Isolation, identification and chitinolytic index of bacteria from rotten tiger shrimp (*Penaeus monodon*) shells

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Abstract. Tiger shrimp shells contain high amount of chitin, protein and mineral that make them easily spoiled. Because of its high chitin content, many of its spoilage microorganisms have chitinolytic properties. The aim of this research was to obtain bacteria with strong chitinolytic activity that can be further used to ferment chitin into glucosamine. Research method used was descriptive method, consisted of decay of shrimp shells, isolation of chitinolytic bacteria that formed purple colour inchitin-containing agar media, chitinolytic index test and identification of the strongest chitinolytic bacteria based on order of rDNA 16S. Results show that there were 17 chitinolytic bacteria isolated from rotten tiger shrimp shells, 8 of which had strong chitinolytic properties. Isolate A12 was chosen as the strongest chitinolytic bacteria with chitinolytic index of 4.42 and lightness of purple colour of 23.88. Isolate A12 was identified as *Providencia stuartii*.
Key Words: chitinolytic bacteria, chitin, spoil, isolate.

Introduction. Based on KKP (2014), export of tiger shrimp (*Penaeus monodon*) in Indonesia is about 24% from the total production. Frozen shrimp are exported without their shells and head, which are about 25-50% of shrimp parts (Dompeipen et al 2016). Based on data from General Directorate of Strengthening Competitiveness of Marine and Fishery Products (Ditjen PDSPKP 2015), Jakarta area produces about 100 tons of dried shrimp shells or equal to 13 tons of chitins, which is about 15-20% of total shrimp shells (Paul 2015).

Chitin is a linear chain of long polysaccharides which comprises of N-acetyl-D-glucosamine monomers that are linked by β-1.4 linkage (Einbu 2007). Depolymerization of chitin by hydrolyzing its chitin glycosidic linkage produces glucosamine, an amino sugar, which are a valuable product in health because of its function as main component of human joints' cartilage (McColl 2004). Chitinase enzyme from microorganism has been widely used for waste processing and biocontrol agent of plant pests. Besides, the results of chitin hydrolysis can be used as an antitumor, a supplement, to control blood glucose level, basic material of suture and anti-inflammatory agent (Pratiwi et al 2015).

Hydrolysis can be done by using microorganisms, enzymes or chemical agents. The most commonly method to hydrolyze chitin is by chemical reaction, but it gives many negative impacts on environment as well as on the physicochemical characteristics of chitin itself (Arbia et al 2013; Percot et al 2003). Production of glucosamine using microorganism is an alternative that has gain its popularity nowadays because the process is simpler, productive and environmental friendly (Younes & Rinaudo 2015).

Various chitinolytic microorganisms have been looked for and isolated from several sources, such as liquid and solid waste of shrimp shells (Setia & Suharjono 2015), seafood industrial waste (Krithika & Chellaram (2016), organic waste (Purkan et al 2014),

rhizosphere of pepper plant (Mubarik et al 2010) and product of shrimp fermentation (Puspita et al 2017). However, the isolated microorganisms are still considered not strong enough in hydrolysing chitin. Thus, it is required to search for stronger chitinolytic microorganisms which can break down chitin in materials that contain a lot of chitins, such as rotten shrimp shells.

The present research was aimed to isolate, identify and characterize the strong chitinolytic bacteria isolated from rotten *P. monodon* shells.

Material and Method

Materials. The main material used in the present study was *P. monodon* shells that consisted of shells and head part of the shrimp, obtained from PT Lola Mina, Muara Baru, Jakarta. These shrimp shells were then spoiled and later used as chitin sources in chitinolytic media making. Moreover, supporting materials were Nutrient Agar (NA) and Nutrient Broth (NB) "MERCK", Bromocresol Purple (BCP) indicator, tartaric acid (10%), NaOH solution (3.5% and 10 N) (p.a.), HCl solution (1 M, 10%, dan 37%) (p.a.), aquadest, 0.85% NaCl solution, 70% alcohol (p.a.), Bovine Serum Albumin (BSA) solution, crystal violet solution "MERCK", lugol, safranin "MERCK", alcohol-acetone and KBr platelet.

