Optimization of Agitation Rate in Bioreactor Increases Chitinase Activity of *Serratia marcescens* PT6

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Abstract. *Serratia marcescens* PT6 is a Gram-negative bacteria isolated from shrimp pond sediment that capable of producing chitinase. This study aimed to observe the effect of agitation rate on growth and chitinase activity of *S. marcescens* PT-6 in a bioreactor. The production of chitinase was done in 1.5 l bioreactor using colloidal chitin broth at the condition of pH 7, the temperature of 30°C, aeration of 0.04 vvm, and variation of agitation rate (200, 350, 500 rpm). Bacterial growth was measured by colonies counting in agar medium, while chitinase activity was measured by means of colorimetric every day for four days incubation. The results of ANOVA analysis show that the agitation rate had no effect on bacterial growth, but a significant effect ($P < 0.05$) was observed on chitinase activity. The highest growth and chitinase activity were obtained at 200 rpm, with the highest chitinase activity of 0.006 ± 0.001 U/ml was at day-2. This study implies that the optimized agitation rate in the bioreactor increased the chitinase activity produced by *S. marcescens* PT-6.

1 Introduction

Bioconversion of chitin from shrimp shell waste into N-acetylglucosamine (NAG) is beneficial for the production of a value-added compound derived from seafood waste. NAG is widely used in pharmaceutical and cosmetics [1]. Chitinase is an enzyme used in the biological process for NAG production. *Serratia marcescens* is one of the most effective microorganisms to degrade chitin [2]. *S. marcescens* PT-6 was isolated previously from shrimp pond sediments and showed the chitinase activity of 0.0002 U/ml [3, 4]. Optimization of *S. marcescens* PT-6 growth environment was carried out to increase the production of chitinase [5, 6]. An increase in chitinase activity of 103.5 times was observed by the addition of starch and yeast extract in the production medium [6]. These experiments were conducted

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using shaken flasks with a production volume of 100 ml. A lab-scale bioreactor is often used to increase the production volume of microbial metabolite products.

In a bioreactor system, the fermentation process is easily regulated to provide the optimal condition for bacterial growth and production of metabolites. Agitation in bioreactor plays a vital role in providing the best environment for bacteria as it regulates the rate of oxygen solubility and accelerates the transfer of nutrients and oxygen into microorganism cells [7]. The chitinase activity of *S. marcescens* 990E in bioreactor increased with the optimal agitation and aeration (300 rpm; 0.04 vvm), reaching 34 U/ml [8]. The optimal agitation rate in 2 l bench-top bioreactor increased the chitinase activity of *Lecanicillium muscarium* CCFEE 5003 fungi by 23% compared to shaken flasks. This study aimed to determine the effect of agitation rate in the 1.5 l bioreactor on chitinase activity of *S. marcescens* PT-6.

2 Materials and methods

2.1 Medium and inoculum preparation

Chitin was prepared from shrimp shell [9], and colloidal chitin was prepared by the addition of HCl [10]. The chitin broth for chitinase production was made of KH₂PO₄ (0.03%), K₂HPO₄ (0.07%), MgSO₄·5H₂O (0.05%), ZnSO₄ (0.0001%), MnCl₂ (0.0001%), starch (1.5%), yeast extract (0.15%) and colloidal chitin (1.5%). Chitin agar was prepared using the same ingredient to chitin broth except for the addition of bacto agar (2%). The pH of the chitin broth and agar medium was adjusted to 7. Nutrient broth (NB) and nutrient agar (NA) (Oxoid) medium were used for the preparation of inoculum and cell count, respectively.

S. marcescens PT-6 used in the research was isolated from shrimp pond sediment [3]. *S. marcescens* PT-6 cultured in NB for 24 hours at 30°C of 0.6 ml was transferred into 30 ml NB and incubated in water bath shaker at 30°C and 100 rpm reciprocal agitation for another 8 hours to reach logarithmic phase. The inoculum was ready to be transferred to the production medium.

