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Isolation of chitinolytic bacteria and optimization of chitin fermentation process for glucosamine production using RSM method

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Abstract

Chitinolytic bacteria originating from shrimp shells can be used for the production of glucosamine by fermentation. The aim of this research was to isolate and identify chitinolytic bacteria from shrimp shells that were spoiled in a closed container and to optimize chitin fermentation using the strongest chitinolytic bacteria. Bacteria isolation was done on shrimp shells that were rotten in a closed container and then identify the strongest chitinolytic bacteria using Polymerase Chain Reaction (PCR) method. The strongest chitinolytic bacteria isolate was optimized for its glucosamine production using Response Surface Methodology (RSM) method with pH and fermentation period. Results show that fermented chitin has moisture content of 6.69%, ash content of 1.42% and deacetylation degree of 35.16%. The strongest chitinolytic bacteria isolate has a clear zone diameter of 8.26±0.13 mm and identified as *Bacterium* 4H106 which was rod-shaped, Gram-positive bacteria using 16S molecular identification. Chitin fermentation process that produced the optimum glucosamine was at pH of 6.2 and fermentation period of 10.1 days, i.e. produce N-acetyl-glucosamine concentration of 239,429.96 ppm. Bacterium isolate 4H106 possessed a strong chitinolytic activity and had an optimum pH of 6.2 in degrading chitin into glucosamine through fermentation.

1. Introduction

Chitin is a homopolymer from N-acetyl-D-glucosamine residue that is bound by β-1,4 glycosidic bonds. An abundant amount of chitin allows its use in biotechnology and industrial field (Arif *et al*., 2013). Chitin can be utilized directly or by derivatization into oligomer of chitin and monomer of glucosamine. Chitin derivatives are widely used in several areas, such as in health and medicine as an immunoadjuvant (non-specific stimulator of immune response) and material for suture (Knorr, 1991; Subasinghe, 1999). In beauty areas, chitin derivatives can be used as ingredients for cosmetics, toothpaste, body cream and hair care products. In the textile area, they are widely used as a coating material for cellulose fiber, nylon, cotton and wool (Kaban, 2009). Moreover, glucosamine is used in osteoarthritis treatment and food supplement (Sashiwa *et al.,* 2012; Mirunalini *et al*., 2015; Nurjannah *et al*., 2016).

Shrimp shells are one of the chitin sources. Shrimp shells contain about 15-20% chitin, but the amount of chitin depends on the types of shrimp (Foucher *et al*., 2009). In Indonesia, shrimp shells are by-products or waste of shrimp processing that reach about 30-35% of total shrimp weight (Darmawan *et al*., 2007). Thus, shrimp shells are potential to be used as a source of chitin and its derivatives, such as chitosan and glucosamine.

One of chitin derivatization processes into glucosamine is by fermentation of chitin by microorganisms (Purnomo *et al*., 2012). The microorganism used in fermentation to produce glucosamine from shrimp shells is a microorganism that produces chitinase. It is believed that microorganism is the naturally main mediator in chitin degradation. Soil is a source of chitinolytic bacteria; it is shown by the increase in chitin hydrolysis rate as the number of bacteria colonies increases in the soil (Powthong and Suntornthiticharoen, 2017). Other than bacteria, fungi are also important hydrolyzing agents. In the aquatic system, bacteria are also main chitin hydrolyzing agents (Beier and Bertilsson, 2013). Furthermore, another source of chitinolytic bacteria is organic waste (Purkan *et al*., 2014), in peat and palm oil bunch compost (Hutauruk

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et al., 2016), marine waste (Krithika and Chellararm, 2016), marine sediments (Nagaseshu *et al.*, 2016) and marine environment (Thirumurugan *et al*., 2015).

