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Occurrence of chitinolytic bacteria in shrimp *rusip* and measurement of their chitin-degrading enzyme activities

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Abstract. *Puspita ID, Wardani A, Puspitasari ROA, Nugraheni PS, Putra MMP, Pudjiraharti S, Ustadi. 2017. Occurrence of chitinolytic bacteria in shrimp* rusip *and measurement of their chitin-degrading enzyme activities. Biodiversitas 18: 1275-1281.* The objectives of this research were to isolate chitinolytic bacteria from shrimp *rusip* (an Indonesian traditional fermented shrimp product), identify bacterial isolates showing high chitinolytic activity, and determine the chitinolytic activity of these isolates. There were 44 chitinolytic bacteria isolated from shrimp *rusip*: 39 isolates of Gram-positive bacteria and 5 isolates of Gram-negative bacteria. The quantitative method we used to evaluate chitin-degrading enzyme activity measured the amount of N-acetylglucosamine produced from the reaction of crude enzyme and colloidal chitin. Seven isolates showing highest chitinolytic activity were *Bacillus cereus* (KKT 1, KKT 14, and KKT 19), *Bacillus thuringiensis* (KKT 6), *Enterobacter cloacae* (LCK 20), *Pseudomonas stutzeri* (LCK 17), and *Stenotrophomonas maltophilia* (THK1). The highest chitinolytic activity showed by KKT 1, KKT 6, KKT 14, KKT 19, and THK 1 were 0.0016 U/mL (at 72 hours); 0.0018 U/mL (at 48 hours); 0.0019 U/mL (at 48 hours); 0.0033 U/mL (at 48 hours); and 0.0032 U/mL (at 24 hours), respectively.

Keywords: chitinase activity, chitinolytic bacteria, shrimp *rusip*

INTRODUCTION

Indonesia is one of the main producers of shrimp and crab among Southeast Asian countries. According to data released by the Ministry of Marine Affairs and Fisheries Republic of Indonesia (2015), Indonesia's total volume of export of shrimp and crab (2010-2015) averaged 164,473 and 25,840 ton/year, respectively. These commodities are exported in the form of frozen peeled products. Shell waste from shrimp and crabs, which comprise about 60-80% of their total body weight (Wang et al. 2011), has the potential to cause an environmental problem if not treated properly. Fishery Statistics Data reveal that Indonesia produces shell waste averaging 56,200 ton/year (Ministry of Marine Affairs and Fisheries Republic of Indonesia 2000). The abundance of shell waste in Indonesia requires processing technology to utilize waste by converting it into high value-added products. Crustacean shells are a source of chitin, a polysaccharide composed of N-acetyl-glucosamine bonded with a ß- (1,4) bond. Partial de-acetylation of chitin produces chitosan (Younes and Rinaudo 2015). Chitin, chitosan, and its hydrolysate have been intensively studied with respect to production, characteristics, and function. However, chitin has a lower solubility at neutral pH than does chitosan, limiting its applications. The improvement of its solubility can be achieved by reduction of its molecular weight by depolymerization into an oligomeric form.

Hydrolysis of chitin and chitosan into lower molecular weight oligomers can be carried out using a bacterial chitinolytic enzyme, which offers an advantage over acid hydrolysis because it minimises chemical waste production and the reaction is more controllable. In terms of bioactivity, chitin/chitosan oligomer has been reported to have antimicrobial, anti-tumor, anti-hypertensive, antioxidant, hypocholesterolemic, and immunostimulant activity (Prashanth and Tharanathan 2007). Considering the important potential role of chitinolytic bacteria in the bioconversion of chitinous waste into bioactive molecules, bioprospecting of chitinolytic bacteria from various type of environments has become a crucial step in obtaining isolates that exhibit good performance in the bioconversion process.

Microbial prospecting is usually carried out in extreme environments to obtain microbes possessing unique metabolic characteristics (Gohel et al. 2006). Bioprospecting of chitinolytic bacteria has been reported from soil (Das et al. 2010; Kuddus et al. 2013), the rhizosphere (Someya et al. 2011), volcanic mountains (Soeka and Sulistiani 2011), seawater and plankton (Souza et al. 2009), freshwater shrimp ponds (Shu-Chen et al. 2004), intestinal tracts of fish (Itoi et al. 2006) and animal rumens (Kopečný et al. 1996). A distinctive habitat where chitinolytic bacteria are also possibly to be found is in crustacean fermented products.

Fermented fishery products provide unfavorable conditions for the growth of bacteria, such as high salt/ sugar concentration, low pH, high temperatures during processing, predominance of proteolytic enzymes, and high competitiveness for nutrient access. The fermentation conditions favor the predominant growth of anaerobic/ facultative anaerobic, acid tolerant, and salt tolerant bacteria. Most studies on bacterial populations in fermented shrimp have focused on the isolation of lactic acid bacteria (Kobayashi et al. 2003; La Anh 2015; Lee et al. 2014). The isolation of chitinolytic bacteria has been reported from *terasi* (Zilda and Chasanah 2005) and *petis* (Orinda et al. 2015), which are traditional fermented shrimp pastes from Indonesia. Shrimp *rusip* is another traditional fermented shrimp paste from Indonesia composed of small shrimp, salt, and spices (Irianto and Irianto1998). *Rusip* has a low pH value and a high concentration of salt (Kusmarwati et al. 2011; Koesoemawardani 2007).

