 150 rpm. The number

of bacteria tends to increase at the early period of incubation and then decreased afterward. An increase in the cell number on day-1 and day-2 showed that bacteria were in the logarithmic phase. In the logarithmic phase, bacteria divided two times faster so that the number of bacteria increased and reached the highest number compared to the other phases [26].

Figure 1B shows the highest chitinase activity of 0.0076 U/ml was achieved at 200 rpm on day-2. In general, chitinase activity at an agitation rate of 200 rpm is higher than 150 rpm. The previous study [16] uses an agitation rate of 200 rpm, 350 rpm and 500 rpm, and optimum chitinase activity was achieved at 200 rpm agitation. The results showed an agitation of 350 rpm and 500 rpm was too high for *S. marcescens* PT-6 to produce enzymes. Too high agitation rates resulted in a low chitinase activity due to shear stress and shear force caused by a thin blade around impeller in bioreactor. The stress conditions in cells have a negative impact on growth and enzyme stability [27]. Shear force or shear stress may cause cell lysis, inhibit growth, inhibit product synthesis, change cell morphology, and cause cell wall thickening [28]. Chitinase activity at 150 rpm shows that the agitation was too low and decreased enzyme production. Too low agitation speed increased broth viscosity which resulted in a decrease in mass transfer efficiency [29]. According to [30], high viscosity of broth increased broth heterogeneity and limited oxygen availability. The limited oxygen and nutrients in the production process inhibited the process oxygen transfer from the liquid phase into the cells. This had a direct impact on the yield and growth of microorganisms.

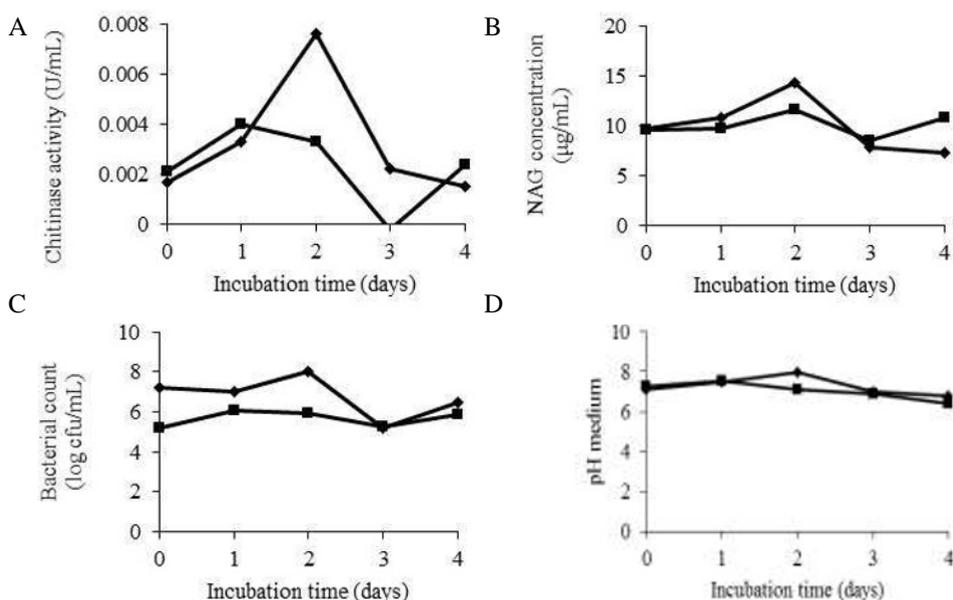


Fig. 1. Chitinase activity (U/ml) (A), NAG concentration ($\mu\text{g/ml}$) (B), bacterial count (log cfu/ml) (C), and pH medium (D) of *Serratia marcescens* PT-6 culture with an agitation rate of 150 rpm (■) and 200 rpm (◆) in a 1-L bioreactor system.

Figure 1C shows the results of NAG concentration ($\mu\text{g/ml}$) in the medium. NAG is a product of chitin decomposition which provides a source of carbon and nitrogen for microorganisms [31]. In general, the concentration of NAG at 200 rpm was higher than 150 rpm. Chitinase activity increased on day-2, then decreased afterward. The optimum result for NAG concentration was 14.36 $\mu\text{g/ml}$ achieved at 200 rpm and 2 days incubation. High production of chitinase was followed by a high concentration of NAG in the medium.