Chitinolytic microbes isolated from organic waste are *Pseudomonas* sp., *Aeromonas* sp., *Xanthomonas* sp., *Serratia* sp., *Cytophaga* sp., *Arthrobacter* sp. and *Bacillus* sp. (Purkan *et al*., 2014). *Serratiamarcescens* are widely researched as chitinolytic bacteria because they produce chitinase and isoenzymes (Apriani, 2008; Agrawal and Kotasthane, 2012). Several chitinolytic bacteria which were found in organic waste in aquatic system consisted of *Cytophaga-Flavobacteria*, *Chitiniphilusshianonensis, Acinetobacter* sp., *Bacillus badius, Acinetobactervenetianus* and *Brevibacillus borstelensis*. Microorganisms specifically isolated from shrimp processing waste were *Acinetobacter johnsonii, Bacillus* sp. and *Pseudomonas* sp. (Beier and Bertilsson, 2013; Krithika and Chellaram, 2016). There were also six strains of *Bacillus* that have been identified, i.e. *B. amyloliquefaciens, B. subtilis, B. licheniformis, B. mojavensis, B. pumilus* and *B. cereus* (Setia and Suharjono, 2015). *Bacillus subtilis* is a chitinolytic bacterium that also has antifungal activity (Velusamy and Das, 2014).

The selection of sources for chitinolytic microorganisms should be adjusted for their purpose. If chitin is degraded from shrimp shells, then the source of microorganisms can be obtained from shrimp shells. There is no research about chitinolytic microorganisms that were isolated directly from chitin obtained from rotten shrimp shells. It is expected that microorganisms isolated directly from chitin destruction will have high chitinolytic activity. Thus, the research about the isolation of chitinolytic bacteria from chitin obtained from rotten shrimp shells and its application in chitin fermentation should be done.

2. Materials and methods

2.1 Materials

Materials used as a chitin source were Vanamei shrimp shells obtained from a frozen fresh shrimp processing company in Sidoarjo, East Java, Indonesia. Supporting materials for chitin isolation and fermentation included HCl, KH_2PO_4, NaOH, $MgSO_4.5H_2O$, $FeSO_4.7H_2O$, $ZnSO_4$, $MnCl_2$, K_2HPO_4, CH_3COONa, Plate Count Agar (PCA), Nutrient Agar (NA), Phenyl isothiocyanate (PITC), Glucosamine standard, methanol and aquadest. The equipment used in this research were glassware, petri dish, autoclave, magnetic stirrer, reaction tubes, vacuum filter, tray, pH meter, refrigerator, vacuum dryer, analytical balance, blender, micropipette, UV-VIS spectrophotometer and incubator.

2.2 Research method

The methods used in this research were descriptive method and experimental method. Descriptive method was used in isolation and identification of chitinolytic bacteria from shrimp shells. Experimental method was used for optimization of glucosamine production by fermentation using chitinolytic bacteria isolate.

2.2.1 Preparation of chitin from shrimp shells

Preparation of chitin from shrimp shells was based on spectrophotometry method (Puspawati and Simpen, 2010). The process of chitin preparation consisted of three stages, i.e. preparation of shrimp shells powder, demineralization step using HCl and deproteination step using NaOH. Preparation of shrimp shells powder was performed by washing Vannamei shrimp shells from dirt, sun drying and size reducing using a blender, followed by sieving using a 60-mesh sieve. The sieving result was Vannamei shrimp shells powder.

Vannamei shrimp shells powder was added with 1.5 M HCl with a ratio of 1:15 (w/v). The mixture was heated at 70-80^oC for 4 hrs while stirred at 50 rpm and then filtered. Solid obtained was washed using aquadest to eliminate the remaining HCl, followed by drying using 70^oC oven for 24 hrs and cooling down to obtain demineralized shrimp shells powder.

Demineralized shrimp shells powder was put inside beaker glass and added with 3.5% NaOH with a ratio of 1:10 (w/v) and then heated at 65-70^oC for 4 hrs while stirred using magnetic stirrer at 50 rpm. The mixture was then filtered and cooled down to obtain solid or chitin residue. The chitin residue was then washed using aquadest until neutral pH was reached and dried using the oven at 40^oC.

2.2.2 Isolation and identification of chitinolytic bacteria from rotten shrimp shells

Isolation of chitinolytic bacteria was done on Vannamei shrimp shells that were spoiled. The process was done by putting fresh shrimp shells in a closed container and left to rot naturally. The decay process was done for 7 days at room temperature.

