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Chitinolytic Assay and Identification of Bacteria Isolated from Shrimp Waste Based on 16S rDNA Sequences

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Abstract

Shrimp waste contains 20% - 60% chitin and possible to be source of chitinolytic bacteria. Chitinolytic bacteria are capable of hydrolyzing of chitin progressively to produce N-acetylglucosamine monomer which can be used to overcome the shrimp waste. The objectives of this research were to identify species of bacteria with high activity of chitin degradation in shrimp waste and to analyze their potency as chitin degradation agent. The research consists of screening of chitinolytic bacteria based on chitinolytic index, activity assay of chitinase using colorimetric method, and molecular identification of bacteria based on 16S rDNA sequences. Two of eighteen isolates of chitinolytic bacteria (PBK 2 and SA 1.2 isolates) showed the highest chitinolytic index, which were 2.069 and 2.084, whereas chitinase activity was 0.213 and 0.219 U/ml respectively. Based on 16S rDNA sequences, isolate of PBK 2 was identified as *Acinetobacter johnsonii* 3-1, whereas SA 1.2 was identified as *Bacillus amyloliquefaciens* GR53 with 99.78% similarity.

Keywords

Chitinolytic Bacteria, Chitin, Shrimp, Shrimp Waste

1. Introduction

Shrimp is one of the important export commodities of Indonesian fishery; therefore many fishery industries provide processed shrimp products to be exported [1]. According to Sachindra *et al.* [2], generally frozen shrimp are exported in headless form or without shells (peeled), so shrimp production will produce a lot of organic waste. Shrimp waste contains 20% - 60% of chitin as major structural component of shrimp exoskeletons and

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other crustacea, mollusca, insects, and arthropods [3]. Chitin is also contained in most of fungi cells walls [4].

Chitin ($C_6H_9O_4 \cdot NHCOCH)_n$ is linier homopolysaccharide which consists of 2000 - 3000 monomers of β-1,4 linked N-acetyl-D-glucosamine). It is second-most abundant organic compound after cellulose [5]. Chitin is water insoluble and degraded naturally by microorganism such as chitinolytic bacteria using chitinase as hydrolytic enzyme [6] [7]. Chitinase is secreted to outer cells of bacteria and bind to chitin molecule to break chitin into N-acetylglucosamine monomer [8]. Chitinolytic bacteria are capable of producing chitinase and hydrolyzing chitin progressively to produce GlNAc (N-acetylglucosamine) monomer through enzymatic reaction [9]. These bacteria are found in soil, marine, lake, or chitinous waste such as industrial shrimp waste [10]. The objectives of this research were to identify species of bacteria with high activity of chitin degradation in shrimp waste based on 16S rDNA and to analyze their potency as chitin degradation agent.

2. Materials and Methods

2.1. Preparation of Colloidal Chitin

Colloidal chitin was prepared according modified method as described by Faramarzi *et al.* [11]. Ten grams of chitin from shrimp shell flake were added into 100 ml concentrated HCl (37%) and kept in vigorous stirring for 2 h at room temperature or until chitin completely dissolved. The suspension was precipited by slowly added to 500 ml of ice-cold absolute ethanol. Then pH of suspension was neutralized with 10 N NaOH. Suspension was centrifuged at 8000 rpm for 10 min and the precipitate was ready to use as medium substrate.

2.2. Sample Collection and Isolation of Chitinolytic Bacteria

Solid waste (shrimp-shells) and shrimp wastewater aseptically collected from PT. Bumi Menara Internusa (08˚13'03.08"S, 112˚44'49.1"E). Chitinolytic bacteria were isolated using spread plate method in CCA medium (Colloidal Chitin Agar) which consist of (g/L): Na_2HPO_4 (6); KH_2PO_4 (3); NH_4Cl (1); NaCl (0.5); yeast extract (0.05); agar (15) and colloidal chitin 0.5% (w/v). Chitinolytic bacteria showed by clear zones surrounding colonies after 72 h of incubations at 30˚C [8].

2.3. Primary Screening of Chitinolytic Bacteria

Primary screening was performed by disc diffusion modified method of Jiang [12] with three replications. A loopfull of bacteria inoculated in colloidal chitin broth containing 1% colloidal chitin (w/v) and incubated for 24 h, 180 rpm, 30˚C [9]. Cultures of each isolate (10^7 cells/ml) was inoculated to sterile paper disc (d = 5 mm, Whatmann filter paper No. 1) and put on medium agar surface supplemented with 1% colloidal chitin and incubated at 30˚C [5]. Chitinolytic index data (ratio of clear zone and colony sizediameter) were recorded up to 7 days incubations [11]. Data of chitinolytic index were analyzed using analysis of variance (ANOVA) that continued with *Games-Howel* test ($\alpha = 0.05$) using *SPSS for Windows V*.16.