We expect that chitinolytic bacteria can be recovered from *rusip*. Isolates obtained from *rusip* exhibiting high chitinolytic activity could have potential to be exploited for useful bioconversion processes. Thus, the objectives of the research reported here were to screen bacterial isolates obtained from shrimp *rusip* for their chitinolytic activity and to quantify the extent of their chitinolytic enzyme activity.

MATERIALS AND METHODS

Sample preparation and isolation of bacteria

Three different brands (LCK, KKT, THK) of shrimp *rusip* were obtained from Belitung Island, Sumatera, Indonesia. Samples were serially diluted in NaCl 0.85% (b/v), plated on colloidal chitin agar medium (K_2HPO_4 0.1% (w/v), $MgSO_4.7H_2O$ 0.01% (w/v), NaCl 0.1% (w/v), $(NH_4)_2SO_4$ 0.7% (w/v), yeast extract 0.05% (w/v), colloidal chitin 2% (w/v), agar 1% (w/v) (Nasran et al. 2003)), and incubated at 30°C for 96 h. Colloidal chitin was prepared following Hsu and Lockwood (1975). Bacteria showing a clear zone around their colonies were picked up and streaked on chitin agar medium to obtain pure cultures. All isolates were cultivated in a colloidal chitin broth medium (K_2HPO_4 0.1%, $MgSO_4.7H_20$ 0.01%, NaCl 0.1%, $(NH_4)_2SO_4$ 0.7%, yeast extract 0.05%, colloidal chitin 1%) at 30°C for 96 h and kept frozen in 50% glycerol at -30°C prior to use.

Screening of chitinolytic bacteria

Screening of chitinolytic bacteria was performed by two methods, chitinolytic enzyme activities and spot inoculating. All isolates were cultivated in tryptic soy broth (TSB) at 37°C for 24 h. An inoculum of 50 µL was transferred to 5 mL colloidal chitin broth and incubated further at 37°C for 48 h. The bacterial supernatant (1,500 µL) was collected and centrifuged (6,000 rpm, 4°C, 10 min). The cell-free supernatants were measured for their chitinolytic enzyme activities (Reissig et al. 1955). Selected isolates from previous screening were tested for their chitinolytic index. Inoculum prepared in the same way as previously (2 µL) was spot-inoculated on colloidal chitin agar and incubated at 30°C for 72 h. The chitinolytic index was calculated as the diameter of the clear zone produced by the bacteria, divided by the diameter of the bacterial colony.

Bacterial identification

The cells were cultured in colloidal chitin broth medium at 37˚C for 7 days. Genomic DNA was extracted using a PrestoTM Mini gDNA Bacteria Kit (GeneAid) following the manufacturer's protocol. Universal primer of 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-TAC GGT TAC CTT GTT ACG ACT T-3') was used to amplify the 16S rRNA region gene. The thermal cycle was initiated by pre-denaturation for 3 minutes at 95 ˚C and continued by 30 cycles of denaturation for 30 seconds at 95˚C, annealing for 30 seconds at 55˚C, elongation for 90 seconds at 72˚C and final extension for 5 minutes at 72˚C. The expected size of the PCR amplicon was confirmed by electrophoresis on 1% agarose gel. The partial 16S rRNA was sent to First Base Laboratories, Singapore, for sequence analysis, and matched with the nucleotide database available at GenBank using BLAST tool in NCBI and further processed using MEGA version 5. Additional biochemical tests were performed for several of the isolates to support the results from the molecular identification.

Determination of chitin-degrading enzyme activity

Supernatant was prepared from bacterial incubation in colloidal chitin medium. Cell-free supernatant was obtained by centrifugation at 6,000 rpm and 4°C for 10 min. Supernatant (500 µL) as crude enzyme was incubated with 1 mL colloidal chitin (1.3% in 50 mM phosphate buffer pH 7.4) at 37°C for 30 min. The enzymatic reaction was stopped by heating the mixture in boiling water for 5 min then cooled and centrifuged at 10,000 rpm for 5 min (Wang et al. 2011). The amount of N-acetylglucosamine (GlcNAc) released from the enzymatic reaction was measured following Reissig et al. (1955). Briefly, 0.25 mL of enzymatic reaction supernatant from the previous step was mixed with 0.05 mL potassium tetraborate (pH 9.1), boiled for 3 min, and the mixture allowed to cool down. An amount of 1.25 mL p-Dimethylaminobenzaldehyde (DMAB) reagent was added to the mixture and incubated at 37ºC for 20 min in a water bath. The color produced by the DMAB reaction was detected by spectrophotometer at 584 nm. Solutions of N-Acetylglucosamine (GlcNAc) at several concentrations (0; 0.5; 1; 1.5; 2; 2.5 µg/mL) were used as the standard solutions. One unit of chitinase activity was defined as the amount of GlcNAc (µmol) released by enzyme per minute at the condition mentioned.

