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Utilization of Crude Intracellular Chitinase Enzyme from *Providencia stuartii* for Glucosamine Production from Shrimp Shells

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Abstract

Chitin hydrolysis using enzyme is one of the methods to produce glucosamine in shorter time compared to using microbial cells, but the ability to produce glucosamine at enzyme's optimum condition is influenced by substrate concentration and fermentation time. The objective of this research was to determine the optimum substrate concentration and fermentation time of shrimp shells' chitin to produce glucosamine at the optimum pH and temperature of crude intracellular chitinase enzyme from Providencia stuartii. Method used was experimental method, started by extraction of intracellular enzyme from P. stuartii, followed by determination of optimum pH and temperature of enzyme. The optimum condition was used for experiment of shrimp shells' chitin fermentation with treatments of chitin substrate concentration (0.5; 1.0; 1.5; 2.0%) and fermentation time (2, 4, 6 and 24 hours). Results showed that optimum enzyme activity occurred at pH of 5.0 and temperature of 40°C, which was about 6.03 U/ml. Concentration of chitin substrate and fermentation time influenced the amount of glucosamine obtained. Fermentation of shrimp shells' chitin using crude intracellular enzyme was optimum at 1.0% substrate concentration and 6 hours fermentation time, which produced glucosamine about 1680.06±58.49 ppm.

Keywords: *intracellular chitinase enzyme, glucosamine, shrimp shells' chitin, P. stuartii*

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INTRODUCTION

Chitin is a linier polysaccharide that consists of N-acetyl glucosamine, connected through β-1,4-N-acetyl glucosamine linkage (GlcNAc) (Arif *et al.*, 2013). The main sources of chitin are shrimp shells, lobster shells, crab shells, jellyfish shells, insect's exoskeleton and cell walls of molds and bacteria (Chang *et al.*, 2004).

Chitin from shrimp shells are usually obtained through two stages, i.e. demineralization and deproteination. Demineralization is a process to eliminate organic compounds from shrimp shells by adding HCl. Deproteination is a process to eliminate

protein content from shrimp shells by adding hot NaOH (Kurniasih and Dwiasi, 2007).

N-acetyl glucosamine can be obtained from chitin by two methods, i.e. enzymatic hydrolysis and chemical hydrolysis. Chitin hydrolysis chemically can be done using concentrated acid, such as HCl or H_2SO_4. Chemical hydrolysis produces acid glucosamine depends on types of acid used and produces chemical waste that can be harmful for the environment. Moreover, acid glucosamine is considered as unnatural (Pratiwi *et al.*, 2015). Enzymatic hydrolysis can be done using molds or bacteria that produce chitinase enzyme (Taherzadeh and Karimi, 2007), or using chitinase enzyme from microorganisms directly. The product obtained from chitin degradation enzymatically is natural glucosamine, in form of N-acetyl glucosamine. N-acetyl glucosamine is also a major compound of GAG of cartilage and synovial fluid that plays role in lubricating bones (Kralovec and Barrow, 2008; Kardiman, 2013).

Chitinase can be obtained from various microorganisms, such as bacteria, virus, molds, as well as from animal, plant and insect that has the ability to hydrolyze chitin (Tomokazu *et al.*, 2004). Microorganisms that are able to hydrolyze chitin into glucosamine using chitinase enzyme is often called as chitinolytic microorganisms.

Providencia stuartii is one of strong chitinolytic bacteria that was successfully isolated from Tiger shrimp shells (Halim *et al.*, 2018). *Providencia stuartii* is a Gram-negative bacterium from *Enterbacteriaceae* group, that is motile, lives anaerobic facultatively, grows at 37ºC and has greyish white color (Manos and Belas, 2006). Chitinolytic index of *Providencia stuartii* (4.08) is higher compared to other chitinolytic bacteria isolated from shrimp shells, such as *Acinetobacter johnsonii* (chitinolytic index = 2.06) and *Bacillus amyloliquefaciens* (chitinolytic index = 2.08) (Setia dan Suharjono, 2015). Thus, *Providencia stuartii* has stronger chitinolytic power to degrade chitin faster and produce higher amount of natural glucosamine.

