Optimum Condition for the Production of N-acetylglucosamine from Tiger Shrimp Shells using Semi Pure Extracellular Chitinase Enzyme Produced by *Mucor circinelloides*

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Abstract: Chitin is a biodegradable polysaccharide, commonly found in shrimp shells and further processed into its derivatives, such as glucosamine that is extensively used in dietary supplements for the treatment of osteoarthritis, knee pain and back pain. This research was conducted to determine the optimum pH, temperature, substrate concentration and fermentation time for semi pure extracellular chitinase enzyme from *Mucor circinelloides* to be used in N-acetylglucosamine production. The optimum pH was determined at different pH of 3, 4, 5, 6, 7, 8 and 9 and optimum temperature was determined at 30, 40, 50, 60, 70 and 80°C by measuring chitinase activity. Substrate concentration varies from 0.5, 1.0, 1.5 and 2.0% and fermentation time varies from 2, 4, 6 and 24 hours were used to determine the optimum condition for N-acetylglucosamine production. Results showed that optimum pH of extracellular chitinase enzyme produced by *Mucor circinelloides* with colloidal chitin as a substrate was 8 with chitinase activity of 5.76 ± 0.17 U/ml and optimum temperature was 50°C with chitinase activity of 6.78 ± 0.13 U/ml. The optimum substrate concentration of extracellular chitinase enzyme with chitin as substrate was 1.5% chitin with concentration of N-acetylglucosamine produced of 1285.73 ± 66.19 ppm and the optimum fermentation time was 2 hours with concentration of N-acetylglucosamine produced of 1322.71 ± 45.43 ppm.

1 INTRODUCTION

Chitin is a non-toxic biodegradable polysaccharide that is commonly found in shrimp shells. Chitin is hard to be absorbed by human body because chitin has low solubility and large molecular size. Therefore, it is commonly further processed into its derivatives, such as glucosamine and chitosan (Haliza and Suhartono, 2012). Glucosamine is a derived chitin monomer and an important precursor in the biosynthesis of glycolipids, glycoproteins and proteoglycans proven to be involved in maintaining joint health (Kardiman, 2013). Glucosamine can be found naturally in the human body and is a precursor for the biochemical synthesis of glycosaminoglycans found in cartilage. Glucosamine is extensively used in dietary supplements for the treatment of osteoarthritis, knee pain and back pain (Benavente *et al.,* 2015). The conventional production of glucosamine using chemical treatment has many disadvantages as it is not environment friendly due to acidic wastes, the yield is low and hard to control (Sashiwa *et al.,* 2002). Enzymatic hydrolysis method as an alternative treatment using chitin directly from crab or shrimp shells is faster, simpler and more environmentally friendly compared to chemical treatment (Krokeide *et al.*, 2007). Glucosamine produced by enzymatic hydrolysis is in the form of N-acetylglucosamine.

Mucor circinelloides is one of the filamentous fungi that produce chitinase enzyme to degrade chitin into glucosamine (Shubakov and Kucheryavykh, 2004). In this research, chitin degradation into glucosamine is done using semi pure extracellular chitinase enzyme produced and collected from *Mucorcircinelloides* because of the ability of *Mucor circinelloides* to secrete chitinase enzyme extracellularly (Luong *et al.,* 2010) and higher purity of chitinase enzyme increases the chitinase enzyme activity (Suryadi[a] *et al.*, 2013). The aim of this research was to utilize Tiger shrimp (*Penaeus monodon*) shells to produce N-acetylglucosamine enzymatically using extracellular semi pure chitinase

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enzyme from *Mucor circinelloides*. Several parameters that contribute to enzymatic reaction, such as pH, temperature, substrate concentration and fermentation time were also determined.