2.4. Secondary Screening of Chitinolytic Bacteria

Chitinase activity was determined using colorimetric method as described by Monreal and Reese [13] with three replications. Reaction mix consist of 1 ml crude enzyme, 1.5 ml of 1% colloidal chitin substrate in 200 mM potassium phosphate buffer (pH 6.0). The mixture was incubated at 30˚C for 2 h, boiled for 10 min to stop reaction, and centrifuged at 8000 rpm for 20 min. Then 1 ml of test supernatant was added to 1 ml of DNS (Dinitrosalicylic Acid), boiled for 5 min, and cooling down at room temperature. Released of GlcNAc (N-acetylglucosamine) was measured at 540 nm. Standard curve of GlcNAc was plotted between GlcNAc concentration and GlcNAc absorbance. One unit of chitinase activity was described as the amount of enzyme which liberate 1.0 mg GlcNAc per hour from chitin substrate under reaction condition. Data of chitinase activity were analyzed with *Independent Sample T-test* analysis statistic ($\alpha = 0.05$) using *SPSS for Windows V*.16.

2.5. DNA Extraction of Chitinolytic Bacteria

Two loopfull of chitinolytic bacteria was inoculated in Luria Bertani broth (Merck) and incubated for 24 hours, 120 rpm, 30˚C. Whole genome extraction was carried out by Ausubel method [14].

2.6. Amplification and Sequencing of 16S rDNA

Sequences of 16S rDNA was amplified with universal primers 27F (5'GAGAGTTTGATCCTGGCTCAG3') and 1492R (5'CTACGGCTACCTTGTTACGA3') [15]. PCR master mix solution was prepared as described by Intron Biotechnology [16] as shown in **Table 1**, PCR reaction condition was based on method of Mulhardt [17] and Zarei *et al.* [18] as shown in **Table 2**. Amplicon of 16S rDNA was separated by 1% electrophoresis agarose gels [10]. Amplicon of partial 16S rDNA was purified and sequenced for further process in First Base, Malaysia. Sequencing was carried out using same primers. Partial sequences was matched with nucleotide database in Gene Bank (http://www.ncbi.nlm.nih.gov) using BLASTN to identify bacteria isolate based on highest % nucleotide similarity.

2.7. Phylogeny Tree Construction

Sequences of 16S rDNA was matched with sequences of reference strains (**Table 3**) in *GenBank* database (http://www.ncbi.nlm.nih.gov) and was aligned through *Clustal W Multiple Alignment* tool. Phylogeny tree was constructed using phylogeny tool in *MEGAV*.6 *program*, inferenced using *Maximum Likelihood* method, and analyzed with evolutionary distance using *Tamura-Nei* model [19]. Outgroup of phylogeny tree was choosed according to similar genus and has been reported as chitinolytic bacteria.

3. Results and Disscussion

3.1. Chitinase Activity of Shrimp Waste Chitinolytic Bacteria

Eighteen isolates with different morphology of colonies were isolated from shrimp waste, 14 isolates from wastewater, and 4 isolates from shrimp-shells waste. Wastewater aeration can support more bacterial growth. During aeration, bacteria will grow and survive by using oxygen to breakdown wastewater compound and combine with organic matter in wastewater to form an activated sludge [20]. Based on primary screening, two of 18 isolates showed the highest chitinolytic index (CI > 2), there were isolates PBK 2 and SA 1.2 with chitinolytic index 2.069 dan 2.084 respectively. Clear zone surrounding the colony indicates chitinase activity to breakdown chitin compound in medium [8]. From primary screening result, isolate PBK 2 and SA 1.2 were selected for chitinase activity assay.

As shown in **Figure 1**, isolate PBK 2 and SA 1.2 showed almost similar chitinase activity (0.213 and 0.219 U/ml respectively). Chitinase activity is amount of product which is produced from chitin substrate derivation.

Table 1. PCR master mix composition.

Composition	Volume (µl)	Concentration
ddH_2O	6	-
2x PCR *Master Mix* (i-Taq™)	15	-
Primer 1 (27f)	3	10 pmol/µl
Primer 2 (1495r)	3	10 pmol/µl
DNA template	3	1 µg

Table 2. PCR reaction condition.

Reaction	Temperature (˚C)	Time (minutes)
Pre-denaturation	94	5
35 cycles : denaturation	94	0.5
Annealing	55	0.5
Extension	72	1.5
Post extension	72	5

Table 3. Reference strains for phylogeny construction based on 16S rDNA sequences.