A strong chitinolytic power of *Providencia stuartii* is potential to produce glucosamine through chitin fermentation process using cells or enzyme. Fermentation process using microorganisms directly requires longer time, i.e. several days or weeks (Hardoko *et al*, 2017), but fermentation using enzyme requires shorter time, i.e. in several hours (Sashiwa *et al.*, 2002).

Enzyme used in fermentation can be in form of pure enzyme or crude enzyme. To obtain pure enzyme, longer process is required, whereas crude enzyme can be obtained in shorter process. The shorter process to obtain crude enzyme is more advantageous in producing natural glucosamine enzymatically. Furthermore, optimum conditions, such as pH, temperature, substrate concentration and fermentation time are required for enzyme to be able to work optimally.

The aim of this research was to determine the optimum substrate concentration and fermentation time of shrimp shells' chitin to produce glucosamine at the optimum pH and temperature of crude intracellular chitinase enzyme from *Providencia stuartii.*

MATERIALS AND METHODS

Materials and Equipment

Materials used in this research were Tiger shrimp shells (*Penaeus monodon,* Fabricius) obtained from PT. Lola Mina, Muara Baru, Jakarta, Indonesia, *Providencia stuartii* culture obtained from isolation by Halim *et al.* (2018), media (MERCK) Nutrient Agar, Nutrient Broth, Bromocresol Puple indicator (MERCK). pH buffer solutions of 3, 4, 5, 6, 8, 9 (MERCK), chemical ingredients (MERCK), i.e. $MgSO_4.7H_2O$, KH_2PO_4, K_2HPO_4 , $(NH_4)_2SO_4$, NaCl, HCl 1 N solution, NaOH 3.5% solution, alcohol 70%, ethanol, and SIGMA chemical, i.e 3,5 Dinitrosalicylic acid (DNS), Coomassie Blue, Bovine Serum Albumin (BSA).

Equipment used in this research were Petri dishes, micropipette and tips, inoculating loop, waterbath (MEMMERT K-072-02-T), vortex (THERMOLYNE), Bunsen burner, Quartz cuvette, heater, dry blender (PANASONIC MX-GX-1462), cabinet dryer (WANGDI W), laminar air flow (ESCO ECH-4), autoclave (HIRAMAYA HL 36 AE), centrifuge (HETTICH EBA 200), incubator (MEMMERT INE 800), UV-Vis spectrophotometer (THERMO-SCIENTIFIC GENESYS 20), Whatmann no.1 filter paper, table balance (OHAUS), analytical balance (OHAUS U-1800 AR 2140), oven (MEMMERT) and glasswares.

Extraction of Crude Intracellular Chitinase Enzyme

Production of crude intracellular chitinase enzyme from *Providencia stuartii* was done by inoculating one loop of bacteria into Nutrient Broth media and then incubated for 18 hours (log phase of bacteria growth curve) at 37ºC. After incubation period was achieved, bacteria stock containing bacteria was added into chitin fermentation media (Nutrient Broth added with K_2HPO_4; $MgSO_4.7H_2O$; $(NH_4)_2SO_4$ and 2% colloidal chitin). The mixture was then incubated using shaker incubator with speed of 120 rpm, at 37ºC for 20 hours. Afterwards, fermentation media was centrifuged with speed of 3500 rpm for 10 minutes and the pellets obtained were added with lysis buffer. The mixture was then further centrifuged, and supernatant obtained was crude intracellular chitinase enzyme of *Providencia stuartii.*

Enzymatic Fermentation to Produce N-acetyl Glucosamin from Shrimp Shells' Chitin

Enzymatic fermentation process to produce glucosamine was done with treatments of substrate concentration (0.5 %, 1,0 %, 1.5%, 2.0%) and fermentation time (2, 4, 6 and 24 hours). Chitin substrate was prepared with concentration of 0.5 %, 1,0 %, 1.5%, 2.0% based on the volume of optimum pH buffer (pH 5). Each substrate concentration was added with 1 ml of crude intracellular chitinase enzyme. The solution was incubated at optimum temperature (40ºC) for 2, 4, 6 and 24 hours. After fermentation time was achieved, the solution was centrifuged with speed of 2000 rpm for 10 minutes and supernatant obtained was filtered using Whatman no.1 filter paper. Afterwards, N-acetyl glucosamine concentration was measured.