2 MATERIALS AND METHODS

The materials used in this research were chitin isolated from Tiger shrimp shells (Penaeus monodon) obtained from PT. Lola Mina, Muara Baru, Jakarta, Mucor circinelloides culture isolated from Tiger shrimp shells, Potato Dextrose Agar (PDA) "MERCK" and Potato Dextrose Broth (PDB) "DB Filco", N-acetylglucosamine standard "SIGMA ALDRICH", 3-5 dinitro salicylic acid "MERCK", bovine serum albumin (BSA), dipotassium phosphate (K2HPO4), potassium dihydrogen phosphate (KH2PO4), magnesium sulphate heptahydrate (MgSO4.7H2O), ammonium sulphate ((NH4)2SO4), disodium hydrogen phosphate (Na2HPO4), potassium sodium tartrate (Na-K-tartrate), phosphoric acid (H3PO4), distilled water, NaOH solution (3.5%, 10 N), HCl solution (37%, 1 M), tartaric acid 10% and ethanol 96%. The equipment used in this research were incubator shaker "HEIDOLPH 22UNIMAX 1010", analytical balance "OHAUS U-1800 AR 2140", oven "MEMMERT", centrifuge "MPW-223e", microcentrifuge "HETTICH ZENTRIFUGE EBA 20", microscopic camera "OLYMPUS DP21", UV-VIS spectrophotometer "THERMO SCIENTIFIC GENESYS 10S", pH meter "METROHM 913", quartz cuvette "HELLMA Analytics", micropipette and glassware.

2.1 Colloidal Chitin Preparation (Setia and Suharjono, 2015)

Colloidal chitin was prepared as substrate in optimum pH and optimum temperature determination and added in culture media to induce the chitinase production. Ten grams of isolated chitin was added with 140 ml of 37% HCl and stirred with magnetic stirrer for 2 hours to dissolve the chitin. The mixture was added with 500 ml of absolute ethanol and filtered with Buchner funnel. The residue obtained was added with 5 N of NaOH until the pH reached neutral and centrifuged with speed 4000 rpm for 5 minutes. The precipitate obtained was collected as the colloidal chitin.

2.2 Production of Semi Pure Chitinase (Jenifer et al., 2014)

About 5 ml of Mucor circinelloides spore culture was added into 250 ml Potato Dextrose Broth (PDB) media containing 0.5% colloidal chitin, 0.5% Na2HPO4 and 0.5% MgSO4.7H2O. The mixture was incubated for 2 days in incubator shaker at room temperature. The grown culture suspension was centrifuged at 3300 rpm for 10 min at 4°C and the supernatant obtained was the extracellular crude chitinase. The extracellular crude chitinase was precipitated with 90% ammonium sulphate while stirred at 4°C. This suspension left for 24 hours at 4°C then centrifuged at 3300 rpm for 10 minutes. The precipitate was taken and dissolved in 8 ml of 0.05 M phosphate buffer solution (pH 8) per 150 ml of grown culture suspension (Lawati, 2013). The precipitate taken was the extracellular semi pure chitinase enzyme and was stored at 4°C prior to usage in fermentation.

2.3 Determination of Optimum pH and Temperature (Jenifer et al., 2014)

1 ml of 0.5% colloidal chitin in each pH buffers (pH 3-9) were added by 1 ml of semi pure chitinase enzyme then incubated in room temperature for 1 hour. The optimum pH was determined from highest chitinase activity. Furthermore, 1 ml of 0.5% colloidal chitin in the optimum pH buffer were added by 1 ml of semi pure chitinase enzyme then incubated in temperature of 30, 40, 50, 60, 70 and 80°C for 1 hour. The optimum fermentation temperature was determined from highest chitinase activity.

2.4 Determination of Optimum Substrate Concentration and Fermentation Time (Herdyastuti et al., 2009)

Different levels of chitin concentration of 0.5, 1, 1.5 and 2% chitin were added to the optimum pH buffer. 1 ml of different concentration of chitin in the optimum pH buffer were added by 1 ml of semi pure chitinase enzyme then incubated in the optimum temperature for 2, 4, 6 and 24 h. The optimum substrate concentration and fermentation time were determined by the highest N-acetylglucosamine concentration.