Equipment used in the present study was dry-blender "PANASONIC MX-GX1462", waterbath, vortex, heater, incubator, laminar air-flow, autoclave, pH-meter "METROHM 913", sieve-shaker, furnace, centrifuge, refrigerator, analytical balance, cabinet dryer, dessicator, oven, UV-Vis spectrophotometer, light illumination microscope "OLYMPUS", microscope camera "OLYMPUS DP21", software "OLYMPUS STREAM START", chromameter "KONICA MINOLTA CR400", ashing dish, PCR, FTIR spectrophotometer, magnetic stirrer, glass slides, vacuum pump, Buchner funnel, thermometer, Vernier caliper, cuvette, disposable Petri dish, micropipette, hockey stick, inoculating loop, Whatman no. 1 and glasswares.

Methods. This research used descriptive method with several stages, i.e. decay of shrimp shell powder, isolation of chitinolytic bacteria, characterization of isolates macroscopically and microscopically, chitinolytic index measurement and identification of bacteria with highest chitinolytic index.

Decay of P. monodon shell and isolation of chitinolytic bacteria. *P. monodon* shells (shells and head part) were washed and sun dried. Dried shrimp shells were then grinded to powder. These shrimp shells powder were placed into plastic container, wetted with little amount of water and spoiled for 4 days at the park near Universitas Pelita Harapan pond, until they became smelly and had blackish colour. Rotten shrimp shells powder was then stirred until homogenous and about 25 g were taken to be placed into 225 mL of physiological saline solution and mixed. About 1 mL of sample solution resulting from 10^{-3} to 10^{-7} dilution was taken and poured into Petri dish and added with NA media that were added with 2% colloidal chitin, 10% tartaric acid to obtain pH of 4.7, 0.01% BCP indicator (Agrawal & Kotasthane 2012), 0.07% KH_2PO_4, 0.03% KH_2PO_4, 0.01% $MgSO_4.7H_2O$ and 0.7% $(NH_4)_2SO_4$ mineral salt (Halimahtussadiyah et al 2017; Woo et al 1996). Sample was then incubated at 37^{o}C incubator for 48 hours. The colonies that formed purple zone were then isolated, given codes and observed for their colony characteristics.

Colloidal chitin preparation from *P. monodon* shells powder as a substrate added into NA media, was based on method by Thiagarajan et al (2011). Chitin powder was prepared according to method of Agustina et al (2015) through demineralization and deproteination processes.

Purification and morphological characterization. Purification of isolates was performed based on method by Febrinanda (2015), i.e. quadrant streak plate method on NA-colloidal chitin media until uniform single colony was obtained. Pure isolates were then observed for their morphological characteristics macroscopically and microscopically (cell). Macroscopic morphologes include colour, shape, margin, surface, bacterial colony elevation. Microscopic morphologies include shape observation and Gram staining.

Analysis of chitinolytic index of bacteria isolates. Chitinolytic index analysis was done using well diffusion method (Zou et al 2002 with modification) on NA-2% colloidal chitin media, added with 0.01% BCP and 10% tartaric acid until pH 4.7 was reached. Addition of BCP as an indicator of color change of media from reddish yellow into purple by change of pH media caused by chitin hydrolysis into glucosamine. Well diffusion method was done by placing 60 µL of working culture (chitinolytic bacteria isolate) that has been incubated for 24 hours into a well with 0.5 cm diameter. These bacteria were then incubated at 37^{o}C for 48 hours. Chitinolytic properties of bacteria was expressed by measuring isolate's chitinolytic index (CI), i.e. ratio between diameter of clear zone (purple colour) and diameter of bacterial colony. The hydrolysis zone of media was also measured for its colour using chromameter to obtain L, a, b and ^{O}Hue.