Isolation of chitinolytic bacteria was done by diluting rotten shrimp shells until $10^{-7}$and growing them inside the chitin-containing media by pour plate method to obtain separated colonies that formed a clear zone (Apriani, 2008). Chitin media was prepared from NA media added with 2% colloidal chitin, 10% tartaric acid to reach pH of 4.7 (Agrawal and Kotasthane, 2012), and also added with mineral salts 0.03% KH_2PO_4, 0.07%

K_2HPO_4, 0.01% $MgSO_4.7H_2O$, and 0.7% $(NH_4)_2SO_4$ (Woo *et al.,* 1996; Halimahtussadiyah *et al.,* 2017). The separated colonies which formed clear zone was taken as chitinolytic bacteria isolates, streaked on slant agar, purified using quadrant streak method to obtain pure chitinolytic bacteria isolate. The isolates were measured for its clear zone diameter and identified molecularly using PCR (Polymerase Chain Reaction) (Gibbs, 1990).

2.2.3 Optimization experiment of glucosamine production using RSM

Chitinolytic bacteria used in the research that was also optimized for their glucosamine production were the isolates that produced the largest clear zone diameter. The optimum response was calculated from free variables, i.e. pH (A) and fermentation period (B). Experimental level range for pH was 4.5 (low level (-1)), 6.0 (center point (0)) and 7.5 (high level (+1)), whereas experimental level range for fermentation period was 5 days (low level (-1)), 10 days (center point (0)) and 13 days (high level (+1)). Experimental matrix obtained from Software design expert can be seen in Table 1.

Table 1. Experimental design using RSM

Run	pH	Time (days)
1	3.88	10.00
2	7.50	5.00
3	6.00	10.00
4	6.00	17.07
5	6.00	17.07
6	3.88	10.00
7	4.50	5.00
8	4.50	15.00
9	6.00	10.00
10	6.00	2.93
11	4.50	15.00
12	7.50	5.00
13	7.50	15.00
14	6.00	10.00
15	8.12	10.00
16	7.50	15.00
17	6.00	2.93
18	6.00	10.00
19	6.00	10.00
20	4.50	5.00
21	8.12	10.00

2.2.4 Glucosamine analysis using spectrophotometer

Glucosamine analysis was done based on analysis spectrophotometry method (Utami, 2014). Stages of analysis started with the preparation of PTH (Phenylthiourea) standard solution, followed by measurement of glucosamine absorbance in the sample at a wavelength of 584 nm and calibration of measurement results using PTH standard.

Preparation of PTH standard solution consisted of two stages, i.e. preparation of N-acetyl-D-glucosamine standard solution and PITC solution. Preparation of N-acetyl-D-glucosamine standard solution was done by weighing 0.01 gram of N-acetyl-D-glucosamine standard and diluting it into 10 ml of 0.25 M CH_3COONa. The solution was left at room temperature to stabilize it and 1000 ppm of N-acetyl-D-glucosamine standard stock solution was obtained. This stock solution was then diluted into 3, 6, 9 and 15 ppm solution. PITC solution was prepared with similar steps of the preparation of N-acetyl-D-glucosamine standard solution, with methanol as its solvent. After these two solutions were prepared, 10 ml of N-acetyl-D-glucosamine standard solution was mixed with 10 mL of PITC solution and then the mixture was homogenized by shaking it for about 5 minutes. This process was repeated in each concentration of solutions.

The reaction between N-acetyl-D-glucosamine and PITC produced a derivate compound, i.e. phenylthiourea (PTH), that can be measured for its absorbance at wavelength of 584 nm on each concentration. Linear regression between absorbance and glucosamine concentration resulted in glucosamine standard curve or equation which was later used to count the glucosamine concentration obtained from the fermentation process.