RESULTS AND DISCUSSION

Isolation and screening of chitinolytic bacteria from *rusip*

Rusip is a fermented shrimp or fish from Sumatera, Indonesia, that is processed traditionally. The whole, small-size shrimp or fish are rinsed and decanted, and then salt (25%), and palm sugar (10%) is added. Some producers

also use an additional carbon-source such as rice or starch. Then, the mixture is kept in a sealed container and left for several days at room temperature (Koesoemawardani 2007). A spontaneous fermentation occurs. As a result, the product appears viscous with a brown to black color and a distinct acid, fishy, tasty odor. Similar products are also found in Malaysia and Thailand, called *cencalok* and *jaloo*, respectively (Hajeb and Jinap 2012). The pH of *rusip* ranges from 4.7 to 6.37 (Irianto and Irianto 1998; Kusmarwati et al. 2011) with a salt concentration of 17-30% (w/w) (Koesoemawardani 2007).

From the three brands (LCK, KKT, THK) of shrimp *rusip* from Belitung Island, we isolated a total of 44 strains of chitinolytic bacteria. All isolates showed a clear zone on chitin agar medium on day-2 to -4 of incubation at 30°C. Most of the isolates (86%) were Gram-positive bacteria (Table 1). The highest number of chitinolytic bacteria isolates was obtained from KKT (52.3%), followed by LCK (43.2%) and THK (4.5%). The difference in number of chitinolytic bacteria isolated from the three samples is associated with variation in their characteristics, such as consistency, composition, raw material quality, fermentation stage, and storage period.

Among the 44 isolates, only 21 isolates showed a positive result (> 0 U/mL) for chitinase activity when tested quantitatively in chitin liquid medium after 48-hour incubation (data not shown). The undetectable level of chitinolytic activity from other 23 isolates after 48 hours incubation in liquid medium is possibly due to a requirement for a longer incubation period in order to produce chitinolytic enzyme of detectable level in a liquid medium, even though most isolates had exhibited a clear zone in the chitin agar medium. A larger size of clear zone produced on chitin agar medium is not always in accordance with higher production of chitinolytic enzyme in a liquid medium (Howard et al. 2003). Time is considered as an important parameter; the faster the time, the more efficient the production process. Therefore, only isolates showing high chitinolytic activity during the 48-hour incubation were chosen for further screening of their chitinolytic index. Seven isolates were selected representing the three *rusip* samples, namely KKT 1, KKT 6, KKT 14, KKT 19, LCK 20, LCK 17, and THK 1. The highest chitinolytic index was shown by LCK 17 (Table 2).

Bacterial identification

Molecular identification based on BLAST results on 16S rRNA (27F-1492R) sequences in GenBank showed that four isolates of Gram-positive (KKT 1, KKT 6, KKT 14, and KKT 19) were all from the genus *Bacillus*, while three isolates of Gram-negative bacteria (LCK 20, LCK 17, and THK 1) were from different genera (Table 2).

Among the four isolates of *Bacillus*, three (KKT 1, KKT 16, and KKT 19) were identified as *Bacillus cereus*, while KKT 6 was from a different clade, and was identified as *Bacillus thuringiensis* (Figure 1). *B. cereus* is a Gram-positive, rod-shaped, facultative anaerobic, motile bacterium. The natural habitat of *B. cereus* is soil, but it is also found in plant cells as an endophyte. Its ability to produce endospores and its simple nutrient requirement results in *B. cereus* inhabiting many types of environment, including fermented foods (Kotiranta et al. 2000).

Some different biochemical characteristics were observed among the three isolates of *B. cereus* obtained in this experiment. Isolate KKT 14 gave a negative result on a catalase test; while the two other isolates showed a positive result. Isolate KKT 19 showed a positive result on an oxidase test and mannitol fermentation test; while the two other isolates showed a negative result on the oxidase test and failed to ferment mannitol. The variation in biochemical characteristic among the *B. cereus* isolates indicated that they were of different strains.

Some strains of *B. cereus* are known to be pathogenic bacteria due to their ability to produce enterotoxin, hemolysin BL (HBL), and non-haemolytic enterotoxin, causing diarrhea. Pathogenic *B. cereus* also produces an emetic toxin causing headache and nausea (Kotiranta et al. 2000). Contamination of pathogenic *B. cereus* in food has been reported in fresh produce (fruits, vegetables, and meat), dairy products, dried foods (cereal, pasta, and grains), minimally processed foods, and ready-to-eat foods (Valero et al. 2002). Isolation of pathogenic *B. cereus* was also reported in fresh and processed seafood products (Rahmati and Labbe 2008). However, the non-pathogenic strain of *B. cereus* has also been found widely in the food environment. Kamar et al. (2013) isolated non-pathogenic *B. cereus* from vegetables, fruits, and pasteurized milk.