2.5 Analysis of Chitinase Enzyme Activity (Rahmansyah and Sudiana, 2003)

Chitinase activity was measured using DNS (dinitrosalicylic) colorimetric method. The determination of chitinase activity used colloidal chitin as the substrate. 1 gram of 3,5-dinitrosalicylic acid (DNS) was dissolved in 20 ml distilled water then added with 1 gram of NaOH, 0.2 gram of phenol and 0.05 gram of sodium sulphite. The mixture was then transferred and diluted into 100 ml volumetric flask. The mixture was then centrifuged at 3300 rpm for 5 minutes. 1 ml of supernatant was taken and added with 2 ml of modified DNS and 1 ml of 4% potassium sodium tartrate. The mixture then heated for 15 minutes at boiling temperature. The mixture then was observed using spectrophotometer at 540 nm. Chitinase activity were calculated using the formula:

$$\text{Chitinase Activity (U/ml)} = \frac{\text{N} - \text{acetylglucosamine concentration x 1000 x enzyme}}{\text{N} - \text{acetylglucosamine molecular weight x incubation period (h)}} \quad (1)$$

2.6 N-acetylglucosamine Concentration Quantification (Rahmansyah and Sudiana, 2003)

Glucosamine standard curve was prepared for quantification of N-acetylglucosamine concentration. Blank was first prepared by adding 2 ml of modified DNS and 1 ml of 4% potassium sodium tartrate into 1 ml of distilled water then heated for 15 minutes in boiling temperature. N-acetylglucosamine standard was prepared in concentration of 200, 400, 600, 800 and 1000 ppm. 1 ml from each concentration was added with 2 ml of modified DNS and 1 ml of 4% potassium sodium tartrate. The mixture heated for 15 minutes in boiling temperature. The heated mixture of blank and each concentration of glucosamine standard were observed by spectrophotometer at 540 nm. N-acetylglucosamine content calculated by using the linear equation of glucosamine standard. 1 ml of N-acetylglucosamine from the fermentation was added with 2 ml of modified DNS and 1 ml of 4% potassium sodium tartrate. The mixture was heated at boiling temperature for 15 minutes and the absorbance observed using spectrophotometer at 540 nm.

2.7 Data Analysis

The experimental design used was Completely Randomized Factorial Design with 1 factor for optimum pH or temperature determination and 2 factors for optimum substrate concentration and fermentation time determination. All data obtained were analyzed using SPSS version 22.

3 RESULT AND DISCUSSION

3.1 Characteristics of Isolated Chitin

Chitin used in this research was isolated from Tiger shrimp shells through demineralization and deproteination processes and then analysed for its yield, moisture content, protein content, ash content and degree of deacetylation. The results can be observed on Table 1.

Table 1: Characteristics of isolated chitin.

Parameter	Content
Yield (% db)	8.85 ± 0.33
Moisture content (% wb)	4.59 ± 0.32
Ash content (%)	0.46 ± 0.03
Protein content (%)	1.74 ± 0.07
Degree of Deacetylation (%)	28.07

Yield of isolated chitin obtained in this research was lower compared to the previous study by Hossain and Iqbal (2014) that showed the yield content of chitin from shrimp shell is in range of 13.12-17.36%. The moisture content of isolated chitin is comparable to the previous studies (Arif et al., 2013; Isa et al., 2014; Liu et al., 2013; Sanusi, 2004) that showed the moisture content of chitin is fewer than 10%, i.e. about 8.70%, 7.64% and 5.22%, respectively. The ash content of chitin is lower compared to the previous studies by Arif et al. (2013), Isa et al. (2014) and Liu et al. (2013) that was about 2%, 5.60% and 1.59%, respectively. This shows that the demineralization process in this research was more effective to remove the minerals from shrimp shells. Furthermore, protein content of isolated chitin was lower compared to by Arif et al. (2013) that showed that the protein content of isolated chitin was 4.16%. This result also shows that the deproteination process in this research was more effective. Degree of deacetylation is an indicator of chitin purity (Sanusi, 2004). The degree of deacetylation (DD) of isolated chitin obtained was 28.07%, in accordance with previous researches (Arif et al., 2013; Younes and Rinaudo, 2015) that mentioned degree of deacetylation of chitin was 15 - 70% and less than 50%, respectively.

3.2 Effect of pH on Semi Pure Extracellular Chitinase Activity

Statistical analysis using ANOVA shows that pH gave significant effect on enzyme activity of chitinase produced by Mucor circinelloides. Results also show that the optimum pH for semi pure extracellular chitinase activity is at pH 8, with enzyme activity of 5.76 ± 0.17 U/ml. The results can be observed on Figure 1.