Analysis of Glucosamine Concentration

Analysis of glucosamine concentration was based on 3,5 dinitrosalycilic acid (DNS) method, done by Rahmansyah and Sudiana (2003) and Ifnawati (2013). Standard curve was prepared by making series of glucosamine concentration and each concentration was added with 2 ml of DNS reagent and 1 ml of Na-K tartrate reagent. The mixture was then homogenized using vortex and heated at 100ºC for 15 minutes. 1 ml of mixture was taken and added with 4 ml of aquadest. The sample was then measured for its absorbance using spectrophotometer at wavelength of 540 nm. The correlation between absorbance and glucosamine concentration was made to obtain standard curve. Afterwards, sample from fermentation that has been centrifuged was added with the same reagent and treatment used in standard curve preparation. The samples' absorbance obtained was put into the standard curve to obtain the glucosamine concentration of samples.

RESULTS AND DISCUSSIONS

Characteristics of Chitin and Chitinase Enzyme

Characteristics of chitin and crude intracellular chitinase enzyme used in fermentation to obtain glucosamine can be seen on Table 1. Chitin that was used as a substrate for fermentation was prepared according to method used by Kurniasih dan Dwiasih (2007). Chitin obtained is categorized as good quality because it fulfilled the standard of chitin and when compared to other researches, the moisture content, ash content, protein content and degree of deacetylation are lower. Quality standard of good quality of chitin are contains protein <7% (Bastaman *et al.*, 1990) and degree of deacetylation <45 (Arif *et al.*, 2013). According to Azhar *et al.* (2010), chitin that has undergone further process into chitosan has degree of deacetylation more than 40%. Furthermore, moisture content of chitin from other researches, such as from Martati *et al.* (2002) was 10%, Kurniasih and Dwiasi (2007) was 5.39%, and Qin *et al.* (2010) was 8.7%.

Tabel 1. Characteristics of chitin substrate and enzyme activity of crude intracellular chitinase enzyme

Parameter	Amount
Moisture content (%)	4.58±0.43
Ash content (%)	0.45±0.02
Protein content (%)	1.74±0.07
Yield (%)	23.15±0.25
Degree of deacetylation	28
Enzyme activity (U/mL)	6.03±0.24

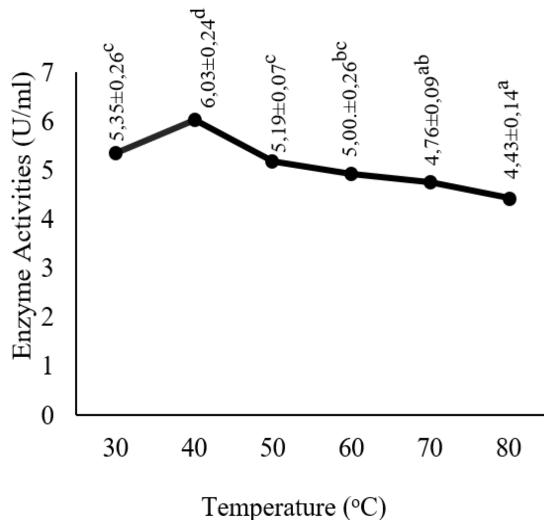
Low moisture content influences the chitin's shelf life (Poeloengasih *et al.*, 2008). As content of chitin based on research from Febriani *et al.* (2016) was 3.78%, Arif *et al.* (2013) was 0.55%, and Kurniasih and Dwiasi (2007) was 2.66%. Lower ash content of chitin indicates the better quality of chitin obtained. According to Agustina *et al.* (2015), yield of chitin from shrimp shells was >20%, but several other researches obtained higher chitin yield, such as Dompeipen *et al.* (2016) with yield of 50%, dan Arif *et al.* (2013) with yield of 29.38%.

According to Lehninger *et al.* (2004), generally chitinase enzyme has the optimum activity at pH of 4-8 and temperature of 40-50oC. Enzyme activity of crude intracellular chitinase obtained in this research (Table 1) is higher compared to research from Suryadi *et al.* (2013), which was about 0.059 U/ml (chitinase enzyme from *Bacillus cereus* at optimum pH of 8) and chitinase activity of *Aspergillus niger* molds, which was about 0.155 U/ml at optimum pH of 6. These results show that enzyme activity is also influenced by the source of enzyme or the microorganisms used as the source of enzyme.