Measurement of glucosamine concentration obtained from fermentation (sample) was done by taking 1 gram of sample and diluting it with 0.25M CH_3COONa to obtain the concentration of 10,000 ppm. This mixture was further diluted subsequently to obtain a concentration of 100 ppm and then reacted with PITC, homogenized for about 5 mins and read for its absorbance. The absorbance of the sample was then calibrated using PTH standard curve and its linear regression.

3. Results and discussion

3.1 Characteristics of shrimp shells' chitin

Chitin that was used in fermentation by chitinolytic bacteria isolates has several characteristics, i.e. moisture content of 6.69%, ash content of 1.42% and deacetylation degree of 35.16%.

Deacetylation degree obtained shows the purity of chitin, in which the higher the deacetylation degree of chitin shows more acetyl group that is released and freer active amine ($-NH_2$), resulted in lower purity (Afridiana, 2011). However, chitin used in fermentation has deacetylation degree of 35.16% and can be concluded that it is in accordance with commercial chitin standard (Subasinghe, 1999) and laboratory standard of 15-70% (Bastaman *et al.*, 1990) and suitable to be used in fermentation to obtain glucosamine. According to Martati *et al.* (2007), the deacetylation degree is influenced by the demineralization process, i.e. time and temperature. The higher the temperature and the longer

the process of demineralization with alkaline (NaOH) can increase deacetylation so that chitin turns into chitosan. The degree of deacetylation of 35.16 chitin of shrimp skin showed that the chitin content was still high and had not yet become chitosan.

Based on moisture content, chitin obtained also meets the requirement of food grade commercial chitin, i.e. less than 10% (Subasinghe, 1999; Bastaman *et al.*, 1990). Moisture content of chitin was lower than dried shrimp shells powder. It is because immersion in the strong base and acid causes the damage of water holding ability of certain protein structure (Novian, 2005; Saleh *et al*., 1994).

Ash content of chitin has also met the requirement of food grade commercial chitin, i.e. mineral content less than 2.5%. Knorr (1991) stated that the main mineral in shrimp shells are $CaCO_3$ and $Ca_2(PO)_4$ in a lesser amount. The mineral was discarded from the matrix by HCl because of reaction between mineral and HCl, causing it to be easily dissolved and removed.

3.2 Identification of chitinolytic bacteria isolates

Chitinolytic bacteria is characterized by clear zone formed surrounding the colonies. A clear zone was formed because there is a bacterial activity in degrading chitin into N-acetyl glucosamine monomers (Purkan *et al.,* 2014; Pratiwi *et al.,* 2015). In shrimp shells that were spoiled in a closed container, there were three colonies found that could form clear zone and were then isolated to measure its chitinolytic activity based on clear zone formed after 24 hrs of incubation. Isolate 101CU is a rod -shape, Gram-positive bacteria which forms clear zone diameter of 8.26±0.13 mm, whereas isolate 102CU is rod -shaped, Gram-negative bacteria which form clear zone diameter of 7.55±0.07 mm and isolate 103CU is also rod -shape, Gram-negative bacteria which form clear zone diameter of 7.09±0.56 mm. The isolate with the largest clear zone formed was the molecularly identified and then optimized for its glucosamine production by fermentation method.

Based on the clear zone diameter formed, isolate 101CU was chosen to be molecularly identified based on 16S rDNA, using electrophoresis and PCR (Polymerase Chain Reaction). 16S rDNA gene fragments isolate 101CU measuring 1380 bp were sequenced using *Sequence Scanner software* and matched with databases from gene banks. The sequencing of the 16S rDNA fragment was uploaded through the *Basic Local Alignment Search Tool* (BLAST) program on the ncbi.nlm.nih.gov website to obtain sequence alignments to determine its homology with 16S rDNA sequences of other bacteria that had been registered in the gene bank database. The results of electrophoresis 16S rDNA gene sequence of isolate 101CU can be seen in Figure 1.