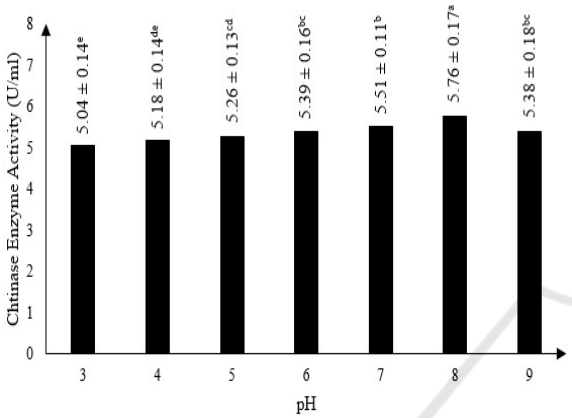

Figure 1: The effect of pH on chitinase enzyme activity (Note: Different letter notations indicates a significant difference at p≤0.05).

This result is in accordance with a previous study who stated that the chitinase activity may increase and decrease because of the difference in pH as a factor (Purkan et al., 2014). Chitinase enzyme produced by *M. circinelloides* that is optimum at pH 8, is similar to chitinase enzyme produced by *Moniliophthora perniciosa* (Galante et al., 2012), *Aeromonas* sp. (Haliza and Suhartono, 2012) and Bacillus cereus (Suryadib et al., 2013) that were also optimum at pH 8.

3.3 Effect of Temperature on Semi Pure Extracellular Chitinase Activity

Statistical analysis using ANOVA shows that temperature of reaction gave significant effect on enzyme activity of chitinase produced by *Mucor circinelloides.* Results also show that the optimum temperature for semi pure extracellular chitinase activity is at 50^{O}C, with enzyme activity of 6.78 ± 0.13 U/ml. The results can be observed on Figure 2.

Temperature affects the kinetic energy of molecule which can accelerate enzyme hydrolysis reaction with substrate when the temperature is raised (Murray *et al.,* 2005). However, after optimum temperature is reached, the chitinase enzyme activity decrease as the temperature is raised because enzyme can be denatured in high temperature (Lehninger *et al.,* 2004). The optimum temperature of 50°C obtained in this research is similar to chitinase enzyme produced by *Aspergillus tereus* (Farag *et al.,* 2016) and *Serratia marcescens* (Zeki and Muslim, 2010). Chitinase enzyme produced by *M. circinelloides* can be considered as thermostable.

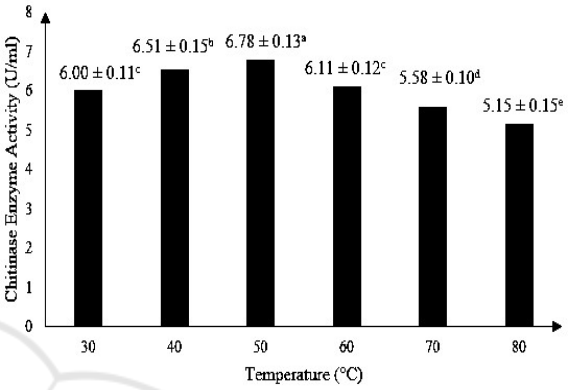

Figure 2: The effect of temperature on chitinase enzyme activity (Note: Different letter notations indicates a significant difference at p≤0.05).

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3.4 Effect of Substrate Concentration and Fermentation Time on N- acetylglucosamine Production

Statistical analysis using Univariate shows that interaction between substrate concentration and fermentation time did not affect the N-acetylglucosamine (NAG) concentration produced by chitinase enzyme. However, substrate concentration and fermentation time affected the NAG concentration produced by chitinase enzyme. These results can be observed on Figure 3 and Figure 4.