Optimum Temperature and pH of Crude Intracellular Chitinase from *P. stuartii*

Optimum pH and temperature are the condition in which enzyme can produce the highest activity to catalyze chemical reaction (Purkan *et al.,* 2014). 1 unit of chitinase enzyme activity is defined as the amount of N-acetyl glucosamine produced per hour by enzyme in certain temperature and pH (Orinda *et al.*, 2015). Suryadi *et al.* (2013) and Pratiwi *et al.* (2017) stated that optimum pH and temperature can be determined based on the highest enzyme activity value (U/ml) at various temperature and pH that have been set. The optimum temperature for crude intracellular chitinase can be seen on Figure 1, whereas its optimum pH can be seen on Figure 2.

Figure 1 and Figure 2 show that optimum enzyme activity occurred at temperature of 40ºC and pH of 5 with enzyme activity of 6.03±0.24 U/ml and 6.02±0.16, respectively. Generally, chitinase enzyme has optimum activity at temperature of 40-50 ºC and optimum pH at pH of 4-8 (Lehninger *et al.*, 2004).

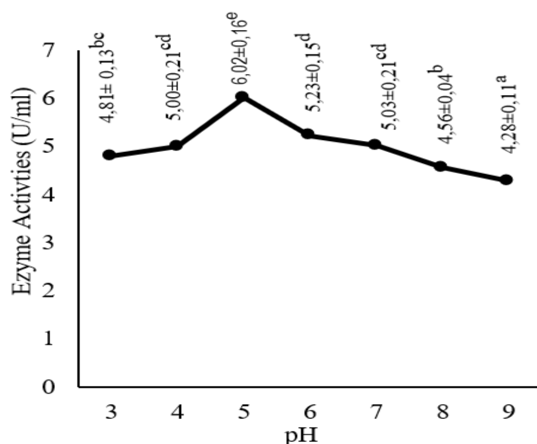


Note: different alphabetic notation indicates significant difference at $p<0.05$

Figure 1. Optimum temperature of crude intracellular chitinase enzyme activity from *Providencia stuartii*

Enzyme activity of crude intracellular chitinase from *P. stuartii* in this research is higher compared to chitinase from *Bacillus cereus* (0.070 U/ml at optimum temperature of 37°C and 0.059 U/ml at pH of 8) (Suryadi *et al.* (2013), *Aspergillus niger* (0.137 U/ml at optimum temperature of 40 °C and 0.155 U/ml at optimum pH of 6).

Enzyme activity occurs at optimum temperature of 40 °C because there is an increase of kinetic energy between enzyme and substrate that causes rotational, vibrational and transitional motions from enzyme and substrate becomes more frequent. However, too high temperature can decrease enzyme activity because denaturation occurs that can inhibit the linkage between enzyme and substrate (Pratiwi *et al.*, 2015). Enzyme denaturation damages enzyme



Note: different alphabetic notation indicates significant difference at $p<0.05$

Figure 2. Optimum pH of crude intracellular chitinase enzyme activity from *Providencia stuartii*

structure, therefore secondary, tertiary and quaternary structures of protein molecules change and as a result, enzyme cannot work optimally (Simanjorang *et al.*, 2012).