2.2 Chitinase production of *S. marcescens* PT-6 in various agitation rate

The inoculum of 37.5 ml was added to 1.5 l chitin broth in a 2 l bioreactor (Eppendorf New Brunswick Scientific Model 115). The fermentation process occurred at 30°C, pH 7, 0.04 vvm aeration rate, and variation of agitation rate (200, 350, and 500 rpm). Each treatment was done duplicate. Every day for four days incubation, the culture medium was harvested and measured for the bacterial growth and chitinase activity. Cell morphology from inoculum, 0-day culture of 500 rpm, and 4-day culture of 500 rpm was observed using Scanning Electron Microscope (SEM).

Bacteria growth was observed by measuring total plate count on NA. Chitinase activity was observed from crude enzyme prepared by centrifugation of 1 mL culture medium at 10,000 rpm and 4°C for 1 minute. The crude enzyme of 500 µl was added with 1.3% colloidal chitin (in 50 mM phosphate buffer pH 6) and incubated in water bath shaker for 30 minutes at 37°C and 100 rpm agitation [11]. N-acetylglucosamine (NAG) resulted from the enzymatic reaction was measured based on the absorbance detected on spectrophotometer UV-Vis at 584 nm [12]. The absorbance of the standard NAG solution at various concentrations (5-50 µg/ml) was also measured. Negative control was prepared from heated crude enzyme and treated in the same procedure as sample. A unit of chitinase activity was defined as the amount of NAG (µmol) released per minute. The cell specimen for morphology observation was prepared by centrifugation of culture medium at 5,000 rpm 4°C for 10 minutes and

washed for several times. A small amount of cell pellet was put on the carbon tip, coated in the ion sputter machine (10 mA, 50 sec), and observed with SEM (JEOL JSM 5310 LV).

3 Results and discussion

The Anova analysis showed that the agitation rate had no significant effect ($P<0.05$) on *S. marcescens* PT-6 growth. However, Figure 1 indicates that bacterial growth in 200 rpm tended to be higher than other treatments. Moreover, the decrease in cell number was observed in 350 and 500 rpm. The higher agitation rate tended to increase the rate of cell number decline. Cells were subjected to shear stress in the system with high agitation rate [13]. Shear stress cause lysis and morphological change of the cells that inhibit growth [14]. The high agitation rate decreased cell size, pellet density, and total biomass [15].

Fig. 1. The cell number of *S. marcescens* PT-6 cultured in a bioreactor (1.5 l, pH 7, 30°C, 0.04 vvm) with various agitation rate (●: 200 rpm; ■: 350 rpm; ▲: 500 rpm).

The observation was carried out to find out the changes in cell morphology after fourth-day incubation in a bioreactor at the agitation rate of 500 rpm. The rod-shaped cells of *S. marcescens* PT-6 were easily found in inoculum culture (Fig. 2a) and in the initial culture before incubation (Fig. 2b), meanwhile intact cell was very few in the culture at 500 rpm after fourth-day incubation. Change in cell morphology was reported in *Trichoderma virens* UKM1, chitinase-producing fungi, in which the number of fragmented mycelium increased as the agitation rate increased [15].

The adverse effect of agitation on bacterial growth is also related to the oxygen tolerance of bacteria. Agitation helps to increase the amount of dissolved oxygen in the system. *S. marcescens* are facultative anaerobic bacteria [16]; therefore, the high concentration of dissolved oxygen in the medium might be a limiting factor for its growth. Oxygen plays a vital role in aerobic microorganisms as it serves as a substrate to produce energy [15]. Aerobic respiration resulted in the production of radical oxygen species as its by-products. Oxygen tolerance of facultative anaerobic bacteria related to the ability of bacteria producing superoxide dismutase and catalase, two key enzymes to detoxify radical oxygen species [17].

Fig. 2. Scanning electron micrograph of *S. marcescens* PT-6 (a) inoculum (15,000x magnification), (b) at 0-day incubation (15,000x magnification), (c) at 4-day incubation of 500 rpm.