Figure 3 shows the highest NAG produced by chitinase enzyme of *M. circinelloides* with isolated chitin as substrate is at 1.5% substrate concentration, i.e. about 1285.73 ± 66.19 ppm. The optimum substrate concentration of 1.5% is according to a previous study that stated chitinase activity reached maximum activity at chitin concentration of 1.5%, in which optimum chitinase activity maximizes the production of NAG (Karthik *et al.,* 2014). Optimum substrate concentration for chitinase isolated from *M. circinelloides* of 1.5% is also in accordance with optimum substrate concentration of chitinase isolated from *Streptomyces viridificans* (Gupta *et al.,* 1995) and *Aeromonas* sp. (Huang *et al.,* 1996).

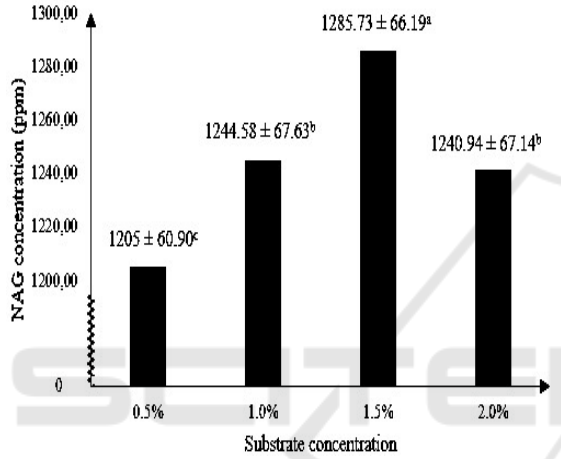

Figure 3: The effect of substrate concentration on NAG concentration (Note: Different letter notations indicates a significant difference at p≤0.05).

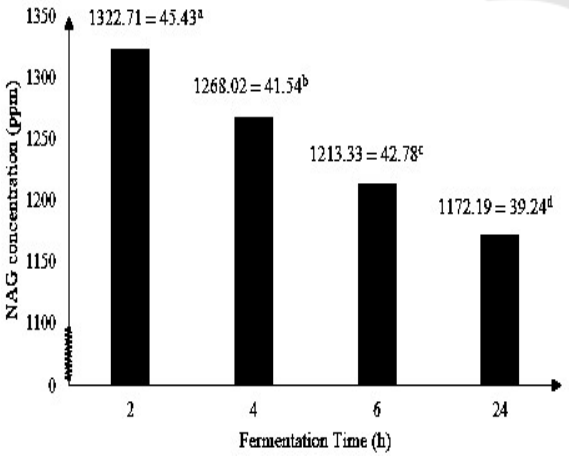

Figure 4: The effect of fermentation on NAG concentration (Note: Different letter notations indicates a significant difference at p≤0.05).

Figure 4 shows that the highest NAG concentration produced by chitinase enzyme of *M. circinelloides* is at 2 hours of fermentation, i.e. about 1322.71 ± 45.43 ppm. The ability of chitinase to degrade chitin structure affected the time needed to produce NAG (Wulandari, 2009). Chitinase activity isolated from *Aeromonas* sp. reached maximum chitinase activity of 8.7 U/ml at 72 hours of incubation (Younes *et al.,* 2013), *Trichoderma harzianum* reached maximum chitinase activity of 5.4 U/ml after incubation of 72 hours (Sandhya *et al.,* 2005) and *Streptomyces rubiginosus* reached maximum chitinase activity of 2.2 U/ml after 72 h of incubation (Jha *et al,* 2016). This also means that chitinase produced by *Mucor circinelloides* reaches its maximum activity and NAG production much faster compared to chitinase produced by *Aeromonas* sp., *Trichoderma harzianum* and *Streptomyces rubiginosu.*

4 CONCLUSIONS

This research confirmed the potency of extracellular semi pure chitinase enzyme produced by *Mucor circinelloides* as an alternative method to produce N-acetylglucosamine. The optimum pH of extracellular semi pure chitinase from *Mucor circinelloides* is 8 with chitinase activity of 5.76 ± 0.17 U/ml and the optimum temperature is 50°C with chitinase activity of 6.78 ± 0.13 U/ml. The optimum substrate concentration to produce N-acetylglucosamine is 1.5% of chitin with 1285.73 ± 66.19 ppm of N-acetylglucosamine produced and the optimum fermentation time is 2 hours with concentration of N-acetylglucosamine produced is about 1322.71 ± 45.43 ppm.

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