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Research Article

Immobilization of *Providencia stuartii* Cells in Papaya Trunk Wood for N-acetylglucosamine Production from *Pennaeus vannamei* Shrimp Shells

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Abstract

Chitin is a natural compound found abundantly in shrimp shells. Chitin can be degraded to produce N-acetylglucosamine, which has wide applications in the food and pharmaceutical fields. Fermentation using chitinolytic microorganisms can be used to produce N-acetylglucosamine from shrimp shells' chitin. One of the strong chitinolytic bacteria that was isolated from previous research was *Providencia stuartii*. To provide better stability and efficiency in fermentation, *P. stuartii* cells were immobilized using entrapment method in papaya trunk wood. The aims of this research were to determine the optimum papaya trunk wood size, ratio of papaya trunk wood and growth medium, as well as the optimum fermentation cycle to produce N-acetylglucosamine from *P. vannamei* shrimp shells using submerged fermentation method. The research used experimental method with treatment of different sizes of papaya trunk wood (1 x 1 x 1 cm^3, 1.5 x 1.5 x 1.5 cm^3, and 2 x 2 x 2 cm^3), different ratio of papaya trunk wood and growth medium (1:10, 1:15 and 1:20), and 4 fermentation cycles. Results showed that papaya trunk wood with size of 1 x 1 x 1 cm^3 and ratio (w/v) of 1:10 could immobilize 87.08±2.05% of *P. stuartii* cells and produce the highest N-acetylglucosamine concentration, which was 238177.78±3153.48 ppm. The highest N-acetylglucosamine production was obtained from first fermentation cycle and decreased over the last three cycles, but still produced high concentration of N-acetylglucosamine. Therefore, it is possible to perform continuous N-acetylglucosamine production from shrimp shells using *P. stuartii* cells immobilized in papaya trunk wood.

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1. Introduction

Approximately 70% of the captured fisheries are utilized for food processing and a significant amount of the processed fisheries resulted as waste (Kim and Mendis, 2006). In crustaceans such as crab and shrimp, the waste makes up approximately 45% of the crustaceans. These chitinous crustacean waste are hazardous to the environment due to the high perishability and high polluting effect if discarded as it is without any processing. In the sea, the crustacean waste rapidly lead to high biochemical oxygen demand (BOD), while on land the waste quickly become colonized by pathogens and spoilage organisms resulted in environmental issue and public health concerns (Kim, 2011). One of the most commonly shrimp species found in Indonesia is *P. vannamei* or white leg shrimp (Uno *et al.,* 2010). Its cultivation became popular in Indonesia because it is more disease-tolerant, it has better survival and grow more rapidly compared to other species (Amelia *et al.,* 2021). Nowadays, shrimp waste is only used as animal feed supplementation or simply discarded (Hongkulsup *et al.*, 2016). Meanwhile, shrimp and crab shells are the main natural sources of chitin or poly β-(1-4)-N-acetyl-D-glucosamine, an important natural polysaccharide, which have been used widely for biomedical research and pharmaceutical applications, such as for wound healing, drug delivery system, and cancer diagnosis (Younes and Rinaudo, 2015; Elieh-Ali-Komi and Hamblin, 2016). Several researches have utilized shrimp shells as source of chitin (Hongkulsup *et al.,* 2016; Liu *et al.,* 2020).

About half of shrimp shells is composed of chitin (Bhattachrya *et al*., 2007). N-acetylglucosamine is a derivative from chitin that is commercially used to treat joint damage or osteoarthritis (Kubomura *et al*., 2017) and is widely researched as a potential candidate to treat inflammatory bowel disease (IBD) (Chen *et al*., 2010). It also has wide applications in food, cosmetics, and pharmaceutical industries (Liu *et al*., 2013; Zhou *et al*., 2019). Traditionally, N-acetylglucosamine is produced from chitin by chemical hydrolysis using strong acids, which can cause severe environmental problems and has potential risk of allergic reactions (Liu *et al.*, 2019; Kim *et al.*, 2018; Arbia *et al*., 2013). Enzymatic method to produce N-acetylglucosamine has also been done. However, using enzyme is costly and as an alternative, fermentation method can be used, because during the fermentation, chitinolytic microorganisms can produce chitinase (Brzezinska *et al*., 2014). Microbial fermentation offers high reproducibility in shorter time, simpler process manipulation, smaller solvent consumption, lower energy input, and environmental-friendly (Younes and Rinaudo, 2015). Furthermore, to increase the fermentation efficiency, immobilized cells are preferred compared to free cells. For industrial purposes, the use of immobilized cells using entrapment method in continuous fermentation is more advantageous compared to batch fermentation (Niknezhad *et al.*, 2016) as it offers better stability, reusability, and adaptability for scale-up production (Adinarayana *et al.*, 2005; Jiang *et al*., 2013)

Previous research by Hardoko *et al.* (2020) has successfully isolated some chitinolytic bacteria from rotten shrimp shells, with the strongest chitinolytic properties possessed by *P. stuartii*. Immobilization of *P. stuartii* cells can be used as an alternative way to suppress the cost of using enzyme to produce N-acetylglucosamine and allows the repeated use of *P. stuartii* cells for N-acetylglucosamine production from shrimp shells' chitin.

Cell or enzyme immobilization has been performed using several supports, such as agar, calcium alginate (Cheba, 2011; Niknezhad *et al*., 2016), cellulose, starch, agarose, chitosan, and proteins such as gelatin and albumin (Malmiri *et al.,* 2012). However, the use of natural polymeric gels is limited because of their mechanical strength, resulting in leakage and release of cells in the medium (Cheba, 2011; Iqbal and Saeed, 2005). This suggests the importance of finding natural structural materials as supports for cell immobilization, as they offer better mechanical strength and open spaces inside the matrix for cell growth (Iqbal and Saeed, 2005). Several natural supports have been used to immobilize cells for fermentation purpose, such as corn cobs (Genisheva *et al*., 2011), biochar (Kyriakou *et al*., 2020) for ethanol production, and grapefruit peel for beer fermentation (Estela-Escalante *et al*., 2020).

Trunk wood of plants, such as papaya can be used for the cell immobilization using entrapment method. According to Iqbal and Saeed (2005), papaya trunk wood has a structural fibrous network (SFN) with promising potential to be the material for the cell entrapment. Additionally, papaya trunk wood is very cheap and abundantly available. Several microorganisms have been immobilized in papaya trunk wood, i.e., *Aspergillus niger* (Osho *et al.,* 2014), *Aspergillus tamarii* (Maduka *et al*.,

2019), and *Aspergillus tereus* (Iqbal and Saeed, 2005). However, the use of papaya trunk wood for bacterial cell immobilization has never been done before.