Table 1. Total number of chitinolytic bacteria isolated from shrimp *rusip*

Sample	Gram-positive	Gram-negative	Total
Rusip 1 (LCK)	17	2	19
Rusip 2 (KKT)	20	3	23
Rusip 3 (THK)	1	1	2
Total	38	6	44

Table 2. Identification of seven chitinolytic bacteria isolates from *rusip*, together with their chitinolytic index values

Isolate	Description	Ident.	Accession number	Chitinolytic index
KKT 1	*Bacillus cereus* strain MHS 16s rDNA gene, partial sequence	100%	KR1325556.1	1.06
KKT 6	*Bacillus thuringiensis* strain YWC2-8, complete genom	100%	CP013055.1	1.05
KKT 14	*Bacillus cereus* strain MHS 16s rDNA gene, partial sequence	100%	KR1325556.1	1.03
KKT 19	*Bacillus cereus* strain MHS 16s rDNA gene, partial sequence	100%	KR1325556.1	1.03
THK 1	*Stenotrophomonas maltophilia* Strain Roi_3A	99%	JN644502.1	1.22
LCK 20	*Enterobacter cloacae* strain 34978, complete genom	100%	CP12165.1	3.45
LCK 17	*Pseudomonas stutzeri* strain AMH-5 16s rDNA gene, partial sequence	99%	KP174140.1	3.66

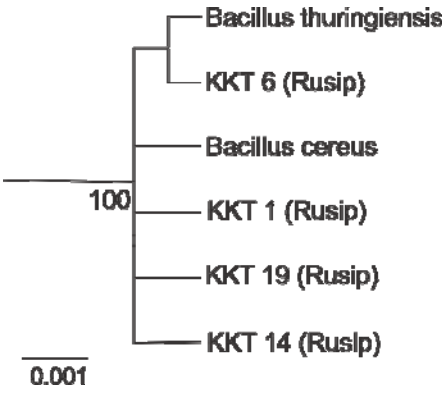

Figure 1. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences for the chitinolytic bacteria KKT1, KKT6, KKT14, and KKT 19 isolated from *rusip*, and related *Bacillus* species. The tree was constructed by MEGA 5.0 software. The scale bar represents an evolutionary distance of 0.001

Among *Bacillus* isolates, KKT 6 showed close similarity to *Bacillus thuringiensis*, and exhibited characteristics distinct from the other three *Bacillus* isolates; as indicated by its cubical crystal shape under malachite-green staining. Another distinctive biochemical characteristic shown by isolate KKT 6 was its negative result on lactose and xylose fermentation tests. *B. thuringiensis* is a member of *B. cereus* sensu lato (Jensen et al. 2003). A distinctive habitat of *B. thuringiensis* is in the digestive track of insects: it may be released into the soil when the insect dies and grow afterwards under favorable conditions. Isolation of *B. thuringiensis* from fermented food has also been reported in grape wine (Bae et al. 2004), fermented soybeans and locust bean (Sarkar et al. 2002).

Isolate THK 1 showed 99% similarity to *Stenotrophomonas maltophilia*. Growth characteristic comparisons between *S. maltophilia* isolate THK 1 and *S. maltophilia* as described by Brooke (2012) showed that THK 1 has several distinct features such as the ability to grow under anaerobic condition, ability to grow at 41^{o}C and a negative result for maltose utilization. Although *S. maltophilia* is known as a non-halophilic bacterium, the recovery of *S. matophilia* from salted food environments such as salted cod (Rodrigues et al. 2003), brined anchovy (Köse et al. 2008), and cheese (Todaro et al. 2011) has been reported, indicating that some strains are salt-tolerant.

LCK 20 was identified as *Enterobacter cloacae*. Like the other members of the family Enterobacteriaceae, *E. cloacae* inhabits a diversity of environments, ranging from plants, soil, water, to humans and animals (Grimont and Grimont 2006). Colonies of *E. cloacae* LCK 20 on chitin agar medium were white in color, typically adherent, hard, and dry. The internal part of an old colony turned black. *E. cloacae* has been isolated from lobster surimi (Yuli et al. 2008), fermented olive (Bevilacqua et al. 2010), fermented sausage (Bover-Cid et al. 2001), cured sausage (Roig-saguez et al. 1996), salted mackerel (Tsai et al. 2005), and salt anchovies (Hernandez-Herrero 1999).

Isolate LCK 17 showed similarity to *Pseudomonas stutzeri* (99%). Like *E. cloacae* LCK 20, *P. stutzeri* LCK 17 exhibited a distinctive shape and consistency of colony on chitin agar medium. LCK 17 was observed as a wrinkled white, adherent, hard, and dry colony. *P. stutzeri* is a member of the genus *Pseudomonas* sensu stricto in the class Gammaproteobacteria. An important characteristic of *P. stutzeri* is its denitrifying ability (Lalucat et al. 2006). *P. stutzeri* widely inhabits water environments, such as freshwater aquaculture farm environments (Kumar and Surendran 2005), sediment (Sikorski et al. 2002), and wastewater from catfish ponds (Diep et al. 2009). There have been no previous reports of *P. stutzeri* recovered from fermented food, but recovery from fresh food has been reported in seafood (Kumar and Surendran 2005) and in refrigerated raw milk (Martin et al. 2006).