Figure 1D shows the pH medium during the fermentation process. In general, the pH at the agitation level of 200 rpm was higher than 150 rpm. The pH value of *S. marcescens* PT-6 culture during the incubation period ranged from 6.40 to 7.96. The range was appropriate with the growth characteristics of *S. marcescens* that ranged at pH 5 – 9. Environmental pH is one of the factors that influence chitinase production by bacteria [31]. This preliminary study shows that the agitation rate of 200 rpm resulted to the higher chitinase compare to 150 rpm. Therefore, the agitation rate of 200 rpm was used for the next step of optimization in the main research.

3.2 Determination of incubation time and sampling period

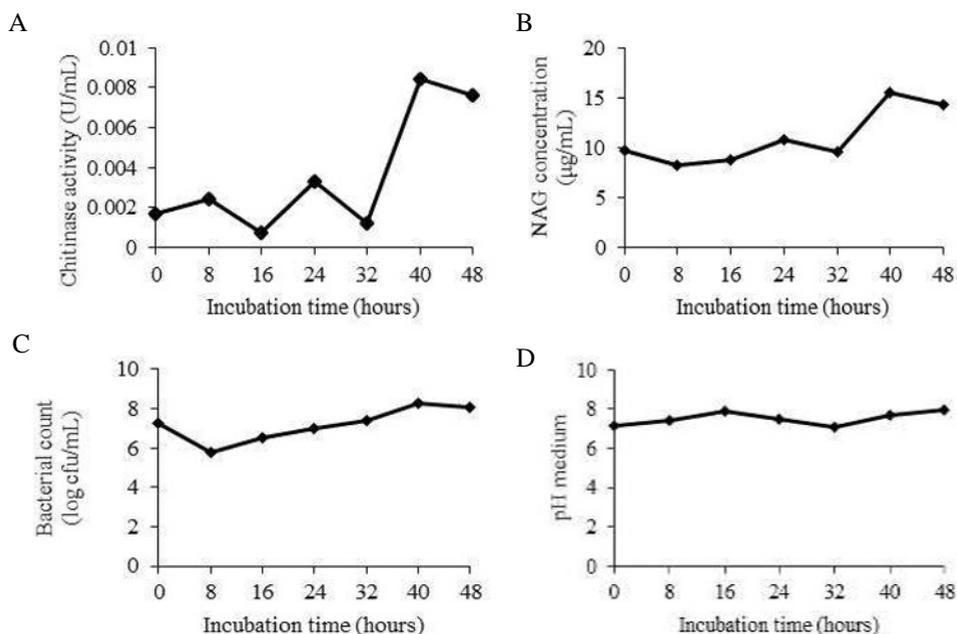


Fig. 2. Chitinase activity (U/ml) (A), NAG concentration (µg/ml) (B), bacterial count (log cfu/ml) (C), and pH medium (D) in the culture of *Serratia marcescens* PT-6 with an agitation rate of 200 rpm

Figure 2A shows that the highest cell number was achieved at the 40 h with the value of 8.29 log cfu/ml, meanwhile at the same period, the highest chitinase activity and NAG concentration in the medium were achieved with the value of 0.0085 U/ml (Fig. 2B) and 15.551 µg/ml (Fig. 2C), respectively. The pH value remained stable during the incubation period (Fig. 2D). Chitinase activity obtained at 40 h was higher than the optimum chitinase activity of 200 rpm obtained from preliminary study which showed the activity of 0.0076 U/ml at 48^h. These results indicate that the incubation time and the interval of sampling period were possible to be reduced. Therefore, optimization step to observe the effect of aeration rates on chitinase activity was conducted for 60 h with the interval of sampling period of 8 h.

3.3 Chitinase activity of *S. marcescens* PT-6 at various rates of aeration

The growth of *S. marcescens* PT-6 (log cfu/ml) in bioreactor with various levels of aeration rate is shown in Figure 3. Aeration rates had no significant effect ($P \geq 0.05$) on bacterial growth. In general, the number of bacteria increased in 40 h incubation and decreased afterwards. This result indicates that *S. marcescens* PT-6 reached its logarithmic phase during 40 h of incubation. The logarithmic phase of *S. marcescens* was characterized by the alteration in the medium color turning into red as the red pigment called prodigiosin was released [32]. The number of bacteria in 0.6 vvm was higher than 0.4 vvm and 0.8 vvm.