This research was conducted based on the fact that shrimp shells are available abundantly and often discarded as waste while actually they contain high amount of chitin that can be further processed into N-acetylglucosamine. In this research, *P. vannamei* shrimp shells were used to produce N-acetylglucosamine using *P. stuartii* cells that were immobilized by entrapment method in papaya trunk wood using repeated submerged fermentation. The purpose of this research was to determine the optimum size and ratio of papaya trunk wood and growth medium, as well as the optimum fermentation cycles to produce N-acetylglucosamine through submerged fermentation from *P. vannamei* shrimp shells.

2. Materials and Methods

2.1 Materials

The main materials used in this research were the shells of *P. vannamei* shrimp bought from PT. First Marine Seafood, Jakarta, Indonesia, papaya trunk wood bought from *Kebun Botani* (Lebak, Banten, Indonesia), and the culture of *P. stuartii* which was isolated from rotten shrimp shells from the previous research by Hardoko *et al.* (2020). The chemicals and media used in this research were distilled water (Amidis), N-acetylglucosamine standard (Sigma Aldrich, USA), medium *Nutrient Agar* (NA; Merck, Germany), medium *Nutrient Broth* (NB; Merck, Germany), methylene blue (Merck, Germany), Bovine Serum Albumen (BSA; Merck, Germany), Coomasie Brilliant Blue G-250 (Sigma Aldrich, USA), potassium dihydrogen phosphate (KH_2PO_4; Merck, Germany), dipotassium phosphate (K_2HPO_4; Merck, Germany), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$; Merck, Germany), ammonium sulfate ($(NH_4)_2SO_4$; Merck, Germany), pH indicator universal (Merck, Germany), and pH buffer 7 (Merck, Germany).

The equipment used in this research were miller (FCT-Z200, Fomac, Indonesia), oven (UNE 800, Memmert, Germany), waterbath (WNB 14, Memmert, Germany), pH meter (Metrohm 744, Germany), analytical balance (PA214C, Ohaus, Germany), haemocytometer (Neubauer, Brand, Germany), incubator (BE 600, Memmert, Germany), fume hood (EFH-4A1, Esco, Singapore), autoclave (HVE-50, Hirayama, Japan), muffle furnace (Thermolyne 62700, Thermo Fischer Scientific, USA), micropipette (Finnpipette F2, Thermo Fischer Scientific, USA), laminar air flow (ECH-4, Esco, Singapore), centrifuge (MPW-223e, Poland), UV-Vis spectrophotometer (Genesys 10S, Thermo Fischer Scientific, USA), quartz cuvette (Spectra Cuvette; T-Bota Scietech, China), Buchner pump (DOA-P504-BN; GAST, USA), SEM (Scanning Electron Microscopy) (JSM-6510 LA, JEOL, Japan), and glasswares (IWAKI Pyrex, Japan).

2.2 Methods

2.2.1 Preparation of P. vannamei shrimp shells powder

The preparation of *P. vannamei* shrimp shells powder was performed according to the method by Halim *et al.* (2018). The shells of *P. vannamei* were washed with clean water and dried under the sun for two days. The dried shrimp shells were crushed into shrimp shells powder using miller and sieved with 60-mesh sieve. The shrimp shell powder then was analyzed, i.e., moisture content using oven drying method (AOAC, 2005), yield (Demir *et al.*, 2016), ash content using dry ashing method (AOAC, 2005), and protein content using Bradford method (Nielsen, 2010).

2.2.2 Pre-treatment of papaya trunk wood

Papaya trunk wood was pre-treated according to method by Iqbal and Saeed (2005). Papaya trunk wood was firstly cut into 3 different sizes, which were 1 x 1 x 1 cm^3, 1.5 x 1.5 x 1.5 cm^3, and 2 x 2 x 2 cm^3. Papaya trunk wood cubes were then boiled for 30 min and washed thoroughly 3 times with running water. Afterwards, they were oven-dried at 80°C for 48 h. The pre-treated papaya trunk wood cubes were then treated with alkaline peroxide treatments to remove their lignin content.

The alkaline peroxide treatment was conducted according to Selig *et al.* (2009) with modification. Each 40 g of papaya trunk wood cubes were treated with 10 ml of 30% hydrogen peroxide and 950 ml of distilled water. The mixture was adjusted for its pH until 11.5 with 10 M sodium hydroxide and heated (65°C) for 3 h with periodic stirring. The cubes were then dried in oven for 24 h at 90°C and autoclaved at 121°C for 15 min before used. The treated papaya trunk wood was used as an immobilization matrix for *P. stuartii* cells.

2.2.3 Immobilization of P. stuartii cells in papaya trunk

wood cubes

P. stuartii cells (1 ml) were first inoculated into growth medium. The growth medium was prepared by mixing 300 ml of NB medium with 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, and 0.7% $(NH_4)_2SO_4$ (Halim *et al.*, 2018). Furthermore, pH of medium was adjusted to 7.0 as it is the optimum pH for the growth of *P. stuartii* (Ayangbenro, 2017). Papaya trunk wood cubes with different sizes as stated before were then submerged in the growth medium with ratio (w/v) of 1:10, 1:15, and 1:20. Cubes were then incubated for 2 hours at 37°C in the growth medium. Afterwards, the number of immobilized cells was calculated by subtracting the number of unimmobilized cells from the initial cell numbers. The initial cells number was the number obtained from the bacterial cell directly counted using haemocytometer and the amount of unimmobilized cells were represented by the amount of the living cells left in the growth medium (Saparianti, 2012 with modification). As much as 1 ml of the leftover growth medium was taken and added with 1 ml of methylene blue (1:5). The suspension was homogenized with vortex and rested for 10 minutes. Furthermore, 0.1 ml of the suspension was taken and put into the haemocytometer for direct counting under the microscope.

To determine the optimum size of papaya trunk wood and ratio between papaya trunk wood and growth medium, submerged fermentation using immobilized cells was carried out. Fermentation medium was prepared according to Halim *et al.* (2018) with modification. The medium consisted of 10% of *P. vannamei* shrimp shells powder (w/v), 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, and 0.7% $(NH_4)_2SO_4$ as nitrogen source in 300 ml distilled water. Additionally, fermentation medium was also adjusted with buffer solution until the pH reached 7. The fermentation medium was then autoclaved at 121°C for 15 min. The fermentation was carried out for 4 days at 37°C with periodic shaking.

In order to obtain the N-acetylglucosamine as the fermentation result, the media was heated at 70°C for 45 minutes to stop the fermentation process by inactivating the *P. stuartii* cells. According to Manos and Belas (2006), *Enterobacteriaceae* family will be destroyed when heated at minimum temperature of 63°C for 30 minutes. Afterwards, centrifugation (4000 rpm, 15 min) and filtration using Whatmann No. 1 filter paper were done (Halim *et al.*, 2018). Filtrate containing N-acetylglucosamine was measured for its absorbance using UV-Vis spectrophotometer at wavelength of 324 nm, according to method by Halim *et al.* (2018). Based on the highest N-acetylglucosamine concentration obtained from the fermentation, the optimum papaya trunk wood size and ratio (w/v) between papaya trunk wood and growth medium were determined.