The species *B. cereus* and *B. thuringiensis* are known as foodborne pathogens, while, *S. maltophilia*, *E. cloacae*, and *P. stutzeri* have been reported as emerging important nosocomial pathogens (Brooke 2012; Davin-Regli and Pagès 2015; Lalucat et al. 2006). *S. maltophilia*, *E. cloacae*, and *P. stutzeri* have also been described as biogenic-amine producers in food products (Ben-Gigirey et al. 2000; Bover-Cid et al. 2001; Lavizzari et al. 2010). Moreover, reports of multidrug resistance species of *S. maltophila* (Brooke 2012) and *E. cloacae* (Davin-Regli and Pagès 2015) have been released. Although the pathogenicity of these isolates has to be determined with further tests, the occurrence of these species in *rusip* is likely to be a result of improper sanitation and hygiene during handling and processing. Therefore, recovery of these species from *rusip* in this experiment provides information of microbiological safety concern for *rusip* processing procedures.

Measurement of chitin degrading enzyme activity from bacterial isolates

Among the four isolates of *Bacillus* obtained from *rusip*, *B. cereus* KKT 19 showed the highest chitinolytic activity on colloidal chitin medium at 48 h incubation (0.0033 U/mL) (Figure 2). This activity was lower than reported for chitinolytic activity of *B. cereus* YQ308 after 3.5 days incubation in a shrimp and crab shell powder medium (> 1 U/mL) (Chang et al. 2003). Chitinolytic *B. cereus* has been isolated from mustard plants (*Sinapis* sp.) (Pleban et al.1997), lily plants (Huang et al. 2005), and soil (Chang et al. 2003). Purification using in-gel chitinase assay method has shown that there are five types of extracellular chitinase produced by *B. cereus*; the main activity is exhibited by a 36-kDa protein identified as chitobiosidase (exochitinase) (Wang et al. 2001). The main feature of *Bacillus chitinase* is its antifungal activity against *Botrytis elliptica* (Huang et al. 2005), *Fusarium oxysporum* (Chang et al. 2003), *F. solani* (Chang et al. 2007), and *Pythium ultimum* (Chang et al. 2003).

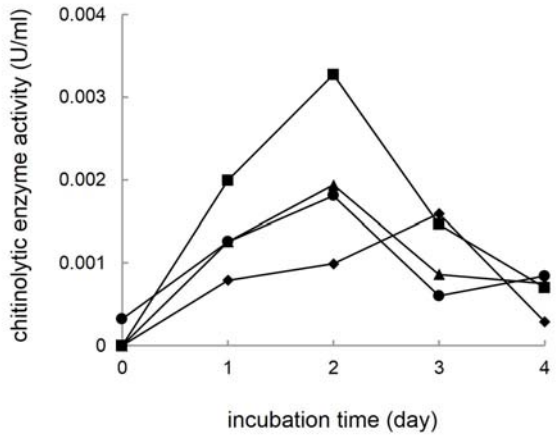

Figure 2. Chitin degrading enzyme activity of Gram-positive isolates when incubated in colloidal chitin medium, 30°C, 100 rpm. *Bacillus cereus* KKT 1 (♦); *Bacillus thuringiensis* KKT 6 (●); *Bacillus cereus* KKT 14 (▲); and *Bacillus cereus* KKT 19 (■).

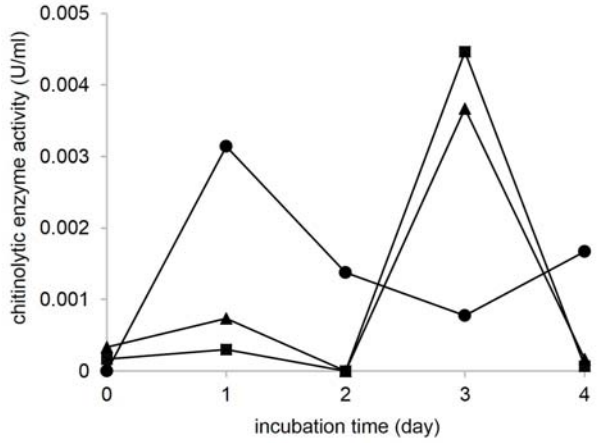

Figure 3. Chitin degrading enzyme activity of Gram-negative isolates when incubated in colloidal chitin medium, 30°C, 100 rpm. *Stenotrophomonas maltophilia* THK 1 (●); *Pseudomonas stutzeri* LCK 17 (▲); and *Enterobacter cloacae* LCK 20 (■).

Among the three isolates of Gram-negative bacteria, *S. maltophilia* exhibited the earliest incubation time to reach maximum chitinolytic activity (Figure 3). Chitinase from *S. maltophilia* is well characterized, as reported by Kobayashi et al. (2002). The highest chitinolytic activity showed by *S. maltophilia* THK 1 was 0.003 U/mL at day-1 of incubation (Figure 3). The activity was lower than has been reported for *S. maltophilia* isolated from bluegrass foliage; which showed activity of 0.05 U/mL after day-6 of incubation and exhibited antifungal activities against *Bipolaris sorokiniana* (Zhang et al. 2001). *E. cloacae* LCK 20 showed highest chitinolytic activity compared to the other isolates with an activity of 0.0045 U/mL after day-3 of incubation (Figure 3). Liu et al. (2013) identified three conserved chitinase genes in *E. cloacae* genome. One of the chitinases produced by *E. cloacae* is identical with chitinase A of *Serratia marcescens*. *In vitro* measurement of chitinolytic activity in *E. cloacae* has not been reported. In fact, a closely related species in the same genus, *E. agglomerans*, showed maximum chitinase activity at 60 h incubation in colloidal chitin medium with specific activity ranged from 2 to 5 U (Chernin et al. 1995); moreover, a fungal antagonistic reaction of chitinase from *E. agglomerans* has also been demonstrated (*ibid*). *P. stutzeri* is also known as a chitinolytic bacterium with potential for fungal biocontrol (Lim and Kim 1990). *P. stutzeri* LCK 17 showed the highest chitinolytic activity at day-3 of incubation, with an activity of 0.0037 U/mL (Figure 3). This value is lower than *P. stutzeri* YPL 1 isolated from ginseng, which showed an activity of 56.2 U/mL at day-5 of incubation (Lim and Kim 1994).