No.	*Acession number*	Species	Strain
1	EU594557	*Acinetobacter johnsonii*	3-1
2	JQ039983	*Acinetobacter johnsonii*	YNB71
3	EU730929	*Acinetobacter johnsonii*	178
4	KJ569367	*Acinetobacter schindleri*	C47EM
5	KJ569366	*Acinetobacter schindleri*	EM21
6	NR025412	*Acinetobacter schindleri*	LUH5832^T
7	AB859678	*Acinetobacter schindleri*	MTCC 9827
8	FJ373024	*Acinetobacter schindleri*	W1-2
9	JX315564	*Acinetobacter schindleri*	URT27
10	HQ689693	*Acinetobacter schindleri*	IBP-SL13
11	Z93438	*Acinetobacter junii*	ATCC 17908^T
12	Z93434	*Acinetobacter calcoaceticus*	ATCC 23055^T
13	EF423606	*Bacillus amyloliquefaciens*	ATCC 15841
14	EF423604	*Bacillus amyloliquefaciens*	ATCC 21556
15	EF423607	*Bacillus amyloliquefaciens*	ATCC 21770
16	NR118950	*Bacillus amyloliquefaciens*	ATCC 23350^T
17	DQ993675	*Bacillus amyloliquefaciens*	ATCC 49763
18	EF423605	*Bacillus amyloliquefaciens*	BCRC 11266
19	EF433406	*Bacillus amyloliquefaciens*	BCRC 11601^T
20	KJ937782	*Bacillus amyloliquefaciens*	GR53
21	KC692168	*Bacillus amyloliquefaciens*	ML265
22	AB679995	*Bacillus amyloliquefaciens*	NBRC 3037
23	NR114581	*Bacillus thuringiensis*	ATCC 10792^T

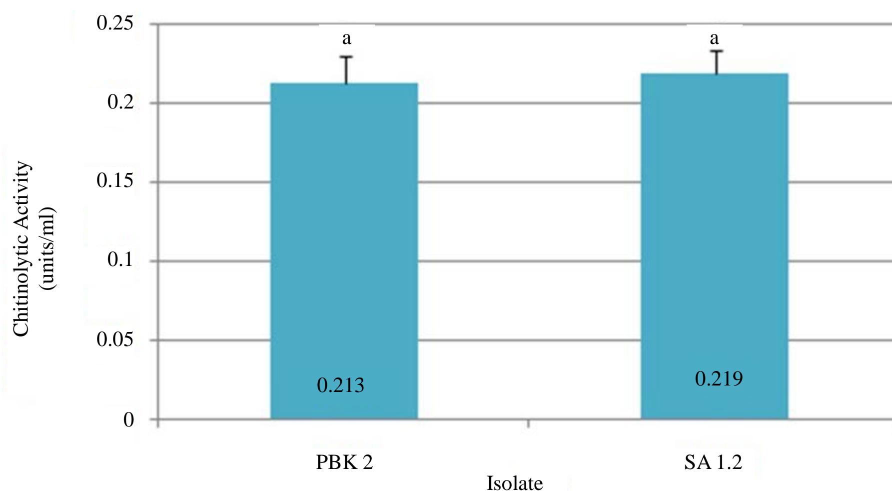

Figure 1. Chitinase activity of PBK 2 and SA 1.2 isolate at pre-stationary phase of growth.

Chitin is substrate which can induce exochitinase and endochitinase formation in microorganisms [21]. Rate of chitin waste degradation is influenced by chitinase activity, enviromental factors, indigenous microorganisms metabolism from carbon utilization as source of energy, protein synthesis, and releasing cell metabolism products [22].

Chitinase activity of microorganisms is different from each other depends on various factors such as time of enzymatic reaction, enzyme and substrate concentation, incubation time, and pH of medium [23]. Based on Lamine *et al.* [24] *Serratia marcescens* DSM 30121^T has the highest chitinase activity (0.556 U/ml) when was cultured in medium containing 1% colloidal chitin concentration, at 30˚C, and pH-value of 6. Based on Saleem *et al.* [25] *Bacillus thuringiensis* isolate CMBL-Bt4 has the highest chitinase activity than 12 of others *Bacillus thuringiensis* (0.23 U/ml) after 4 days incubation at 37˚C with pH value 7 for culture medium. Meanwhile, according to Saadoun *et al*. [26] isolate *Streptomyces* sp. S_{242} has the highest chitinase activity (0.162 U/ml) after 4 days incubation at 30˚C with pH value 7 for culture medium.