2.2.4 Optimum fermentation cycles determination

The optimum papaya trunk wood size and the optimum ratio (w/v) between papaya trunk wood to fermentation medium obtained was used to determine the optimum fermentation cycles. The similar fermentation condition (37°C, 4 days with periodic shaking) was used. At the end of each cycle, papaya trunk wood cubes were separated, and fermentation medium was heated at 70°C for 45 min to stop the fermentation. The fermentation medium was then centrifuged (4000 rpm, 15 min) and filtered using Whatmann No. 1 filter paper to obtain filtrate containing N-acetylglucosamine. The same papaya trunk wood cubes were then used for the next fermentation and the same procedure was repeated up to 4 cycles of fermentation (Halim *et al.*, 2019 with modifications).

2.2.5 Microscopical observation using Scanning Electron Microscopy (SEM)

The presence of *P. stuartii* cells immobilized in papaya trunk wood was observed through SEM analysis. Papaya trunk wood with the size of 1 x 1 x 1 cm^3 was submerged in growth medium containing *P. stuartii* cells with ratio of 1:10 (w/v). It was then incubated at temperature of 37°C for 2 h with periodic shaking every 30 min to allow immobilization of *P. stuartii* cells on the papaya trunk wood. Afterwards, the papaya trunk wood was collected from the growth medium, transferred to a sterile beaker glass, and dried in the incubator at temperature of 37°C for 24 h. The dried sample was then sent to BATAN-PUSPIPTEK for the SEM analysis.

SEM analysis started with size reduction of sample. Size-reduced sample was then put on SEM's specimen holder using carbon double tip and placed inside the vacuum machine. Sample was then vacuumed until 10^{-6} torr and sprayed with argon to increase the conductivity of sample. Sample was then analyzed in SEM with voltage setting of 15kV and work distance of 10-11 mm. The images of sample were taken using two types of detectors, i.e., SE (secondary electron) and BSE (back scattered electron).

2.3 Experimental design

The first stage research utilized the Completely Randomized Factorial Design, involving two factors, which were size of papaya trunk wood cubes (1 x 1 x 1 cm^3, 1.5 x 1.5 x 1.5 cm^3, and 2 x 2 x 2 cm^3) and ratio (w/v) between papaya trunk wood: fermentation media (1:10, 1:15, 1:20). The first stage research was conducted for three replications, with total of 27 samples. The second stage research used the Completely Randomized Factorial Design with one factor which was the optimum fermentation cycles of the immobilized cells (4 cycles). Second stage research was done in five replications, resulting in a total of 20 samples.

2.4 Data Analysis

Data analysis was done statistically with ANOVA by using the SPSS 22nd version software. Post hoc test done was Duncan's Multiple Range Test.

3. Results and Discussion

3.1 P. vannamei shrimp shells powder characteristics

P. vannamei shrimp shells powder that was used for fermentation was first analyzed for its characteristics. Yield of shrimp shells powder obtained was 22.45 ± 0.11% with moisture content of 9.99 ± 0.18% (Table 1), slightly lower than result from Percot *et al.* (2003), which was 11.3 ± 0.4%. Ash content obtained was 19.49 ± 0.34%, lower than the ash content obtained by Sanusi (2004) (27.09%) but comparable to result from Valdez-Pena *et al.* (2010) (17.85 to 26.58% ash content). Difference between the results might be due to different ratio of shrimp shells, as different part of the shrimp shells had different ash content. The protein content of shrimp shells powder was 1.16 ± 0.29%, lower than protein content obtained by Tanasale *et al.* (2006), which was 25.10%. The difference between the results might be due to different methods used to determine the protein content. Kjeldahl method is used to measure the nitrogen content, while Bradford method is used to measure the peptide bond (Nielsen, 2010). As stated by Vasquez *et al.* (2017), shrimp shells contained abundant amount of chitin that consists mainly of nitrogen, this might contribute to overestimation of the result when Kjeldahl method is used (Cheba, 2011).

3.2 Effect of papaya trunk wood size and ratio between papaya trunk wood and growth medium (w/v) on percentage of immobilized P. stuartii cells

To ensure that *P. stuartii* cells were immobilized in papaya trunk wood, number of immobilized cells was measured, and SEM analysis was performed. As an immobilization carrier, papaya trunk wood offers good mechanical properties, reusability, and plenty of open spaces for growing cells. These properties make it an ideal matrix for cell immobilization (Iqbal and Saeed, 2005). Prior to immobilization process, papaya trunk wood was pre-treated to remove its lignin content. It is because papaya trunk wood consisted of 37.33% of lignin (Saeed *et al.*, 2005) and lignin has inhibitory compounds that can affect bacterial growth (Kim *et al.*, 2016; Selig *et al.*, 2009).

Immobilized *P. stuartii* cells cannot be observed clearly at 400x magnification, however the open spaces and porosity of papaya trunk wood can be observed (Figure 1). This indicates that papaya trunk wood provides sufficient spaces for bacterial cells attachment. Moreover, many *P. stuartii* cells were attached to surface of papaya trunk wood, indicating that immobilization was done successfully (Figure 2a and Figure 2b). Based on the results obtained from SEM analysis, it could be concluded that cell immobilization using papaya trunk wood could be done in a simple method, by submerging papaya trunk wood in a growth medium containing *P. stuartii* cells for certain period, in this research was two hours of incubation.

The highest percentage of immobilized *P. stuartii* cells was 87.08±2.05% (Table 2), using papaya trunk wood with size of 1 x 1 x 1 cm^3 and submerged in the growth medium with ratio (w/v) of 1:10. It shows that different size of matrix contributes to different number of immobilized cells, in which result shows that the smallest size of papaya trunk wood could immobilize the highest number of *P. stuartii* cells. Smaller size of immobilization matrix creates larger surface area. Hence, increasing the probability of the bacteria cells to be immobilized (Hrenovic *et al.*, 2009; Wu and Lia, 2008). Other than size of immobilization matrix, the size of pores is also important (Ma *et al.*, 2006). Previous research has reported that bigger pore size of matrix shows better results enzyme immobilization (Li *et al.*, 2010). However, when the pores are too big, it could also cause leakage of the material entrapped inside the matrix (Górecka and Jastrzębska, 2011). Moreover, percentage of immobilized cells (87.08±2.05%) using papaya trunk wood in this research is also higher compared to pumice or other silica-based matrices, i.e., only about 67-72% (Pazarlioğlu and Telefoncu, 2005). Therefore, the existence of pores or open spaces between the fibers of papaya trunk wood (Iqbal and Saeed, 2005) was sufficient to immobilize higher number of cells.