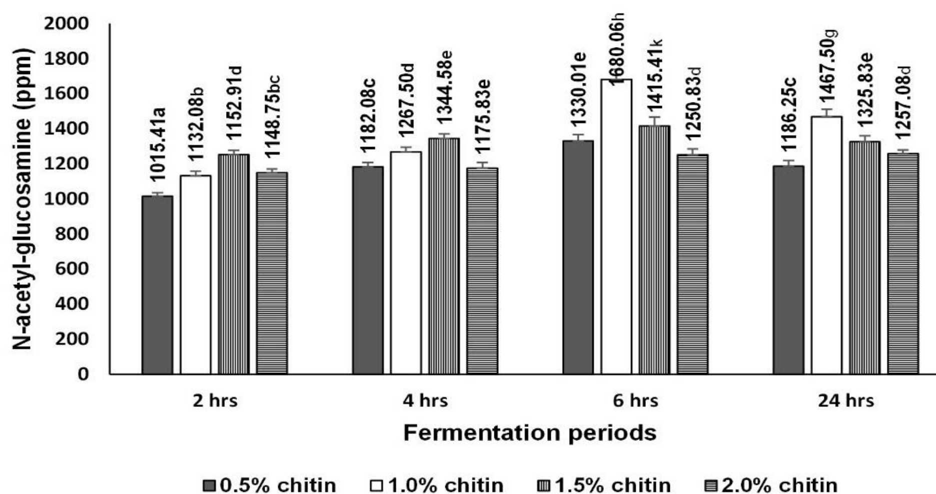
Low enzyme activity at acidic or alkaline pH is caused by enzyme denaturation (Pratiwi *et al.*, 2015). In alkaline solution (pH>9), cysteine residue is damaged and in acidic solution (pH<4), hydrolysis happens on unstable peptide bonds that can inhibit enzyme (Simanjorang *et al.*, 2012). pH also influences ionic properties of amino and carboxyl group that can change catalytic area and enzyme structure.

Glucosamine Production using Crude Chitinase Enzyme from *P. stuartii*

Fermentation process of shrimp shells' chitin by crude intracellular enzyme from *P. stuartii* to produce glucosamine was done at optimum condition, i.e. pH of 5.0, temperature of 40°C and enzyme activity of 6.03 U/mL. Statistical analysis using ANOVA showed that concentration of chitin substrate, fermentation time and interaction of both treatments gave significant difference ($p<0.05$) on glucosamine obtained. The results of glucosamine concentration from Tukey LSD test can be observed on Figure 3.

Figure 3 shows a trend of fermentation time influences the concentration of N-acetyl glucosamine obtained for each chitin concentration used. However, the highest N-acetyl glucosamine production occurred at chitin concentration of 1% and fermentation time of 6 hours ($p<0.05$). Glucosamine concentration obtained at this fermentation condition was about 1680.06±58.49 ppm. Meanwhile, the lowest N-acetyl glucosamine concentration obtained from chitin concentration of 0.5% and fermentation time of 2 hours, with N-acetyl glucosamine concentration of about 1015.41±20.41 ppm.

The highest or optimum point in glucosamine production might correlate with reaction rate between enzyme and substrate, which is slow at the beginning, increases, becomes stationery and then decreases. This enzyme-substrate reaction rate at optimum reaction condition is influenced by concentration of enzyme and concentration of substrate (Lehninger *et al.,* 2004). Poedjiadi (2006) further explained that addition of substrate concentration would increase the rate of reaction catalyzed by enzyme to a certain point when the increase of substrate would not further increase the reaction rate. This is because the enzyme has become saturated with substrate, therefore reaction rate between enzyme and substrate would achieve the optimum condition. Based on kinetics theory, Saropah *et al.*, (2012) also explained that higher substrate concentration would cause the increase of collision between energy and molecule frequency, therefore chitinase enzyme could bind higher amount of substrate.



Note: different alphabetic notation indicates significant difference at p<0.05

Figure 3. Enzymatic fermentation at optimum condition to produce N-acetyl glucosamine

A research from Gomma (2012), which used purified chitinase enzyme from *Bacillus thuringiensis*, the highest concentration of N-acetyl glucosamine was obtained at substrate concentration of 1.5%, with N-acetyl glucosamine concentration of 654.75 ppm and the concentration decreases with higher substrate concenctration. Wirawan and Herdyastuti (2014) also reported that chitinase enzyme from *Pseudomonas* sp. bacteria could produce N-acetyl glucosamine optimally at fermentation time of 36 hours, with concentration obtained was about 164 ppm, and the concentration of N-acetyl glucosamine obtained decreased with longer fermentation time. The difference in optimum fermentation time obtained in this research might because enzyme produced from different bacteria could have different enzyme activity.

CONCLUSIONS

Enzyme activity of crude chitinase used was 6.03±0.24 U/mL at optimum pH of 5.0 and temperature of 40ºC. The highest N-acetyl glucosamine production was at substrate concentration of 1.0% and fermentation time of 6 hours, with crude concentration of N-acetyl glucosamine of 1680.06±58.49 ppm.

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