Molecular identification of chitinolytic bacteria. A selected isolate of bacteria with highest chitinolyic index and lowest lightness value (from purple zone formed) was then identified molecularly based on its 16S rDNA sequence and gene amplification using PCR. The similarity of its sequence was compared using BLASTn website at NCBI to determine its genus and suspected species. Construction of phylogenetic trees was done using ClustalW program.

Results and Discussion

Chitinolytic bacterial isolates. Bacterial colony which has chitinolytic properties is characterized by formation of purple zone around the colony (Agrawal & Kotasthane 2012). If no BCP indicator is added, chitinolytic bacterial colony is characterized by clear zone formation caused by degradation of chitin by bacteria that can hydrolyze chitin into its monomers. However, visualization of clear zone is considered difficult and low (Agrawal & Kotasthane 2012; Vaidya et al 2002), so that it generally requires 3-7 days of incubation to observe the formation of clear zone (Kuddus & Ahmad 2013; Setia & Suharjono 2015; Chasanah et al 2009).

There were 167 colonies of bacteria that had chitinolytic properties obtained from rotten *P. monodon* shells. However, after being grouped, screened and purified based on their morphology and macroscopic characteristics, there were 17 isolates obtained based on their morphology (macroscopically), as can be observed in Table 1, and microscopic characteristics, as can be observed in Table 2.

Several characteristics of colony of chitinolytic bacteria from *P. monodon* shell are similar to the ones reported by Fitri & Yasmin (2011) and Tito (2014), i.e. cocci and white colony. The similarity of colony morphology often causes difficulty in determining the differences among the isolates obtained. Therefore, microscopic observation of morphology, such as cell shape and gram staining, must be done. Most of the isolates of chitinolytic bacteria obtained from rotten *P. monodon* shells are gram positive (10 isolates). Puspita et al (2017) and Ajayi et al (2016) also reported that isolates of chitinolytic bacteria obtained in their research were mostly gram-positive bacteria. According to Beier & Bertilsson (2013), this is because gram-positive bacteria requires component to build peptidoglycan (murein) in their cell wall synthesis, so they require high concentration of N-acetyl-D-glucosamine. On the other hand, gram-negative bacteria only use N-acetyl-D-glucosamine as a substrate of energy acquisition in catabolism reaction.

Table 1

Morphological characteristics of bacterial isolates

No.	*Isolate code*	*Shape of colony*	*Surface of colony*	*Margin of colony*	*Elevation of colony*	*Colony pigmentation*	*Appearance*	*Optical property*	*Size of colony*
1	I21	Circular	Concentric	Curled	Flat	Putih	shiny	Transparent	Small
2	J22	Circular	Concentric	Entire	Flat	Putih	shiny	Transparent	Punctiform
3	I32	Circular	Concentric	Entire	Raised	Putih	shiny	Translucent	Small
4	S11	Circular	Contoured	Curled	Raised	Cream	shiny	Opaque	Large
5	R31	Circular	Smooth	Undulate	Pulvinate	Cream	shiny	Iridescent	Large
6	U13	Circular	Smooth	Entire	Convex	Putih	dull	Transparent	Moderate
7	A12	Circular	Smooth	Entire	Convex	Putih	shiny	Translucent	Moderate
8	F32	Circular	Smooth	Entire	Raised	Putih	shiny	Translucent	Small
9	P34	Circular	Smooth	Undulate	Raised	Cream	dull	Opaque	Large
10	N22	Filamentous	Pinpoint	Filiform	Flat	Putih	shiny	Translucent	Punctiform
11	F12	Irregular	Smooth	Lobate	Flat	Putih	dull	Opaque	Large
12	H14	Irregular	Contoured	Undulate	Umbonate	Cream	dull	Opaque	Moderate
13	H32	Irregular	Radiated	Undulate	Umbonate	Tan	dull	Iridescent	Moderate
14	E11	Irregular	Smooth	Lobate	Convex	Putih kekuningan	dull	Transparent	Moderate
15	B12	Pinpoint	Concentric	Entire	Flat	Putih	shiny	Transparent	Punctiform
16	Q11	Pinpoint	Smooth	Entire	Flat	Cream	shiny	Transparent	Punctiform
17	T11	Pinpoint	Smooth	Entire	Raised	Golden Yellow	shiny	Iridescent	Punctiform