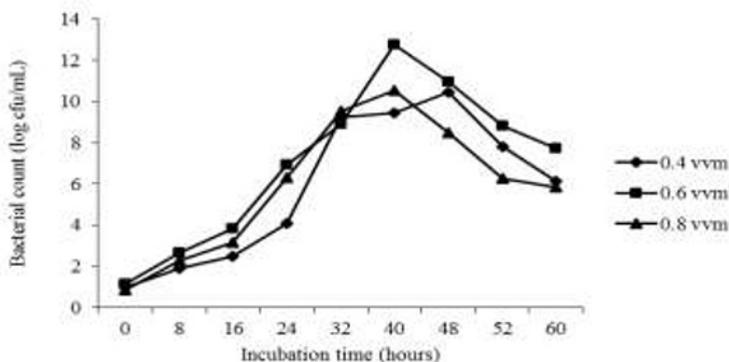


Fig. 3. Bacterial count (log cfu/ml) of *Serratia marcescens* PT-6 in bioreactor (1-L, pH 7, 30 °C, 200 rpm) with various rate of aeration.

S. marcescens PT-6 is a facultative anaerobic microorganism, which the requirement for the available oxygen is low. Too high of oxygen concentration in the environment reduces bacterial growth. The effect of oxygen toxicity in the cell is more severe during the logarithmic phase compare to stationary phase [33]. Excessive oxygen causes oxidative stress to certain microorganisms due to the formation of Reactive Oxygen Species (ROS), such as superoxide and H_2O_2 which are toxic. However, too low oxygen availability is also inhibit cell growth as the metabolism and the enzyme production are hampered. Therefore, determining the appropriate aeration rates in the bioreactor system is crucial to obtain the optimum growth and maximum yield of microbial product.

Chitinase activity (U/ml) of *S. marcescens* PT-6 at various aeration rates is shown in Figure 4. Aeration rate showed a significant effect ($P \leq 0.05$) on chitinase activity. Further analysis using Tukey HSD with $\alpha = 0.05$ showed that significant different was found between 0.6 vvm and 0.8 vvm, but was not different with 0.4 vvm. Similar trend to the bacterial growth curve was observed in the chitinase activity which increased during 40 h of incubation and suddenly decreased afterwards. The optimum chitinase activity was 0.0125 ± 0.0002 U/ml, achieved at 40 h with an aeration rate of 0.6 vvm. This value was higher compared to the previous study using 2-L bioreactor at 0.04 vvm, which resulted to the chitinase activity of 0.006 ± 0.001 U/ml [16]. However, the chitinase activity obtained from this study was lower than the optimal chitinase activity in shaken flask, which reached 0.021 ± 0.006 U/ml after 3 day incubation at 100 rpm reciprocal agitation [15]. A lower chitinase activity produced in bioreactor system compare to shaken flask was probably due to the scaling-up process causes the alteration of environmental conditions that reduce the performance of microorganisms. Similar result of a lower chitinase activity produced in bioreactor compare to shaken flask was reported on *S. marcescens* QMB1466 [12].

Oxygen supply is an important aspect for the growth of microorganisms in aerobic fermentation [29]. Figure 4 shows that the chitinase activity of *S. marcescens* PT-6 at 0.6 vvm was higher than 0.4 vvm and 0.8 vvm. A low rate of aeration causes oxygen depletion in the medium and resulted to the foam formation [29]. The presence of foam may hamper the performance of impeller to homogenize the medium. This condition causes the reduction of bioreactor performance in fermentation system. On the other hand, too high aeration increases oxygen flow and causes flooding. This is the condition where the impeller is surrounded by many air bubbles that interfere the performance of impeller in stirring process [29]. This condition leads to the reduction of oxygen and nutrient transfer from medium into the cell [30].

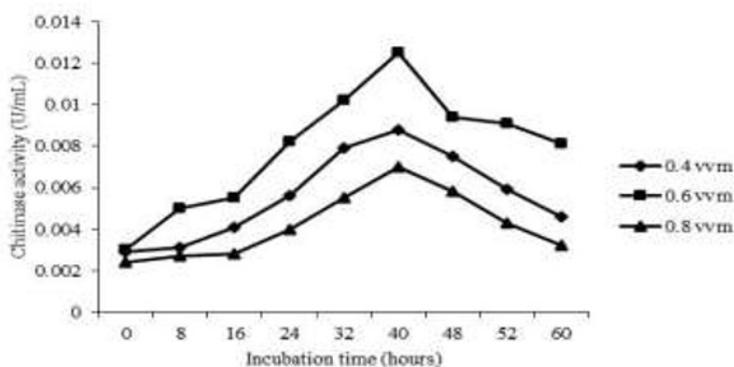


Fig. 4. Chitinase activity (U/ml) of *Serratia marcescens* PT-6 in bioreactor (1-L, pH 7, 30 °C, 200 rpm) with various rate of aeration.