3.2. Taxonomy of Isolate PBK 2 and SA 1.2 Based on 16S rDNA Sequences

Amplicon 16S rDNA sequences of isolate PBK 2 and SA 1.2 on 1% agarose gels showed in 1500 bp (**Figure 2**). According to Nocker *et al.* [27] amplification 16S rDNA sequences of bacteria can produce 1500 - 1600 bp amplicon. Analysis of DNA sequences showed that isolate of PBK 2 was identified as *Acinetobacter johnsonii* 3-1, whereas SA 1.2 identified as *Bacillus amyloliquefaciens* GR53 with 99.78% similarity respectively. Identification criteria at species level consist of strains which have 16S rDNA sequences similarity more than 97%, whereas sequences similarity more than 99% include in strain level [28].

Genus *Acinetobacter* and *Bacillus* were dominant bacteria in industrial wastewater especially in activated sludge flocs which involved in denitrification process with other genus such as *Pseudomonas*, *Spirillum*, *Hyphomicrobium*, *Agrobacterium*, *Propionibacterium*, *Rhizobium*, *Corynebacterium*, *Cytophaga*, *Thiobacillus*, and *Alcaligenes* [29]. Genus *Acinetobacter* is one of the phosporus accumulating microorganisms in wastewater such as *Acinetobacter johnsonii* [30] and *Acinetobacter calcoaceticus* [31]. Those bacteria will accumulate phosphorus as polyphosphate in granules in aerobic condition for cell synthesis and energy transportation, this stage can reduce phosphorus in wastewater 10% - 30%, then inorganic phosphate was released in anaerobic condition [29].

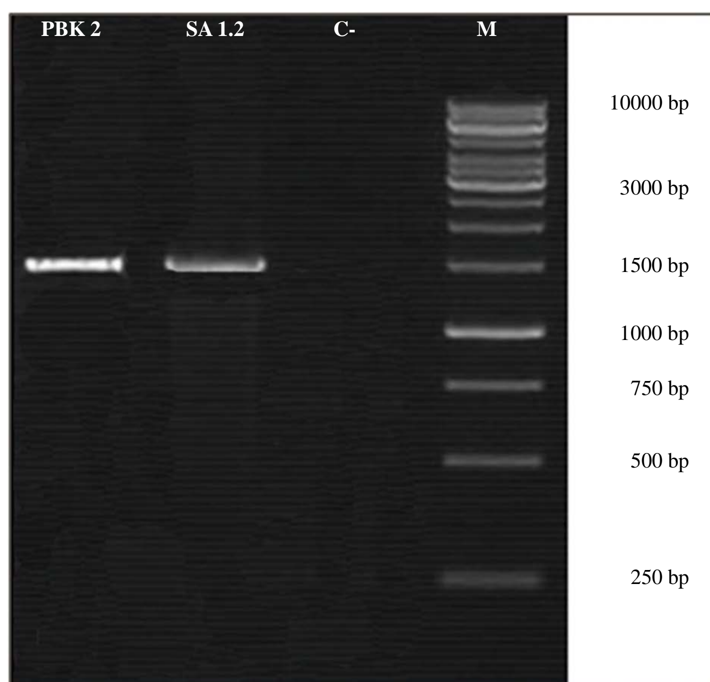

Figure 2. 16S rDNA purified amplicon of bacteria isolate compared with DNA *Ladder Mix* 1 kb (C− = negative control, M = marker).

As shown in **Figure 3**, isolate PBK 2 revealed close phylogenetic relationship with *Acinetobacter johnsonii* 3-1, whereas isolate SA 1.2 revealed close phylogenetic relationship with *Bacillus amyloliquefaciens* GR53. Based on microscopic characterization, isolate PBK 2 was Gram negative bacteria with *coccibacil*-shape and non-endospore forming accordance with *Acinetobacter johnsonii*, meanwhile isolate SA 1.2 was Gram positive, endospore forming, and rod-shape bacteria accordance with *Bacillus amyloliquefaciens*. Chitinase activity of *Bacillus amyloliquefaciens* was reported for the first time by Sabry [32]. Those bacteria can utilize shrimp shell