Table 2

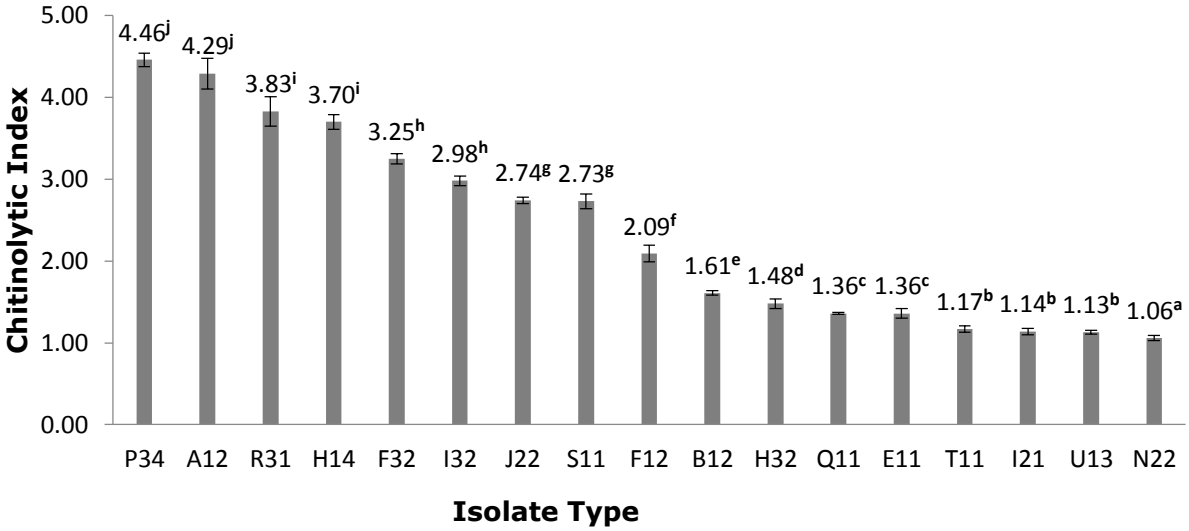
Microscopic characteristics of bacterial isolates

No.	*Isolate code*	*Gram*	*Shape of cell*
1	A12	Negative	Bacilli
2	H14	Negative	Bacilli
3	Q11	Negative	Bacilli
4	I32	Negative	Coccobacilli
5	S11	Negative	Cocci
6	T11	Negative	Cocci
7	U13	Negative	Cocci
8	B12	Positive	Bacilli
9	F12	Positive	Bacilli
10	H32	Positive	Bacilli
11	I21	Positive	Bacilli
12	E11	Positive	Coccobacilli
13	F32	Positive	Coccobacilli
14	P34	Positive	Coccobacilli
15	J22	Positive	Cocci
16	R31	Positive	Cocci
17	N22	Positive	Cocci

The number of chitinolytic bacterial isolates obtained from rotten *P. monodon* shells was different from soil samples collected from rhizosphere of maize, wheat and rice, fish market and pond, i.e. about 58 isolates (Kuddus & Ahmad 2013) and 51 isolates (Shivalee et al 2016); from shrimp ponds, i.e. about 23 isolates (Prabowo & Herdyastuti 2014); from shrimp shells waste, i.e. about 18 isolates (Setia & Suharjono 2015); from lobster shells, i.e. about 4 isolates (Tito 2014). These results show that there is more diversity of chitinolytic bacteria obtained from natural resources compared to indigenous bacteria that grow on certain substrates. Moreover, the difference in place and source of isolation give different bacterial isolates because of the difference in nutrition and ecosystem. The other factors that contribute to these different results are pH of selective media and concentration of colloidal chitin added into the media. pH of media used in the present study was 4.7, whereas the other researches uses media with pH of 7. Concentration of colloidal chitin added in the present study was 2%, according to the previous research of Lamine & Bouziane (2012).