Table 1. *P. vannamei* shrimp shells powder characteristics

Parameter	Amount (%)
Yield (wet basis)	22.45 ± 0.11
Moisture content (wet basis)	9.99 ± 0.18
Ash content	19.49 ± 0.34
Protein content	1.16 ± 0.29

Table 2. Percentage of immobilized *P. stuartii cells*

Papaya trunk wood size	Ratio of papaya trunk wood: growth medium (w/v)	Percentage of immobilized cells (%)
1 x 1 x 1 cm	1:10	87.08±2.05
	1:15	85.14±2.96
	1:20	78.77±2.62
1.5 x 1.5 x 1.5 cm	1:10	82.64±3.14
	1:15	76.84±3.73
	1:20	77.85±2.12
2 x 2 x 2 cm	1:10	71.84±6.44
	1:15	70.26±11.73
	1:20	80.35±6.28

Noted: results of data analysis (significance test of the each treatment should be added)

3.3 Effect of papaya trunk wood size and ratio between papaya trunk wood and fermentation medium (w/v) on N-acetylglucosamine production

In order to determine the optimum papaya trunk wood size and the ratio between the papaya trunk wood and fermentation medium (w/v), the concentration of N-acetylglucosamine as a result from chitin degradation using *P. stuartii* cells was measured. In this research, fermentation was done at the temperature of 37°C with pH of 7.0 for 4 days, as according to Hardoko *et al.* (2020) and Ayangbenro (2017), it was the optimum condition for the growth of *P. stuartii*.

Papaya trunk wood size and ratio between papaya trunk wood and fermentation medium (w/v) significantly affected the concentration of N-acetylglucosamine produced ($p \leq 0.05$). However, there was no significant interaction between papaya trunk wood size and the ratio of papaya trunk wood and fermentation medium (w/v) in the production of N-acetylglucosamine ($p > 0.05$) (Figure 3).

The smallest size of papaya trunk wood and the largest portion papaya trunk wood in the fermentation medium (ratio of 1:10) resulted in the highest concentration of N-acetylglucosamine produced i.e., 238177.78 ± 3153.48 ppm (Figure 3). It is related to the previous results that shows smaller papaya trunk wood size could immobilize higher percentage of *P. stuarti* cells, therefore more chitin is broken down into N-acetylglucosamine. Related to ratio between papaya trunk wood and fermentation medium, this can be explained as larger portion of papaya trunk wood carried more number of *P. stuartii* cells to break down chitin in shrimp shells, resulting in higher concentration of N-acetylglucosamine obtained at the end of fermentation. This result is in line with a previous research by Kuyukina *et al.* (2006), which stated that larger ratio between matrix and its immobilization medium resulted in higher number of viable cells. The fact that *P. stuartii* cells are still capable of degrading chitin to produce N-acetylglucosamine supports the previous finding by Iqbal and Saeed (2005) that papaya trunk wood is not harmful for the growth of cells.

3.4 Effect of fermentation cycles on N-acetylglucosamine production

The immobilization using papaya trunk wood was aimed to observe the possibility of repeated use of *P. stuartii* cells in fermentation to produce N-acetylglucosamine.

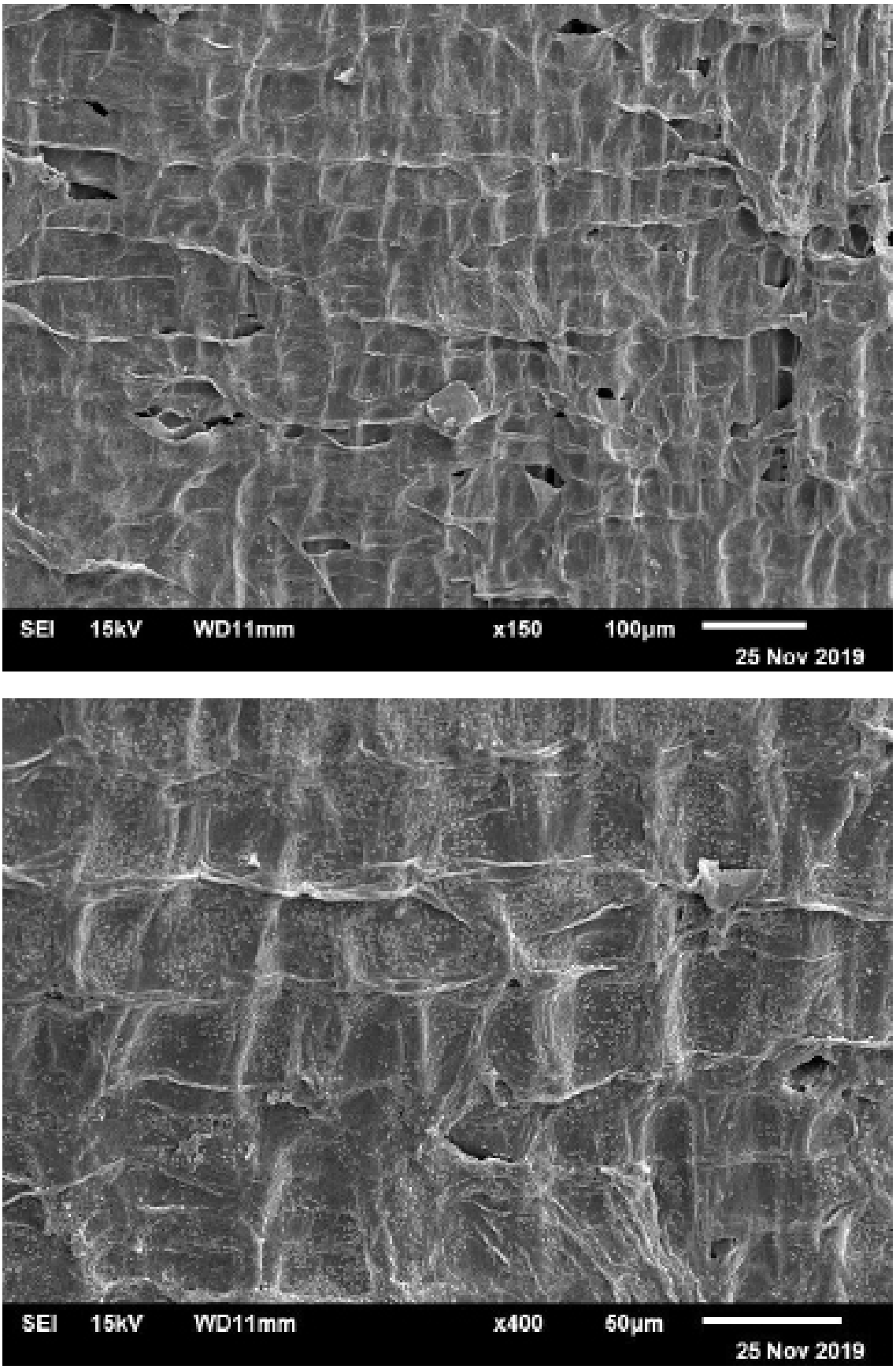

Figure 1. SEM result of papaya trunk wood structure (400x magnification) with size of 1 x 1 x 1 cm^3 and ratio (w/v) of 1:10 between papaya trunk wood and growth medium