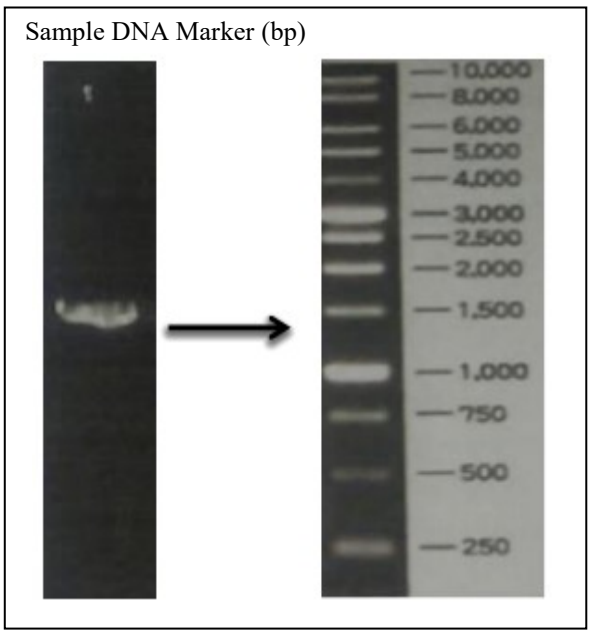

Figure 1. DNA electrophoresis result of isolate 101CU

Figure 1 shows that isolate 101CU has a thick DNA band with a length of 1380 bp which indicates the good DNA purity (Herison *et al.,* 2003). Based on BLAST, isolate 101CU has 100% similarity with 16S ribosomal RNA of *Bacterium* 4H106. Furthermore, 16S rDNA gene sequence data from isolate was compared to several 16S rDNA gene sequence data from other *Bacterium* species to obtain phylogenetic tree of *Bacterium* as can be seen in Figure 2. These microorganisms are similar to bacteria associated with skeletal tissue growth anomalies in the coral *Platygyracarnosus* (Chiu *et al.,* 2012).

Bacterium species increases the amount of chitinolytic microorganisms previously found, such as *Trichoderma harzianum* (Hardoko *et al.*, 2017), *Pseudomonas* sp. TNH-54 (Wirawan and Herdyastuti, 2013), *Pseudomonas* sp*., Aeromonas* sp*., Xanthomonas* sp*., Serratia* sp*., Cytophaga* sp*., Arthrobacter* sp*., Bacillus* sp. (Purkan *et al*., 2014), *Serratia marcescens* (Apriani, 2008), *Cytophaga-Flavobacteria*, *Chitiniphiluss hianonensis, Acinetobacter* sp., *Bacillus badius, Acinetobactervenetianus* and *Brevibacillus borstelensis* (Beier and Bertilsson, 2013; Krithika and Chellaram, 2016).

3.3 Optimization of N-acetyl-D-Glucosamine production by fermentation using bacterium4h106

Optimization of glucosamine production was done by fermentation using isolate 101CU with the treatment of pH (A) and fermentation period (B). N-acetyl-glucosamine concentration data obtained based on pH and fermentation period treatment was processed using *software Design Expert 10.0.* Mathematical equation obtained was Y = 2.38 + 10016.78A + 514.61B +