Chitinolytic index of isolates. Chitinolytic index is the ratio between diameter of purple zone formed and diameter of colony or diameter of well. The highest the chitinolytic index, the highest the chitinolytic properties of an isolate. Each bacterium can show different chitinolytic activity, therefore chitinolytic index are also various, from weak to strong, as reported by Chasanah et al (2009), Halimahtussadiyah et al (2017), Herdyastuti et al (2009), Puspita et al (2017), Kuddus & Ahmad (2013) and Setia & Suharjono (2015). Chitinolytic properties of bacteria is considered strong if the chitinolytic index is above 2.

In the present study, statistical analysis (using ANOVA) shows that there was a significant difference of chitinolytic index of isolates from rotten *P. monodon* shells ($p<0.05$). Post hoc test results using Duncan method are presented in Figure 1.



Different superscript letter behind the values shows a significant difference at α=0.05

Figure 1. Effect of isolate type on chitinolytic index of bacterial isolates.

Based on Figure 1, the highest chitinolytic index were obtained from isolate P34 and A12, which are not significantly different between them ($p>0.05$) but are significantly different from another group with high chitinolytic index ($p<0.05$), i.e. isolate R31 and H14. The next isolate group with high chitinolytic properties (chitinolytic index >2) was isolate F32 and I32, which are significantly different from isolate J22, S11 and F12.

Setia & Suharjono (2015) obtained 14 isolated from liquid waste and 4 isolates from shrimp shells waste, in which only 2 isolates had chitinolytic index (CI)>2, whereas Chasanah et al (2009) obtained 7 isolates with CI>2 from waste of shrimp processing factory. The number of 8 isolates with CI>2 from *P. monodon* shells obtained in the present study is higher compared to previous researches of Setia & Suharjono (2015) and Chasanah et al (2009).

Colour analysis of purple zone of media. According to Longhinotti et al (1998), color-binding mechanism of chitin is through adsorption and depends on pH. In normal condition, amine group of chitins has both neutral charge ($-NH_2$) and cationic ($-NH_3^+$). In acidic condition, amine group (NH_2) is protonated and chitin polymer has positive charge. To maintain neutral condition, chitin polymer will have ion exchange with negative ions from anionic colorant to form electrostatic interaction. According to Agrawal & Kotasthane (2012), the smallest unit of chitin, i.e. chitobiose, which consists of two molecules of N-acetyl-D-glucosamine has two hydroxyl groups, one carbonyl group and one amine group, which is the group that will form reactive linkage with BCP to form colour complex. The purple colour of hydrolysis zone and the lightness of hydrolysis results of chitinolytic bacterial isolates from *P. monodon* shells can be observed in Table 3.

Based on colour measurement of media using chromameter (Table 3), all isolates produce similar colour, which is purplish red (^{o}Hue), whereas control media without isolate has yellowish red colour. Colour difference can also be observed using lightness value, such as in surimi product (Jin et al 2009; Ramadhan et al 2014) which uses lightness to differentiate the colour of the products.