Chitinase activity produced by *Serratia marcescens* PT-6 at various agitation rates is shown in Figure 3. The Anova analysis showed that the rate of agitation had a significant effect ($P<0.05$) on the *S. marcescens* PT-6 chitinase activity. Furthermore, Tukey HSD tests ($\alpha=0.05$) confirmed that chitinase activity at 200 rpm was different from 500 rpm. The highest chitinase activity occurred at 200 rpm on the second-day incubation with a value of 0.006 ± 0.001 U/ml. The decrease in enzyme activity afterward was presumably due to the accumulation of hydrolysis products, which affect enzyme activity [18]. The low chitinase activity of *S. marcescens* PT-6 at 350 and 500 rpm might be also related to the growth inhibition effects at the respective agitation rate.

Fig. 3. The chitinase activity of *S. marcescens* PT-6 cultured in a bioreactor (1.5 l, pH 7, 30°C, 0.04 vvm) with various agitation rate (●: 200 rpm; ■: 350 rpm; ▲: 500 rpm)

Table 1. Regression model of chitinase activity of *S. marcescens* PT-6 cultured in various agitation rate

No	Agitation rate (rpm)	Regression model	R ²	Optimal time ^a (days)	Optimal chitinase activity ^b (U/ml)
1	200	$y = -0.00x^2 + 0.0049x + 0.0002$	0.9725	2.5	0.0062
2	350	$y = 0.00006x^2 + 0.0005x + 0.0005$	0.8907	4.2	0.0037
3	500	$y = 0.000004x^2 - 0.00004x + 0.0005$	0.0327	5	0.0004

a : time (x) obtained when y'= 0 ;
b : chitinase activity (y) obtained at x optimal

The effect of each agitation rate on the *S. marcescens* PT-6 chitinase activity was shown by the R² value (Table 1). The most significant effect was obtained from 200 rpm (R² = 0.9725). Thus, 200 rpm was chosen as the optimal agitation rate in increasing the chitinase activity of *S. marcescens* PT-6. The prediction of the optimal time of chitinase production in the bioreactor was calculated through the regression model when y '= 0. The predicted incubation time to produce optimal chitinase activity of 0.0062 U/ml was achieved on 2.5 days.

The chitinase activity obtained in this study was smaller than previous research using shaken flasks [6]. Cultivation of *S. marcescens* PT-6 in shaken flask resulted in the chitinase activity of 0.021 ± 0.006 U / ml on the day-3 incubation. A lower chitinase activity produced in bioreactor compared to the shaken flask was also reported in *S. marcescens* QMB1466. The scale-up process caused changes in several environmental factors that resulted in the reduction of microbial performance in metabolites production [19].

The optimal agitation rate in the production of bacterial chitinase is related to the characteristics of microorganisms used. Obligate aerobic microorganism, such as fungi, usually requires high agitation rate. *L. muscarium* CCFEE 5003 produced optimal chitinase activity of 0.38 ± 0.008 U/ml at 300 rpm agitation and 1 vvm aeration [14]. *T. virens* UKM1 obtained optimal chitinase activity of 0.004l U/ml at 200 rpm agitation and 0.33 vvm aeration [15]. Chitinase of *S. marcescens* 990E showed the optimal activity of 30 ± 4 U/ml at 300 rpm at a lower aeration rate of 0.04 vvm [8]. Further experiment for optimization of chitinase production by *S. marcescens* PT-6 in lower agitation rate than 200 rpm and the variation of aeration rate is required.

4 Conclusion

Agitation rate significantly affected the chitinase activity of *S. marcescens* PT-6 cultured in a 2 l bioreactor. The optimum agitation rate was 200 rpm resulted in the highest chitinase activity of 0.006 U/ml after two days incubation. The increase in agitation rate caused a lower chitinase activity and a reduction of cell number during fermentation.

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