The concentration of NAG ($\mu\text{g/ml}$) in the culture medium is shown in Figure 5. Aeration rate had a significant effect ($P \leq 0.05$) on the concentration of NAG in the medium. The highest NAG concentration ($\mu\text{g/ml}$) was 24.19, obtained from the treatment of 0.6 vvm at 32 h. The pattern of NAG concentration produce in the medium was similar to the chitinase activity (Fig. 4) and bacterial growth curve (Fig. 3) but the optimum point occurred in an earlier period. Chitin degradation process occurred continuously along with the increasing in chitinase activity, so that the products (NAG) were accumulated and the concentration in the medium increased [34]. However, NAG was used as nutrition by the cells. In the condition of limited nutrition availability in the medium, bacteria tend to use the remaining hydrolysis product (NAG) which is a source of carbon and nitrogen for metabolism, so that the concentration of NAG in the medium decreased [35].

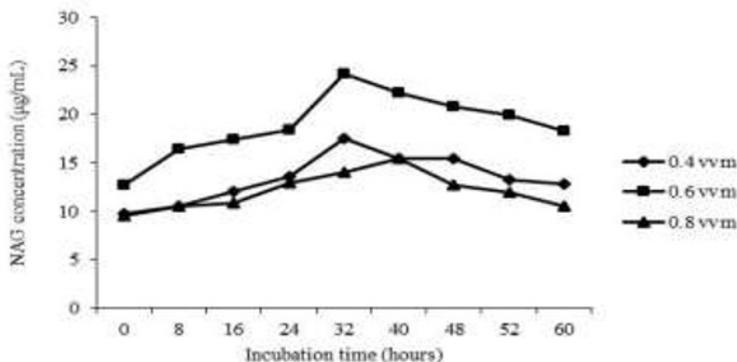


Fig. 5. NAG concentration (µg/ml) in the culture medium of *Serratia marcescens* PT-6 (1-L, pH 7, 30 °C, 200 rpm) with various rate of aeration.

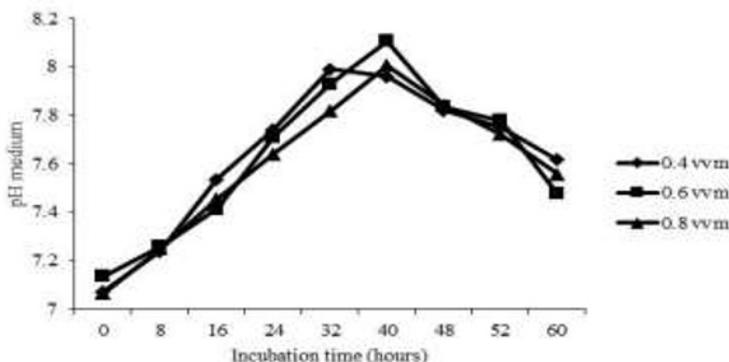


Fig. 6. pH medium of *Serratia marcescens* PT-6 culture in bioreactor (1-L, pH 7, 30 °C, 200 rpm) with various rate of aeration.

Figure 6 shows the pH value of the culture medium of *S. marcescens* PT-6 during 60 h of fermentation. Aeration rates showed no significant effect ($P \geq 0.05$) on the medium pH. In general, the pH value increased during 40 h of incubation and then decreased afterwards. The pH during fermentation process ranged from 7.06 to 8.10. The decreased in pH indicated the accumulation of organic acid substances that were produced during bacterial growth. This condition might be the reason of the declining of cell number after 40 h of incubation (Fig. 3). The appropriate pH helps enzymes to work properly in catalyzing reactions.

4 Conclusion

This paper shows that the aeration rate in bioreactor had a significant effect ($P \leq 0.05$) on chitinase activity and NAG concentration in the medium of *S. marcescens* PT-6 culture. However, aeration rate showed no significant effect ($P \geq 0.05$) on bacterial growth and pH of the medium. The optimum aeration rate in the bioreactor for *S. marcescens* PT-6 to produce chitinase was 0.6 vvm with the highest chitinase activity of 0.0125 U/ml at 40 h. incubation.

5 Acknowledgement

This study was supported by the RTA program 2019.

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