Table 3

°Hue value and lightness of purple zone from isolates

No.	*Isolate*	*°Hue (color of clear zone)*	*Lightness (L*)*
1	A12	-25.10±2.39 (purplish red)	23.88±0.31[a]
2	B12	-43.78±1.34 (purplish red)	24.86±0.09[a]
3	E11	-31.01±1.14 (purplish red)	24.91±0.82[ab]
4	F12	-38.74±5.86 (purplish red)	25.54±0.64[bc]
5	F32	-48.61±2.08 (purplish red)	25.73±0.84[bcd]
6	H14	-40.33±5.07 (purplish red)	25.85±1.21[bcd]
7	H32	-42.87±6.36 (purplish red)	25.97±1.01[bcd]
8	I21	-40.16±5.17 (purplish red)	26.03±1.19[bcd]
9	I32	-38,42±3.17 (purplish red)	26.06±0.72[bcd]
10	J22	-41.63±1.31 (purplish red)	26.07±0.63[bcd]
11	N22	-43.48±8.41 (purplish red)	26.14±0.87[bcd]
12	P34	-25.18±2.90 (purplish red)	26.29±0.52[bcd]
13	Q11	-45.41±8.58 (purplish red)	26.29±0.70[bcd]
14	R31	-23.85±4.26 (purplish red)	26.40±0.51[bcd]
15	S12	-32.65±1.09 (purplish red)	26.47±1.01[bcd]
16	T12	-42.88±9.89 (purplish red)	26.59±0.11[cd]
17	U13	-42.88±6.53 (purplish red)	27.32±0.30[d]
18	Control	72.28±2.06 (yellowish red)	32.84±0.08[e]

Table 3 also shows that isolate P34 and A12 have the lowest lightness on their hydrolysis zone and no significant difference, in terms of lightness, between these two isolates ($p>0.05$) but isolate A12 shows significant different in lightness compared to other isolates ($p<0.05$), except to isolate E11 and P34. Isolates that have low lightness are isolate A12, E11, P34 and B12, which shows significant different with other 13 isolates.

It seems that lightness value of hydrolysis zone is related to chitinolytic index. The higher the chitinolytic index, the lower the lightness, which means the darker the colour of the media. Isolate P34 and A12 have the lowest lightness value.

Identification of bacteria with highest chitinolytic index. Isolate A12 was chosen as bacterial isolate from *P. monodon* shells that had the highest chitinolytic properties, based on its highest chitinolytic index and lowest lightness value of media that had been hydrolyzed. Isolate A12 has several characteristics, which are cocci colony, smooth surface, raised elevation, entire margin, transculent white colour and moderate size colony. The shape of isolate A12 is bacilli and gram negative. This bacterium is aerobic and acid tolerant because it can grow on media with low pH, i.e. 4.7.

Molecular identification based on 16S rDNA gene sequence, isolate A12 was *Providencia stuartii*, with similarity of 99.33% (Figure 2). Shape of *P. stuartii* and its hydrolysis zone can be observed on Figure 3. Kinship of isolate obtained with *Providencia stuartii* can be seen on phylogenetic tree on Figure 2. *P. stuartii* is gram negative, cocci, aerobic bacterium that grow after 24 hours of incubation at 37°C, categorized as genus *Providencia* from *Enterobacteriaceae* family (Buller 2014; O'hara et al 2000). *P. stuartii* is pathogenic. However, since it is mesophilic bacterium, it can be inactivated by thermal treatment (Miller et al 2016).