Based on our knowledge, this is the first paper describing the recovery of chitinolytic bacteria from shrimp *rusip*. Previous reports of bacteria isolated from *rusip* have focused on lactic acid bacteria (LAB). Koesoemawardani (2007) reported 7.62-10.23 log CFU/g of LAB recovered from *rusip*. Kusmawarti et al. (2011) described the LAB from *rusip* from the genera *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. Hajar and Hamid (2013) also reported isolation of a species of LAB, *Staphylococcus piscifermentans*, from the fermented product *cencalok*. Leisner et al. (2008) described LAB showing chitinolytic activity isolated from food; these were of the species *Carnobacteriaum divergens* and *Carnobacterium maltaromaticum*. Our study has revealed that *rusip* also provides a distinct environment for the potentially important chitinolytic bacterial community. Isolates obtained from this study have been investigated further for their chitinase production, for application in bioconversion processing of shrimp shell waste and for possible use in antifungal compounds.

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REFERENCES

Bae S, Fleet GH, Heard GM. 2004. Occurrence and significance of *Bacillus thuringiensis* on wine grapes. Intl J Food Microbiol 94 (3): 301-312.

Ben-Gigirey B, de Sousa JMVB, Villa TG, Barros-Velazquez J. 2000. Characterization of biogenic amine-producing *Stenotrophomonas maltophilia* strains isolated from white muscle of fresh and frozen albacore tuna. Intl J Food Microbiol 57 (1): 19-31.

Bevilacqua A, Cannarsi M, Gallo M, Sinigaglia M, Corbo MR. 2010. Characterization and implications of *Enterobacter cloacae* strains, isolated from Italian table olives "Bella di Cerignola". J Food Sci 75 (1): 53-60.

Bover-Cid S, Hugas M, Izquierdo-Pulido M, Vidal-Carou MC. 2001. Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. Intl J Food Microbiol 66 (3): 185-189.