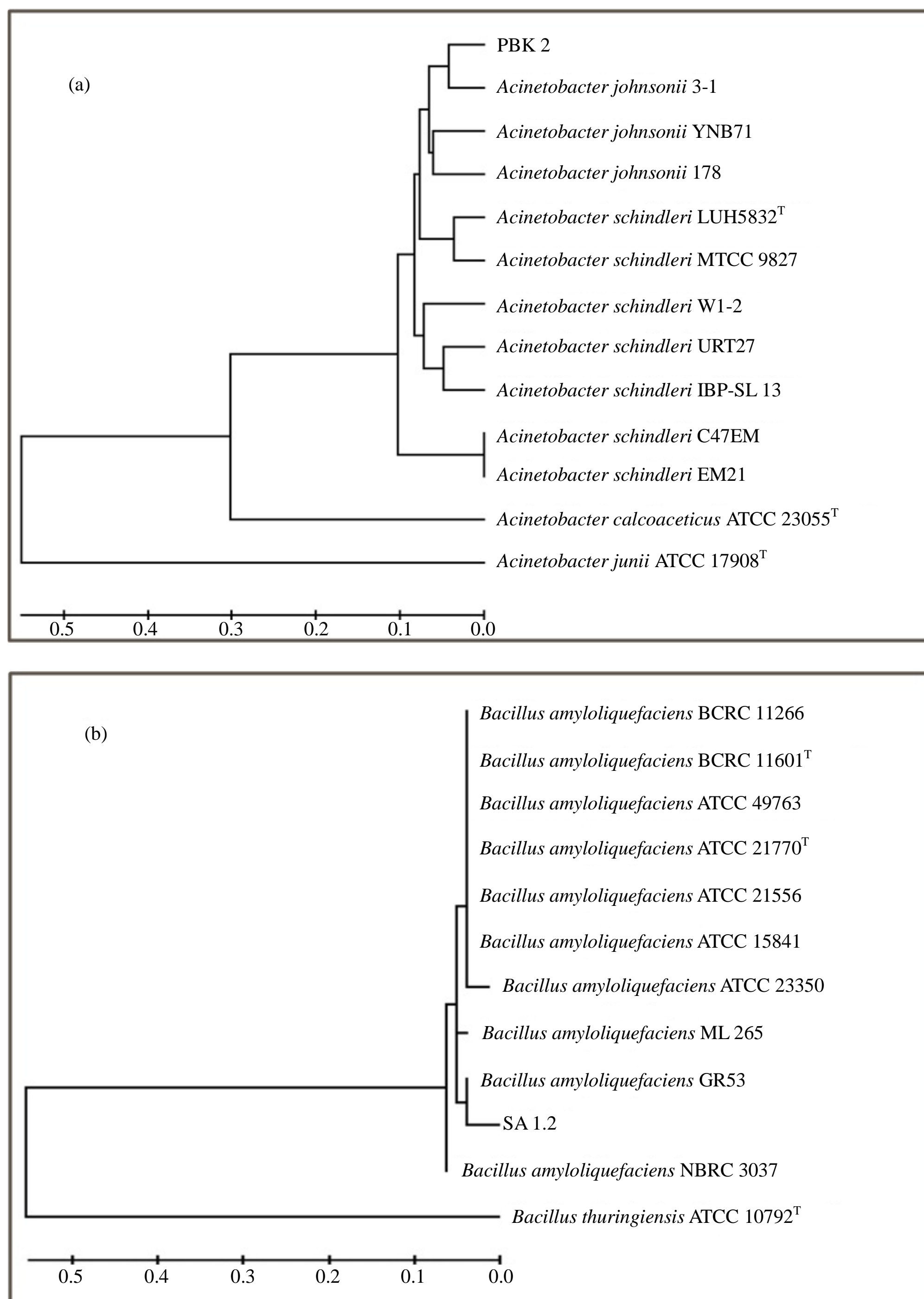

Figure 3. Phylogeny tree of isolate (a) PBK 2; (b) SA 1.2 compared with reference isolate using *Maximum Likelihood* algorithm with *Tamura-Nei* analysis method.

to produce chitinase. According to Wang *et al.* [33]. Gram positive bacteria which identified as *Bacillus amyloliquefaciens* V656 based on analysis 16S rDNA can produced antifungal enzyme. This enzyme showed extracelullar chitinase activities against fungi. *Acinetobacter johnsonii* was reported as one of the chitinase-producing bacteria isolated from proximal intestine of Atlantic Salmon. Genus *Acinetobacter* and *Bacillus* were dominant enzyme-producing bacteria in the intestine of fish with chitin diet treatment [34].

4. Conclusion

Two of eighteen isolates of chitinolytic bacteria (PBK 2 and SA 1.2 isolates) showed the highest chitinolytic index, which were 2.069 and 2.084, whereas chitinase activity was 0.213 and 0.219 U/ml respectively. Based on 16S rDNA sequences, isolate of PBK 2 was identified as *Acinetobacter johnsonii* 3-1 with 99.78% similarity, whereas SA 1.2 was identified as *Bacillus amyloliquefaciens* GR53 with 99.78% similarity.

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