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N-Acetylglucosamine Production by Repeated-Batch Fermentation Using Immobilized Semi-Purified Chitinase Enzyme on Agar

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Abstract

Chitinolytic mold, such as Mucor circinelloidesis can be utilized to produce chitinase enzyme for shrimp shell's chitin hydrolysis into N-acetylglucosamine (NAG). For that purpose, entrapment of chitinase on agar as a carrier could be an alternative way to improve NAG production. This study aimed to investigate the stability of immobilized semi-purified chitinase on agar for multiple cycles fermentation to produce NAG. In this study, 0.6 mL of semi-purified chitinase enzyme was immobilized into 3% of agar matrices and tested for four fermentation cycles to obtain highest NAG concentration and good enzyme activity. The results indicate that the immobilized chitinase could be used for 6 hours fermentation or three fermentation cycles. The NAG concentration produced after three cycle were 1042.22 ± 16.20 ppm. Besides, the immobilized enzyme was considerably stable up to the third cycles with activity value of about 4.74 U/mL.

Keywords: *agar; immobilized;NAG; repeated fermentation*

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INTRODUCTION

Chitin is a polysaccharide which comprises of N-acetyl-D-glucosamine monomers that are linked by β-1.4 linkage (Rinaudo, 2006). Chitin can be hydrolyzed into N-acetyl-glucosamine (NAG), which provide various functional properties and widely used for biomedical applications. The hydrolysis of the chitin is usually carried out using chitinase enzyme. This enzyme used in the bioconversion of chitin-rich materials, such as shrimp shell waste (Rinaudo, 2006; Jayakumar *et al*., 2010).

Microorganisms, such as *Bacillus thuringiensis* NM101-9, *Bacillus licheniformis* NM120-17, *Providencia stuartii* and *Mucor circinelloides* exhibit ability to produce chitinase. Chitinase can function both insides (intracellular) and outside the cell (extracellular). The uses of chitinase provide faster, simpler, and environmentally friendly mechanism in producing chitin-derived compounds

(Gomaa, 2012; Hardoko *et al*, 2020; Inokuma*et al*., 2013). However, chitinase production is costly. Immobilization has been reported to increase cell's stability from which an increased cell productivity can expected (Stoykov*et al*., 2015; Yan and Fong, 2015). According to Soedirga *et al*. (2019), the semi-purified chitinase enzyme produced by *Mucor circinelloides* was successfully immobilized on agar as a carrier. The immobilized semi-purified chitinase enzyme was reported to produce NAG at approximately 1111.667 ppm. Moreover, the chitinase was also considerably stable before and after immobilization (4.78 U/mL). One of the advantages of enzyme immobilization is that the enzyme can be used for several fermentation cycles. Nonetheless, the immobilized enzyme must have stable activity during the repeated fermentation cycle. There have been limited studies on how long the immobilized chitinase enzyme, specifically that derived from *Mucor circinelloides.* This mold is considerably stable in producing high concentration of NAG. Therefore, this research was purposed to determine the acceptable number of fermentation cycles of the immobilized chitinase for repeated-batch fermentation.

MATERIALS AND METHOD

Materials and Equipment

Materials used in this research were chitin and semi-purified chitinase obtained from the previous study (Soedirga *et al*., 2019), commercial powdered agar (Agarpac Swallow Globe), Potato Dextrose Broth (PDB) (Merck P6685 Specification: PRD.4.ZQ5.10000036227), $MgSO_4.7H_2O$ (Merck CAS number:10034-99-8), $(NH4)_2SO_4$ (Merck CAS number 7783-20-2), phosphate buffer solution 0.1 M (Sigma Aldrich P5244 Specification: PRD.1.ZQ5.10000006487), distilled water (Amidis, 5 L gallon), 3-5-dinitrosalicylic acid (DNS) (Sigma Aldrich CAS number 609-99-4), Na-K, tartaric 4% (Merck CAS number 6381-59-5), N-acetylglucosamine standard (Sigma Aldrich A2278 CAS number 134451-94-8). While the equipment used were centrifuge (MPW-223e), centrifuge tube, glassware (Iwaki Pyrex), plastic syringe (Thomas Scientific), autoclave (Hiramaya), water bath (Cimarex), UV-VIS spectrophotometer (Thermo Scientific Genesys 10), quartz glass cuvette (Helma Analytics), pH meter (Methrohm 913), vortex (Cole-Parmer), analytical balance (Ohaus u-1800 ar 2140), filter paper (Sig, micropipette (Thermo fisher), and tip.