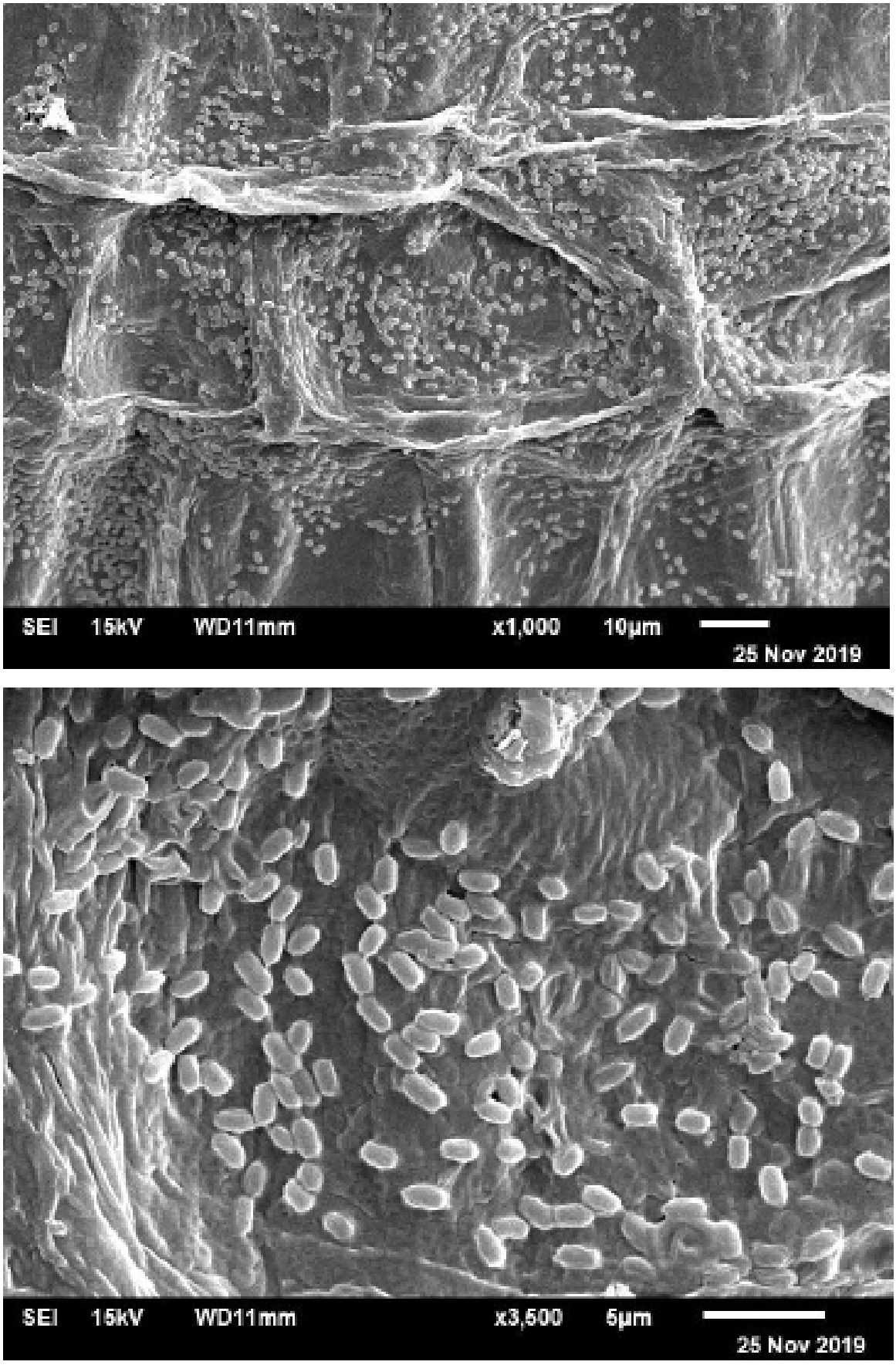

Figure 2. SEM result of P. stuartii cells immobilized in papaya trunk wood (a: 1000x magnification; b: 3500x magnification) with size of 1 x 1 x 1 cm^3 and ratio (w/v) of 1:10 between papaya trunk wood and growth medium

As a result, this could increase the utilization of shrimp shells into products with higher economic value. Related to immobilization, Eş *et al.* (2015) stated that the aims of immobilization are to overcome process restriction, improve the reusability, offer better stability, eliminate unnecessary purification steps, and ultimately to produce the desire products with greater efficiency. To observe this possibility, 4 cycles of fermentation was performed and the amount of N-acetylglucosamine obtained was measured after each cycle. Each fermentation cycle was conducted for 4 days with the temperature of 37°C and pH of 7.0.