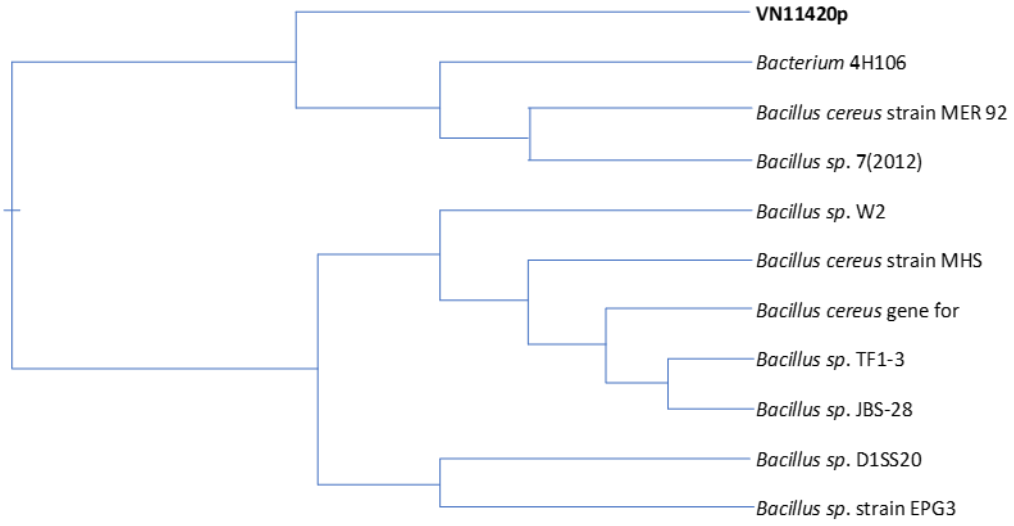

Figure 2. Phylogenetic tree of *Bacterium* 4H106 based on 16S ribosomal RN

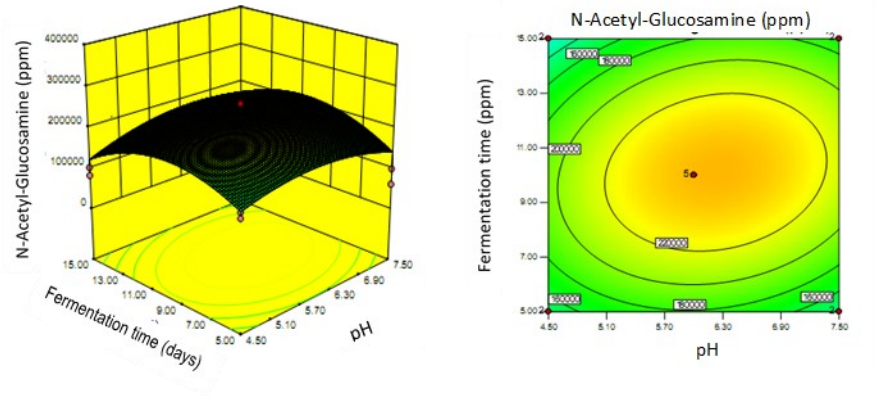

Figure 3. 3D and 2D surface response graphs of N-acetyl-glucosamine concentration

13166.50AB -34020.99A^2 – 59437.62B^2 and surface response graph can be observed in Figure 3.

If the optimum point on Figure 3 is drawn to the pH and the fermentation period axis, it results in the optimum treatment to obtain the highest concentration of N-acetyl-glucosamine. The optimum treatment was a fermentation period of 10 days and a pH of 6.0. If this result is plotted into a mathematical equation from RSM, then glucosamine concentration obtained was about 228,667.00 ppm. Based on RSM optimum equation, the interaction between pH and fermentation period that results in the highest concentration of N-acetyl-glucosamine is at pH 6.2 and fermentation period of 10.1 days with a concentration of 239,429.96 ppm. Meanwhile, the lowest is at pH of 7.5 and fermentation period of 5 days with a concentration of 79,333.50 ppm. Glucosamine concentration obtained in this research is much higher compared to fermentation using *Monascus purpureus*, i.e. about 11,830 ppm (Samuel, 2018) and fermentation using *Trichoderma harzianum*, i.e. about 127,000 ppm (Hardoko *et al.*, 2017). The difference in glucosamine obtained from fermentation can be influenced by several fermentation conditions, such as pH, temperature and fermentation period, and nutrients available for the microorganisms. According to Nasrah *et al.* (2013), the biomass of chitinolytic bacteria's growth depends on nutrients available inside the media. The nutrients that are important to support the growth of chitinolytic bacteria are carbon and nitrogen, which come from sucrose and yeast extract.

4. Conclusion

In shrimp shells that were spoiled in a closed container, there were three chitinolytic bacteria isolates obtained. The isolate that forms the largest clear zone diameter was rod-shape and Gram-positive bacteria, with DNA of 1380 bp, and was identified as *Bacterium* 4H106 based on 16S ribosomal DNA. The optimum point of N-acetyl-glucosamine production from chitin fermentation using *Bacterium* 4H106 was obtained at pH treatment of 6.0 and fermentation period of 10 days, with a concentration of 239,429.96 ppm.

Conflict of Interest

Authors have no conflict of interest.

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