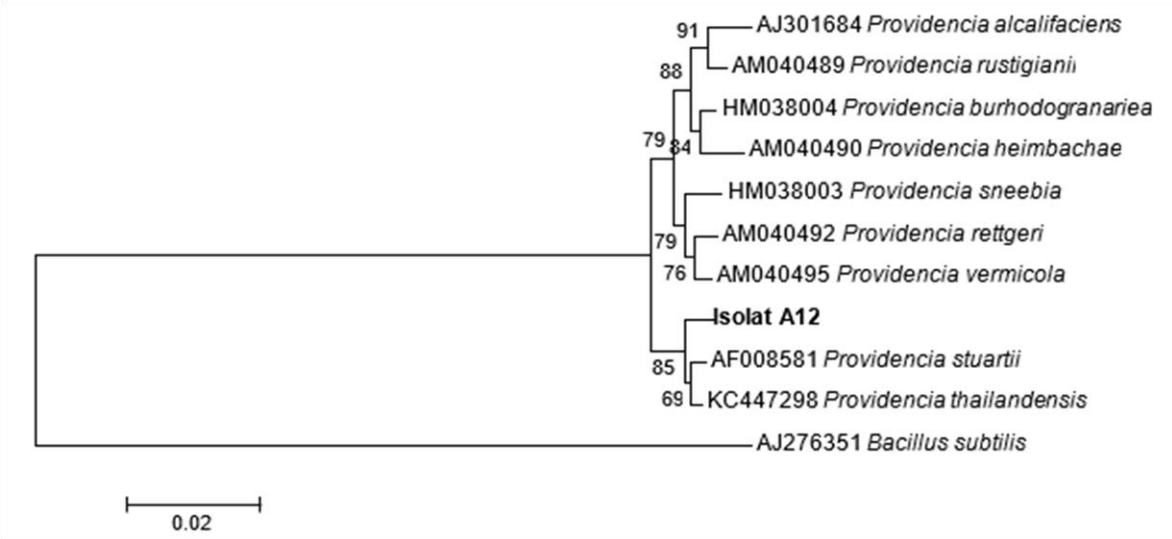
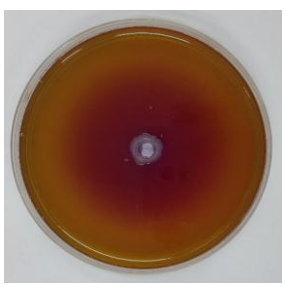
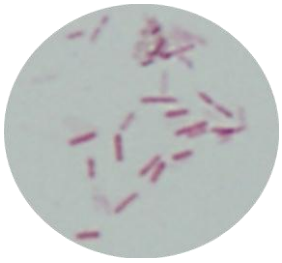


Figure 2. Phylogenetic tree of isolate A12.



Hydrolysis zone (purple)
Colony (white)



Providencia stuartii
(1000x magnification)

Figure 3. Hydrolysis zone and cell of *Providencia stuartii*.

Providencia is often present in water and soil, therefore it can be found in animals that live in water, such as *P. monodon* (Wie 2015; Lo & Chang 2015). Bogomolni et al (2008) found *P. stuartii* in sea mammals. Another species from genus *Providencia,* i.e. *Providencia rettgeri* was isolated from heart, kidney, scale, fin and body surface of fish (Buller 2014; Austin & Austin 2012).

There are many genera from *Enterobacteriaceae* family that have chitinolytic properties, such as *P. stuartii.* This is because habitat of *Enterobacteriaceae* is naturally in water. Donderski & Trzebiatowska (1999) reported that *Enterobacteriaceae* family was a bacteria family that can be found mostly in water which have many arthropods. *P. stuartii* has been reported to have chitinase enzyme type A, C1 and chitobiase enzyme (String 2017). On the other hand, *P. rettgeri* and *Providencia friedericiana* have been reported to have no chitinolytic properties (Buller 2014; Mohamedova et al 2017; Raio et al 2016).

Chitinolytic index of *P. stuartii* reaches about 4.46 after incubation for 48 hours at 37^{o}C, which is higher compared to other chitinolytic bacteria isolated from other sources, such as *Acinetobacter johnsonii* (2.069) and *Bacillus amyloliquefaciens* (2.084), which were isolated from solid and liquid waste of shrimp shells (after incubation for 7 days at 30^{o}C) (Setia & Suharjono 2015); *Pseudomonas pseudomallei* (1.21) which were isolated from organic waste (incubation for 7 days at 37^{o}C) (Purkan et al 2014); *Bacillus cereus* (0.94) which were isolated from rhizosphere of pepper plant (incubation for 2 days at 37^{o}C) (Mubarik et al 2010); *Enterobacter cloacae* (3.45) and *Pseudomonas stutzeri* (3.66) which

were isolated from "rusip", a fermented product from shrimp (incubation for 3 days at 30°C) (Puspita *et al* 2017).

Conclusions. There were 17 bacterial isolates of *P. monodon* shell that had the chitinolytic properties of which 8 had strong chitinolytic properties. Isolate A12 was the isolate with the strongest chitinolytic properties with chitinolytic index of 4.46 and lowest lightness of chitinolytic zone, i.e. 23.88. Isolate A12 was identified as *P. stuartii* and believed as potential bacteria to be used as chitin-hydrolising bacteria for glucosamine production.

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