Brooke JS. 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. Clin Microbiol Rev 25 (1): 2-41.
Chang WT, Chen CS, Wang SL. 2003. An antifungal chitinase produced by *Bacillus cereus* with shrimp and crab shell powder as a carbon source. Curr Microbiol 47 (2): 102-108.
Chang WT, Chen YC, Jao CL. 2007. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. Bioresour Technol 98 (6): 1224-1230.
Chernin L, Ismailov Z, Haran S,Chet I. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl Environ Microbiol 61 (5): 1720-1726.
Das SN, Sarma PVSRN, Neeraja C, Malati N,Podile AR. 2010. Members of Gammaproteobacteria and Bacilli represent the culturable diversity of chitinolytic bacteria in chitin-enriched soils. World J Microb Biot 26 (10): 1875-1881.
Davin-Regli A, Pagès J-M. 2015. *Enterobacter aerogenes* and *Enterobacter cloacae*; versatile bacterial pathogens confronting antibiotic treatment. Front Microbiol 6: 392.
Diep CN, Cam PM, Vung NH, Lai TT. 2009. Isolation of *Pseudomonasstutzeri* in wastewater of catfish fish ponds in the Mekong Delta and its application for wastewater treatment. Bioresour Technol 100 (16): 3787-3791.
Gohel V, Singh A, Vimal M, Ashwini P, Chhatpar HS. 2006. Review-Bioprospecting and antifungal potential of chitinolytic microorganisms. Afr J Biotechnol 5 (2): 54-72.
Grimont F, Grimont PA. 2006. The genus Enterobacter. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds.). The Prokaryotes. Vol. 6. Springer, New York.
Hajar S, Hamid THTA. 2013. Isolation of lactic acid bacteria strain *Staphylococcus piscifermentans* from Malaysian traditional fermented shrimp cincaluk. Intl Food Res J 20 (1): 125-129.
Hajeb P, Jinap S. 2012. Fermented shrimp products as source of umami in Southeast Asia. J Nutr Food Sci S10:006. DOI: 10.4172/2155-9600.S10-006.
Hernández-Herrero MM, Roig-sagués AX, Rodríguez-Jerez JJ, Mora-Ventura MT. 1999. Halotolerant and halophilic histamine-forming bacteria isolated during the ripening of salted anchovies (*Engraulis encrasicholus*). J Food Prot 62 (5): 509-514.
Howard MB, Ekborg NA, Weiner RM, Hutcheson SW. 2003. Detection and characterization of chitinases and other chitin-modifying enzymes. J Ind Microbiol Biot 30 (11): 627-635.
Hsu SC, Lockwood JL. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl Microbiol 29 (3): 422-426.
Huang CJ, Wang TK, Chung SC, Chen CY. 2005. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. BMB Rep 38 (1): 82-88.
Irianto HE, Irianto G. 1998. Traditional fermented fish products in Indonesia. Report of the 26th Session of the Asia Pacific Fishery Commision-FAO. Beijing, 24-30 September 1998.
Itoi S, Okamura T, Koyama Y, Sugita H. 2006. Chitinolytic bacteria in the intestinal tract of japanese coastal fishes. Can J Microbiol 52 (12): 1158-63.
Jensen GB, Hansen BM, Eilenberg J, Mahillon J. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Environ Microbiol 5 (8): 631-640.
Kamar R, Gohar M, Jéhanno I, Réjasse A, Kallassy M, Lereclus D, Ramarao N. 2013. Pathogenic potential of *Bacillus cereus* strains as revealed by phenotypic analysis. J Clin Microbiol 51 (1): 320-323.
Kobayashi DY, Reedy RM, Bick J, Oudemans PV. 2002. Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. Appl Environ Microb 68 (3): 1047-1054.
Kobayashi T, Kajiwara M, Wahyuni M, Kitakado T, Hamada-Sato N, Imada C, Watanabe E. 2003. Isolation and characterization of halophilic lactic acid bacteria isolated from terasi shrimp paste: A traditional fermented seafood product in Indonesia. J Gen Appl Microbiol 49 (5): 279-286.
Koesoemawardani, D. 2007. Sensory analysis of rusip from Sungailiat, Bangka. Jurnal Teknologi dan Industri Hasil Pertanian 12 (2): 36-39. [Indonesian]
Kopečný J, Hodrova B, Stewart CS. 1996. The isolation and characterization of a rumen chitinolytic bacterium. Lett Appl Microbiol 23 (3): 195-198.
Köse S, Üzen F, Tufan B, Koral S, Genç S, Yaşar A. 2008. Investigating some quality parameters of several traditional fish products in relation to food safety. The 59thPacific Fisheries Technologists Annual Meeting, San Fransisco, 3-6 February 2006.
Kotiranta A, Lounatmaa K, Haapasalo M. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbiol Infect 2 (2): 189-198.
Kuddus M,AhmadIZ. 2013. Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. J Genet Eng Biotechnol 11 (1): 39-46.
Kumar R, Surendran PK. 2005. Phenotypic characterization and antibiotic resistance of *Pseudomonas* spp. from seafood and aquaculture farm environments. Fish Technol 42 (2): 203-208.
Kusmarwati A, Heruwati ES, Utami T, Rahayu ES. 2011. Effect of the addition of *Pediococcus acidilactici* F-11 as a starter culture on the quality of rusip. Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan 6 (1): 13-26. [Indonesian]
La Anh N. 2015. Health-promoting microbes in traditional Vietnamese fermented foods: A review. Food Sci Hum Well 4 (4): 147-161.
Lalucat J, Bennasar A, Bosch R, García-Valdés E,Palleroni NJ. 2006. Biology of *Pseudomonas stutzeri*. Microbiol Mol Biol Rev 70 (2): 510-547.
Lavizzari T, Breccia M, Bover-Cid S, Vidal-Carou MC, Veciana-Nogués MT. 2010. Histamine, cadaverine, and putrescine produced in vitro by enterobacteriaceae and pseudomonadaceae isolated from spinach. J Food Prot 73 (2): 385-389.
Lee KW, Park JY, Sa HD, Jeong JH, Jin DE, Heo HJ, Kim JH. 2014. Probiotic properties of Pediococcus strains isolated from jeotgals, salted and fermented Korean seafood. Anaerobe 28: 199-206.
Leisner JJ, Vogensen FK, Kollmann J, AidehB, Vandamme P, Vancanneyt M, Ingmer H. 2008. α-chitinase activity among lactic acid bacteria. Syst Appl Microbial 31 (2): 151-156.
Lim, HS, Kim SD. 1990. The role of chitinase of *Pseudomonas stutzeri* YPL-1 in biocontrol of *Fusarium solani*. Korean J Appl Microbiol Biotech 18 (2): 188-188.
Lim HS, Kim SD. 1994. The production and enzymatic properties of extracellular chitinase from *Pseudomonas stutzeri* YPL-1, as a biocontrol agent. J Microbiol Biotechnol 4 (2): 134-140.
Liu WY, Wong CF, Chung KMK, Jiang JW, Leung FCC. 2013. Comparative genome analysis of *Enterobacter cloacae*. PLoS One 8 (9): 74487. DOI: 10.1371/journal.pone.0074487
Martins ML, Pinto CL, Rocha RB, De Araujo EF, Vanetti MC. 2006. Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. Intl J Food Microbiol 111 (2): 144-148.
Ministry of Marine Affairs and Fisheries Republic of Indonesia. 2015. Marine and Fisheries in Figures 2015. Data Center for Statistics and Information, Ministry of Marine and Fisheries Republic of Indonesia, Jakarta. [Indonesian]
Ministry of Marine Affairs and Fisheries Republic of Indonesia. 2013. Marine and Fisheries in Figures 2013. Data Center for Statistics and Information, Ministry of Marine and Fisheries Republic of Indonesia, Jakarta. [Indonesian]
Nasran S, Ariyani F, Indriati N. 2003. Produksi kitinase dan kitin deasetilase dari *Vibrio harveyi*. Jurnal Penelitian Perikanan Indonesia 9 (5): 33-38. [Indonesian]
Orinda E, Puspita ID, Putra MP, Ustadi, Lelana IYB. 2015. Chitinolytic enzyme activity of isolate SDI23 from petis and the activity of its partially purified enzyme in different pH and temperature. J Fish Sci 17 (2): 96-102. [Indonesian]
Pleban S, Chernin L, Chet I. 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Lett Appl Microbiol 25 (4): 284-288.
Prashanth KVH, Tharanathan RN. 2007. Chitin/chitosan: modifications and their unlimited application potential—an overview. Trends Food Sci Tech 18: 117-131.
Rahmati T, Labbe R. 2008. Levels and toxigenicity of *Bacillus cereus* and *Clostridium perfringens* from retail seafood. J Food Prot 71 (6): 1178-1185.
Reissig JL, Strominger JL,Leloir LF. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J Biol Chem 217 (2): 959-966.
Rodrigues MJ, Ho P, López-Caballero ME, Vaz-Pires P, Nunes ML. 2003. Characterization and identification of microflora from soaked cod and respective salted raw materials. Food Microbiol 20 (4): 471-481.
Roig-Sagues AX, Hernandez-Herrero M, Lopez-Sabater EI, Rodriguez-Jerez JJ, Mora-Ventura MT. 1996. Histidine decarboxylase activity of