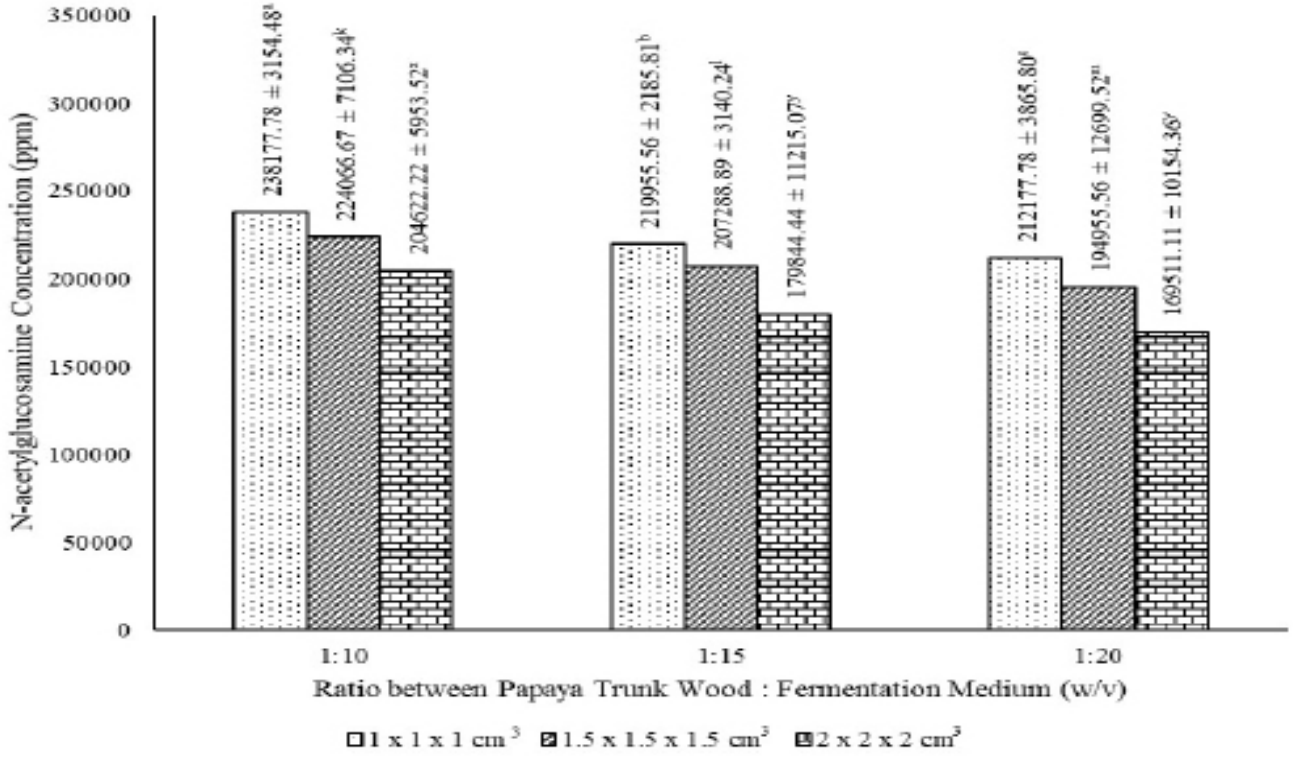

Figure 3. Effect of ratio between papaya trunk wood and fermentation medium (w/v) and papaya trunk wood size on N-acetylglucosamine concentration obtained.
Note: different letter notations on the same pattern show significant difference ($p < 0.05$)

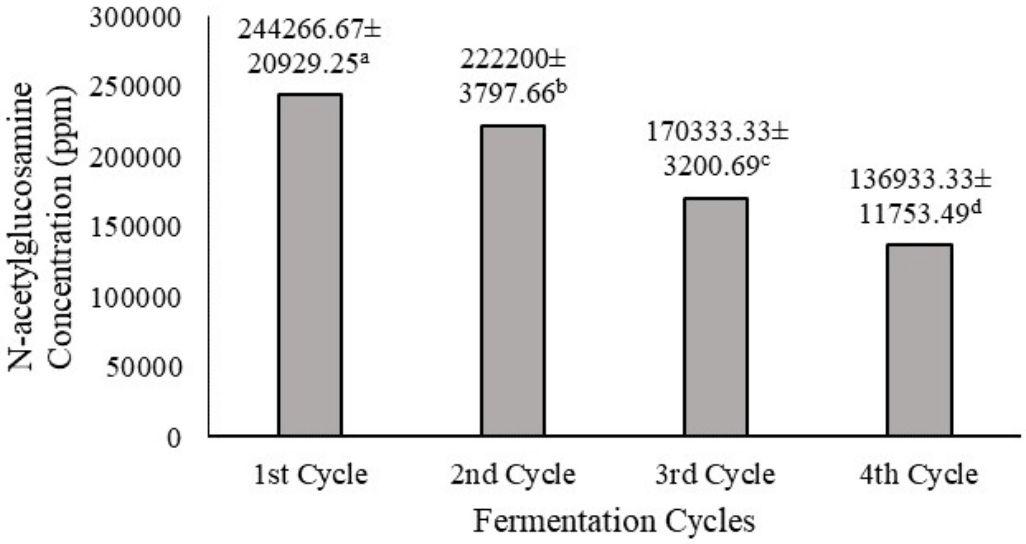

Figure 4. Effect of fermentation cycles on N-acetylglucosamine concentration obtained.
Note: significant difference ($p < 0.05$) is represented by different letter notations

Fermentation cycles significantly affected the N-acetylglucosamine production ($p \leq 0.05$). It can be seen that after 1st cycle of fermentation, the concentration of N-acetylglucosamine obtained has significantly decreased (Figure 4). The highest concentration of N-acetylglucosamine obtained was 244266.67± 20929.25 ppm and the lowest was found in the 4th cycle (136933.33 ± 11753.49 ppm). The similar trend was also observed in previous research by Zagrodnik *et al.* (2013), in which hydrogen production decreased after several cycles of fermentation using *Rhodobacter sphaeroides* bacteria immobilized in non-modified porous glasses. Halim *et al.* (2019) used calcium alginate to immobilize intracellular chitinase from *P. stuartii* and reported the similar result that concentration of N-acetylglucosamine decreased gradually after 1st cycle of fermentation, caused by the leakage of calcium alginate beads. The similar result was also reported by Adinarayana *et al.* (2005) in which calcium alginate was used to immobilize *B. subtilis* cells and enzyme production decreased after 2nd cycle of fermentation, followed by cell leakage. Although papaya trunk wood has good mechanical strength to hold bacterial cells inside, cell leakage might occur when entrapment method is used for immobilization (Martins *et al*., 2013). Cell leakage causes less immobilized cells are present in the next fermentation cycle, hence N-acetylglucosamine produced also decreased.

Although the N-acetylglucosamine produced decreases after each fermentation cycle, the concentration of N-acetylglucosamine after 4th fermentation cycle was still considered high, i.e., 136933.33± 11753.49 ppm. It is comparable to a previous research by Ayu (2019), in which fermentation of shrimp shells using free *P. stuartii* cells resulted in 178752.670 ± 1774.478 ppm of N-acetylglucosamine after 9 days of fermentation, as in this research, each cycle of fermentation was only conducted for 4 days. This result is supported by previous research by Iqbal and Saeed (2005), Jiang *et al.* (2013), and Adinarayana *et al.* (2005), who reported that immobilized cells had higher productivity compared to free cells in repeated batch fermentation.

N-acetylglucosamine produced in this research is also higher compared to Handoyo (2019) which used *Mucor circinelloides* cells immobilized in calcium alginate beads and N-acetylglucosamine produced after 4th fermentation cycle was 1253.33±61.24 ppm. This result suggests that papaya trunk wood could be a better matrix for *P. stuartii* immobilization than polymeric matrices. This result is also comparable to previous researches by Iqbal and Saeed (2005) and Saeed *et al.* (2005) which reported that papaya trunk wood could be used to immobilize *A. terreus* hyphae up to 5 cycles of fermentation. Considering the high amount of N-acetylglucosamine produce, this means that chitin from shrimp shells is still highly available after 4 cycles of fermentation, and it also means that continuous fermentation for more than for 4 cycles could be potentially applied.

4. Conclusion

P. vannamei shrimp shell is a potential source of chitin. Immobilization of *P. stuartii* cells was done successfully in papaya trunk wood and it is potential to be utilized for the continuous production of N-acetylglucosamine from *P. vannamei* shrimp shells. The optimum size of papaya trunk wood for immobilization was 1 x 1 x 1 cm^3 and the optimum ratio (w/v) of papaya trunk wood and the growth medium was 1:10, with percentage of immobilized cells was 87.08±2.05%. The immobilized *P. stuartii* cells could be used up to 4 cycles of fermentation, with the highest concentration of N-acetylglucosamine obtained was 244266.67± 20929.25 ppm and the lowest was found in the 4th cycle, i.e., 136933.33 ± 11753.49 ppm. However, further purification process to obtain pure N-acetylglucosamine is required to be performed in advanced, as well as identification of other compounds obtained from continuous fermentation process.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follows, Yuniwaty; supervised the research, prepared, and edited the manuscript. Hardoko; led the research, designed the experiment, and contributed to critical input for the article. Steven; performed the research, collected and analyzed the data, prepared the article. Ratna; interpreted the data. All authors have contributed to the research and the manuscript.