Immobilization of Semi-Purified Chitinase Enzyme into Agar

The semi-purified chitinase immobilization process was performed according to the procedure of Soedirga *et al*. (2019) and Prakash and Jaiswal (2011). The agar concentration and the amount of enzyme immobilized into the carrier obtained from previous research (Soedirga *et al*., 2019). Approximately 0.6 mL of semi-purified chitinase with 4.798 mg/mL concentration was added with phosphate buffer solution (pH 8) until the total volume was 1 mL. The mixture was then immobilized into 3% of agar solution by injection using a sterile syringe. The mixture was released drop wise from the syringe into the fermentation medium with roughly 0.4 mm diameter. According to Lestari *et al*. (2017), the fermentation medium consists of 0.135 g chitin and 9 mL of phosphate buffer solution (pH 8).

Repeated-Batch Fermentation of Immobilized Semi-Purified Chitinase Enzyme

The repeated fermentation process using immobilized semi-purified chitinase was done according to Yang *et al*. (2005) and Kumar *et al*. (2017) with slight modifications. Chitin was fermented using immobilized semi-purified chitinase in a water bath for 2 hours at 40°C. After 2 hours, the fermentation medium was filtered. The filtrate was then subjected to NAG concentration analysis and the immobilized chitinase was analyzed for its enzyme activity. Whilst the remaining beads were then subsequently used for fermentation with new fermentation medium. This process lasted for four cycles. Each cycle was run for 2 hours and NAG determination was also carried out to each fermentation cycle.

Determination of NAG Concentration

The NAG concentration determination was done according to the DNS method (Soedirga *et al*., 2019). The DNS reagent was prepared by mixing the 3,5-dinitrosalysylic acid, NaOH, phenol, Na_2SO_3, and distilled water. The mixture was stored in a dark bottle to prevent any possible oxidations.

Standard curve was prepared by making a set of glucosamine standard solution with various concentrations. From each glucosamine concentration, DNS and Na-K-tartrate reagents were mixed. The mixture was heated for 15 minutes at 100°C then diluted with distilled water prior to absorbance measurement at 540 nm. While blank sample was prepared by mixing DNS reagent and Na-K-tartaric and the rest of the procedure was similar with the standard making. The absorbances obtained were used to make a standard curve.

About 1 mL of the filtrate obtained from all the cycles of repeated-batch fermentation was analyzed for its NAG concentration. The filtrate was mixed with 2 mL of DNS and 1 mL of Na-K-Tartaric solution to stabilize the color formation of DNS. The heating process was subjected to the mixture for 1 hour before absorbance measurement to accelerate the reaction. The measurement was done using a spectrophotometer at wavelength 540 nm.

Determination of Enzyme Stability

The stability of chitinase was analyzed according to the Miller method (Keharom *et al*., 2016) with necessary modifications. Stability determination of immobilized chitinase before and after immobilization was done by mixing 1.5% of chitin, 1

mL buffer and 1 mL of immobilized enzyme. The mixture was then incubated for 1 hour at 40°C. About 1 mL of this result was taken and mixed with 2 mL DNS reagent and 1 mL of 4% of Na-K-tartrate. The mixture was heated at 100°C for 5 minutes to accelerate the reactions process. After that, the mixture was diluted with distilled water within ratio 1:4 before absorbance measurement at 540 nm. The activity of chitinase in 1 unit expresses the amount of enzyme needed to produce one μmol of NAG in 1 hour, as shown in this formula:

$$Enzyme\ activity\ (U/ml) = \frac{NAG\ concentration \times enzymes\ amount \times 1000}{MR\ of\ NAG\ (221.2) \times incubation\ time} \quad (1)$$

RESULTS AND DISCUSSION

In this research, the fermentation was repeated for four cycles to determine the effect of fermentation cycles on the NAG concentration and enzyme stability. In this research, each fermentation cycle was run for 2 hours, so that in total four fermentation cycles lasted in 8 hours. The statistical evaluation shows that fermentation cycles give a significant effect on the production of NAG ($p < 0.05$). The result from Duncan post-hoc shows that the second and third cycle of fermentation were not significantly different. Nevertheless, the first cycle of fermentation exhibits a significantly higher of NAG concentration as shown in Figure 1.