bacteria isolated from raw and ripened salchichón, a Spanish cured sausage. J Food Prot 59 (5): 516-520.
Sarkar PK, Hasenack B, Nout MJR. 2002. Diversity and functionality of Bacillus and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). Intl J Food Microbiol 77 (3): 175-186.
Shu-Chen C, Wang JT, Vandamme P, Hwang JH. 2004. *Chitinimonas taiwanensis* gen. nov., sp. nov., a novel chitinolytic bacterium isolated from a freshwater pond for shrimp culture. Syst Appl Microbiol 27 (1): 43-49.
Sikorski J, Möhle M, Wackernagel W. 2002. Identification of complex composition, strong strain diversity and directional selection in local *Pseudomonas stutzeri* populations from marine sediment and soils. Environ Microbiol 4 (8): 465-476.
Soeka YS, Sulistiani. 2011. Selection, characterization, and identification of chitinase-producing bacteria isolated from Mount Bromo, East Java. Jurnal Natur Indonesia 13 (2): 155-161. [Indonesian]
Someya N, Ikeda S, Morohoshi T, Noguchi TM, Yoshida T, Sawada H, Tsuchiya K. 2011. Diversity of culturable chitinolytic bacteria from rhizospheres of agronomic plants in Japan. Microbes Environ 26 (1): 7-14.
Souza CP, Burbano-Rosero EM, Almeida BC, Martins GG, Albertini LS, Rivera IN. 2009. Culture medium for isolating chitinolytic bacteria from seawater and plankton. World J Microbiol Biotechnol 25 (11): 2079-2082.
Todaro M, Francesca N, Reale S, Moschetti G, Vitale F, Settanni L. 2011. Effect of different salting technologies on the chemical and microbiological characteristics of PDO Pecorino Siciliano cheese. Eur Food Res Technol 233 (6): 931-940.
Tsai YH, Lin CY, Chang SC, Chen HC, Kung HF, Wei CI, Hwang DF. 2005. Occurrence of histamine and histamine-forming bacteria in salted mackerel in Taiwan. Food Microbiol 22 (5): 461-467.
Valero M, Hernández-Herrero LA, Fernández PS,Salmerón MC. 2002. Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. Food Microbiol 19 (5): 491-499.
Wang SL, Liang TW, Yen YH. 2011. Bioconversion of chitin-containing wastes for the production of enzymes and bioactive materials. Carbohydr Polym 84 (2): 732-742.
Wang SY, Moyne AL, Thottappilly G, Wu SJ, Locy RD,Singh NK. 2001. Purification and characterization of a *Bacillus cereus* exochitinase. Enzyme Microb Technol 28 (6): 492-498.
Younes I, Rinaudo M. 2015. Chitin and chitosan preparation from marine sources. Structure, properties and applications. Mar Drugs 13 (3): 1133-1174.
Yuli H, Robin T, Lay CC, Lee HY, Tang SY,Radu S. 2008. Characterization of *Enterobacter cloacae* isolated from street foods. ASEAN Food J 15 (1): 57-64.
Zhang Z, Yuen GY, Sarath G,Penheiter AR. 2001. Chitinases from the plant disease biocontrol agent, *Stenotrophomonas maltophilia* C3. Phytopathology 91 (2): 204-211.
Zilda DS, Chasanah E. 2005. Screening of chitinolitic bacteria from terasi. The 9th of ASEAN Food Conference Jakarta, 8-10 Agustus 2005.