Conflict of Interest

The authors declare that there is no conflict of

interest.

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References

Adinarayana, K., Jyothi, B., & Ellaiah, P. (2005). Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. *AAPS Pharmaceutical Science Technology*, 6(3):391-397.

Amelia, F., Yustiati, A., & Andriani, Y. (2021). Review of shrimp (*Litopenaeus vannamei* (Boone, 1931) farming in Indonesia: management operating and development. *World Scientific News,* 158:145-158.

Arbia, W., Arbia, L. Adour, L., & Amrane, A. (2013). Chitin extraction from crustacean shells using biological methods - a review. *Food Technology Biotechnology,* 51(1):12-25.

Association of Official Analytical Chemist [AOAC]. (2005). Official methods of analysis of the Association of Official Analytical Chemist. Virginia: Association of Official Analytical Chemist Inc.

Ayangbenro, A. S. (2017). Biodegradation of natural bitumen by *Providencia stuartii* isolated from heavy oil contaminated soil. *NEST Journal,* 19(2):353-358.

Ayu, N. (2019). Produksi N-asetilglukosamin secara fermentasi dari tepung cangkang udang menggu- nakan *Providencia stuartii.* Bachelor Thesis. Tan- gerang, Indonesia: Universitas Pelita Harapan.

Bhattacharya, D., Nagpure, A., & Gupta, R. K. (2007). Bacterial chitinases: properties and potential. *Critical Reviews in Biotechnology,* 27(1):21-28.

Brzezinska, M. S., Jankiewicz, U., Burkowska, A., & Walczak, M. (2014). Chitinolytic microorganisms and their possible application in environmental protection. *Journal of Current Microbiology,* 68(1):71-81.

Cheba, B. A. (2011). Chitin and chitosan: marine biopolymers with unique properties and versatile applications. *Global Journal of Biotechnology & Biochemistry,* 6(3):149-153.

Chen, J-K., Shen, C-R., & Liu, C-L. (2010). N-acetylglucosamine: production and applications. *Mari- ne Drugs,* 8(9):2493-2516.

Demir, D., Fatma, O., Seda, C., & Nimet, B. K. (2016). Extraction and characterization of chitin and chi- tosan from blue crab and synthesis of chitosan cryogel scaffolds. *Journal of The Turkish Chemi- cal Society,* 3(3):131-144.

Elieh-Ali-Komi, D., & Hamblin, M. R. (2016). Chitin and chitosan: production and application of versatile biomedical nanomaterials. *International Journal of Advanced Research,* 4(3):411-427.

Estela-Escalante, W. D., Perez-Escalante, J. J., Fuentes Navarro, E. L., & Pinillos-Miñano, R. M. (2020). The potential of using grapefruit peel as a natural support for yeast immobilization during beer fermentation. *Chemical and Biochemical Engineering Quarterly,* 34(2):105-114.

Eş, I., Vieira, J. D. G., & Amaral, A. C. (2015). Principles, techniques, and applications of biocatalyst immobilization for industrial application. *Applied of Microbiology and Biotechnology,* 99(5):2065-2082.

Genisheva, Z., Mussatto, S. I., Oliveira, J. M., & Teixeira, J. A. (2011). Evaluating the potential of wine-making residues and corn cobs as support materials for cell immobilization for ethanol production. *Industrial Crops and Products,* 34(2011):979-985.

Górecka, E., & Jastrzębska, M. (2011). Immobilization techniques and biopolymer carriers. *Biotechnology and Food Science,* 75(1):65-86.

Halim, Y., Hardoko, H., Handayani, R., & Lucida, V. (2018). Optimum conditions for N-acetylglucosamine production from tiger shrimp (*Penaeus monodon*) shell by *Serratia marcescens*. *Asian Journal of Pharmaceutical and Clinical Research,* 11(12):488-493.

Halim, Y., Hendarlim, B. D., Hardoko, Handayani, R., & Rosa, D. (2019). Imobilisasi kitinase intraseluler *Providencia stuartii* dengan kalsium alginat

dan aplikasinya dalam produksi N-asetilglukosamin. *FaST-Jurnal Sains dan Teknologi,* 3(2):35-44.

Handoyo, B. C. (2019). Produksi N-asetilglukosamin dengan fermentasi menggunakan spora *Mucor circinelloides* terimobilisasi pada kalsium alginat. Bachelor Thesis. Tangerang, Indonesia: Universitas Pelita Harapan.

Hardoko, Josephine, C., Handayani, R., & Halim, Y. (2020). Isolation, identification and chitinolytic index of bacteria from rotten tiger shrimp (*Penaeus monodon*) shells. *AACL Bioflux,* 13(1):360-371.

Hongkulsup, C., Khutoryanskiy, V. V., & Niranjan, K. (2016). Enzyme assisted extraction of chitin from shrimp shells (*Litopenaeus vannamei*). *Journal of Chemical Technology and Biotechnology*, 91(5):1250-1256.

Hrenovic, J., Ivankovic, T., & Tibljas, D. (2009). The effect of mineral carrier composition on phosphate-accumulating bacteria immobilization. *Journal of Hazardous Materials,* 166(2-3):1377-1382.

Iqbal, M., & Saeed, A. (2005). Novel method for cell immobilization and its application for production of organic acid. *Letters in Applied Microbiology,* 40(3):178-182.

Jiang, L., Ruan, Q., Li, R., & Li, T. (2013). Biodegradation of phenol by using free and immobilized cells of *Acinetobacter* sp. BS8Y. *Journal of Basic Microbiology,* 53(3):224-230.

Kim, J. S., Lee, Y. Y., & Kim, T. H. (2016). A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresource Technology,* 199:42-48.

Kim, S-K., & Mendis, E. (2006). Bioactive compounds from marine processing byproducts - a review. *Food Research International*, 39(4):383-393.

Kim, S-K. (2011). Chitin, chitosan, oligosaccharides, and their derivatives: biological activities and applications. Boca Raton: CRC Press.

Kim, T. I., Lim, D. H., Baek, K. S., Jang, S. S., Park, B. Y., & Mayakrishnan, V. (2018). Production of chitinase from *Escherichia fergusonii*, chitosanase from *Chryseobacterium indologenes*, *Comamonas koreensis* and its application in N-acetylglucosamine production. *Interna- tional Journal of Biological Macromolecules,* 112:1115-1121.

Kubomura, D., Ueno, T., Yamada, M., & Nagaoka, I. (2017). Evaluation of the chondroprotective action of N-acetylglucosamine in a rat experimental osteoarthritis model. *Experimental and Therapeutic Medicine*, 14:3137-3144.

Kuyukina, M. S., Ivshina, I. B., Podorozhko, E. A., Lozinsky, V. I., Jeffree, C. E., & Philp, J. C. (2006). Immobilization of hydrocarbon-oxidizing bacteria in poly (vinyl alcohol) cryogels hydrophobized using a biosurfactant. *Journal of microbiological methods*, 65(3):596-603.