The immobilized semi-purified chitinase enzyme can be used up to four fermentation cycles. However, the concentration of NAG decreased proportionally with the increasing number of fermentation cycle, as demonstrated in Figure 1. The highest NAG concentration obtained from the first cycle (1078.89 ± 16.49 ppm), while in the fourth cycle, the NAG concentration decreased significantly to 962.222 ± 15.01 ppm. A similar pattern was also displayed on the immobilized chitinase enzyme from *Bacillus lichenformis* in the production of NAG (El-Sherif *et al.*, 2013). In the first cycle, the NAG produced within the concentration of 3380 ppm, then decreased into 1460 ppm in the fourth cycle of fermentation.

During fermentation, there is a possibility for an increase in the number of free cells due to a decrease in the strength and toughness of the beads' cell walls. It occurred because some of the metabolites produced by microorganisms are acidic in nature.

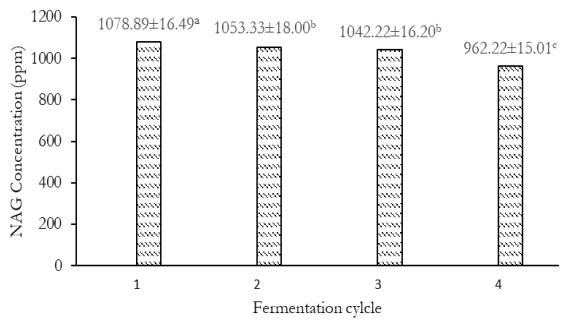

Figure 1. NAG concentration obtained from different fermentation cycle of immobilized semi-purified chitinase enzyme. Different superscript letter on the bar graph indicates significance difference (p <0.05)

The presence of H^+ ions from the acid will replace K^+ so that the matrix degraded and reduced its density. Besides, repeated use of beads for fermentation also caused the beads underwent deformation changes. Deformation changes on beads happened because the continuous diffusion of nutrients will increase bead's diameter and porosity to reduce the beads during microorganism cells' entrapment. Therefore, a decrease in NAG production took place during the repeated fermentation process (Cheba *et al*., 2011; El-Sherif *et al.*,2013).

As shown in Table 1, such increase of the number of fermentation cycle reduced the activity of the immobilized semi-purified chitinase. The activity of chitinase before immobilization was 4.78 U/mL as reported by Soedirga *et al*. (2019). In the first cycle, the enzyme showed the same activity value, which means the enzyme activity after immobilization considerably stable. However, chitinase activity decreased to 4.75 and 4.74 U/mL in the second and third cycle, respectively. This activity decline was about 0.02% of the first cycle. Meanwhile, in the fourth cycle, there is a remarkable decrease of activity of about 0.11% compared to the first cycle. Although immobilized chitinase experienced activity decline, but the enzyme remained potential to be used up to three fermentation cycles. As illustrated in Figure 1, the NAG concentration on the third cycle was not significantly different with the second cycle.

Table 1. Stability of enzyme activity

Fermentation cycle	Enzyme Activity (U/mL)	Decreasing value of enzyme activity compared to the 1st cycle (%)
1	4.86	0.00
2	4.75	0.02
3	4.74	0.02
4	4.35	0.11

Prakash and Jaiswal (2011) stated that the decrease of enzyme activity on each cycle during repeated-batch fermentation is most probably due to enzyme denaturation. Moreover, there will be large possibility of the enzyme to suffer from physical damage. During the immobilization process, the enzyme is trapped into the carrier. The larger pore size of the agar may trigger enzyme leakage during beads washing, which leads to decreasing of enzyme activity (Sharma *et al*., 2014). Consecutive incubation of enzyme could also generate the energy activation for

denaturation that is higher than the activation energy of catalytic reactions. Thus, it tends to be denatured rather than involve in catalytic activities.

CONCLUSION

Based on the results, it can be concluded that the semi-purified chitinase can be immobilized using agar as a carrier and can be used up to 6 hours fermentation time (three cycles) to produce NAG within the concentration of 1042.22 ± 16.20 ppm. The enzyme activity only reduced slightly about 0.02% compared to the first cycle, within the value of 4.74 U/mL. The highest NAG production was obtained from the first cycle of fermentation and the reduction was less than 5% at the third cycle. Prolong fermentation time to 8 hours caused reduction of NAG production by more than 10%. Thus, three fermentation cycles remarkably suitable to be applied for repeated-batch fermentation using immobilized semi-purified chitinase obtained from *Mucor circinelloides*.

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