Kyriakou, M., Patsaloua, M., Xiaris, N., Tsevisc, A., Koutsokeras, L., Constantinides, G., & Koutinasa, M. (2020). Enhancing bioproduction and thermotolerance in *Saccharomyces cerevisiae* via cell immobilization on biochar: application in a citrus peel waste biorefinery. *Renewable Energy,* 155:53-64.

Li, Y., Gao, F., Wei, W., Qu, J. B., Ma, G. H., & Zhou, W. Q. (2010). Pore size of macroporous polystyrene microspheres affects lipase immobilization. *Journal of Molecular Catalysis B: Enzymatic,* 66(1-2):182-189.

Liu, L., Liu, Y., Shin, H., Chen, R., Li, J., Du, G., & Chen, J. (2013). Microbial production of glucosamine and N-acetylglucosamine: advances and perspectives. *Applied Microbiology and Biotechnology,* 97:6149-6158.

Liu, Y., Liu, L., Shin, H., Chen, R. R., Li, J., Du, G., & Chen, J. (2019). Pathway engineering of *Bacillus subtilis* for microbial production of N-acetylglucosamine. *Metabolic Engineering,* 19:107-115.

Liu, Y., Xing, R., Yang, H., Liu, S., Qin, Y., Li, K., Yu., H., & Li, P. (2020). Chitin extraction from shrimp (*Litopenaeus vannamei*) shells by successive two-step fermentation with *Lactobacillus rhamnoides* and *Bacillus amyloliquefaciens. International Journal of Biological Macromolecules,* 148:424-433.

Ma, Y. L., Yang, B. L., & Zhao, J. L. (2006). Removal of H_2S by *Thiobacillus denitrificans* immobilized on different matrices. *Bioresource Technology,*

97(16):2041-2046.

Maduka, N., Emecheta, R. O., Ahaotu, I., Maduka, N., & Odu, N. N. (2019). Proximate composition of date fruits and sensory evaluation of date fruit wine clarified using *Aspergillus* strains immobilized on biomatrix. *Science Park Journal of Applied Microbiology Research*, 4(2):67-79.

Malmiri, H. J., Jahanian, M. A. G., & Berenjian, A. (2012). Potential applications of chitosan nanoparticles as novel support in enzyme immobilization. *American Journal of Biochemistry and Biotechnology,* 8(4):203-219.

Martins, S. C. S., Martins, C. M., Fiuza, L. M. C. G., & Santaella, T. (2013). Immobilization of microbial cells: a promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology,* 12(28):4412-4418.

Manos, J., & Belas, R. (2006). The Genera *Proteus, Providencia*, and *Morganella*. In: M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt (Eds.), The Prokaryotes. (pp 245-269). New York: Springer.

Nielsen, S. (2010). Food analysis (4th ed). New York: Springer Science+Business Media, LLC.

Niknezhad, S. V., Asadollahia, M. A., Zamani, A., & Biria, D. (2016). Production of xanthan gum by free and immobilized cells of *Xanthomonas campestris* and *Xanthomonas pelargonii. International Journal of Biological Macromolecules,* 82:751-756.

Osho, M. B., Popoola, T., & Kareem, S. O. (2014). Immobilization of *Aspergilus niger* ATCC 1015 on bionatural structures for lipase production. *Engineering in Life Sciences,* 14:449-454.

Pazarlioğlu, N. K., & Telefoncu, A. (2005). Biodegradation of phenol by *Pseudomonas putida* immobilized on activated pumice particles. *Process Biochemistry*, 40(5):1807-1814.

Percot, A., Viton, C., & Domard, A. (2003). Optimization of chitin extraction from shrimp shells. *Biomacromolecules,* 4(1):12-18.

Saeed, A., Akhter, M. W., & Iqbal, M. (2005). Removal and recovery of heavy metals from aqueous solution using papaya wood as a new biosorbent. *Separation and Purification Technology,* 45(1):25-31.

Sanusi, M. (2004). Transformasi kitin dari hasil isolasi limbah industri udang beku menjadi kitosan. *Marina Chimica Acta*, 5(2):28-32.

Saparianti, E. (2012). Amobilisasi sel *Pediococcus acidilactici* F11 penghasil bakteriosin pada gel kalsium alginat. *Jurnal Teknologi Pertanian.* 2(1):1-9.

Selig, M. J., Vinzant, T. B., Himmel, M. E., & Decker, S. R. (2009). The effect of lignin removal by alkaline peroxide pretreatment on the susceptibility of corn stover to purified cellulolytic and xylanolytic enzymes. *Applied Biochemistry and Biotechnology,* 155(1-3):94-103.

Tanasale, M. F. J. D. P., Killay, A., & Saily, M. (2006). Kitosan dari limbah udang windu (*Penaeus monodon*) sebagai adsorben fenol. *Alchemy Jurnal Penelitian Kimia,* 5(1):23-30.

Uno, K., Chaweepack, T., & Ruangpan, L. (2010). Pharmacokinetics and bioavailability of oxytetracycline in vannamei shrimp (*Penaeus vannamei*) and the effect of processing on the residues in muscle and shell. *Aquaculture International,* 18(6):1003-1015.

Valdez-Peña, A. U., Espinoza-Perez, J. D., Sando- val-Fabian, G. C., Balagurusamy, N., Hernan- dez-Rivera, A., De-la-Garza-Rodriguez, I. M., & Contreras-Esquivel, J. C. (2010). Screening of industrial enzymes for deproteinization of shrimp head for chitin recovery. *Food Science and Biotechnology,* 19(2):553-557.

Vasquez, J. A., Ramos, P., Miron, J., Valcarcel, J., Sotelo, C., & Perez-Martin, R. (2017). Production of chitin from *Penaeus vannamei* by-products to pilot plant scale using a combination of enzymatic and chemical processes and subsequent optimization of the chemical production of chitosan by response surface methodology. *Marine Drugs,* 15(6):180.

Wu, S. C., & Lia, Y. K. (2008). Application of bacterial cellulose pellets in enzyme immobilization. *Journal of Molecular Catalysis B: Enzymatic,* 54(3-4):103-108.

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources, structure, properties, and applications. *Marine Drugs,*

13(3):1133-1174.

Zagrodnik, R., Thiel, M., Seifert, K., Włodarczak, M., & Łaniecki, M. (2013). Application of immobilized *Rhodobacter sphaeroides* bacteria in hydrogen generation process under semi-continuous conditions. *International Journal of Hydrogen Energy,* 38(18): 7632-7639.

Zhou, D., Jiang, Z., Pang, Q., Zhu, Y, Wang, Q., & Qingsheng, Q. (2019). CRISPR/Cas9-assisted seamless genome editing in *Lactobacillus plantarum* and its application in N-acetylglucosamine production. *Applied and Environmental Microbiology,* 85(21):1-11.