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Molecular identification of bacteria isolated from culture medium of rotifer fed on fishery waste diet

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Abstract. *Wullur S, Napitupulu H, Wantania LL, Ginting EL, Warouw V, Tilaar S, Tallei TE, Rumengan IFM. 2020. Molecular identification of bacteria isolated from culture medium of rotifer fed on fishery waste diet. Biodiversitas 21: 2735-2740.* The aim of this study was 16S-rRNA sequences based molecular identification of bacteria isolated from culture medium of rotifer fed with fishery waste diet (FWD). We cultured rotifer *Brachionus rotundiformis* in sterilized seawater (salinity 25 ppt) using FWD, following the procedure in Patent No. P00201609066. Bacteria from the culture were collected, homogenized, diluted 10 to 1000 fold, spread on agar plates and incubated at 37^0C for 24 to 48 hours. Representative colonies of the bacteria according to their morphologies were isolated for further characterization. Genomic DNA of the isolates were extracted, and the 16S rRNA gene of the isolates were amplified. Polymerase Chain Reaction (PCR) product of each isolate was sequenced and queried against the NCBI GenBank database. Six different isolates based on size, color, elevation, margin, and colony were observed during 24-48 hours incubation at 37^0C. The 16S rRNA genes of the six isolates were successfully amplified and produced DNA band at 1300-1500 bp, with quality value equal to or greater than 20 (QV20+) of each entire sequence around 941-1253 bases. Basic Local Alignment Search Tool (BLAST) queries in the NCBI GenBank and EzBioCloud database using the 16S-rRNA gene sequences showed that the six isolates belong to four different genera, i.e: *Bacillus*, *Staphylococcus*, *Vibrio*, and *Alteromonas.*

Keywords: Bacteria, diet, fish-waste, identification, rotifer, 16S rRNA

INTRODUCTION

The use of rotifer (*Brachionus plicatilis* species complex) as live-food for newly hatched fish larvae was initiated in early 1960s (Hagiwara et al. 2014; Hagiwara et al. 2017). Today, most hatcheries use the rotifers as indispensable live-food for fish larviculture (Kotani et al. 2017; Wullur 2017) due to their benefits such small body size, rapid reproductive rate, high population growth, relatively slow in motility, mass culture possibility and its nutritional value (Wullur et al. 2011; Wullur et al. 2013; Hagiwara et al. 2014; Rumengan et al. 2016; Wati and Imanto 2018; Wullur et al. 2018; Lee et al. 2019).

Procedure used today for culturing the rotifers uses microalgae i.e. *Chlorella* sp. *Nannochloropsis oculata*, etc (Hagiwara et al. 2017). In fact, production cost of microalgae is high due to the high investment costs and running expenses (Acien et al. 2017). For this reason, cheap food sources based on baker's yeast were introduced (Hagiwara et al. 2014; Hagiwara et al. 2017), but it provided less nutritional value particularly for eicosapentaenoic acid (20:5 n-3) and docosahexaenoic (22:6 n-3) to support growth of fish larvae. Also, the baker's yeast often was a source of unstable culture or even culture crashes due to the rapid decline of water quality of the culture and therefore it is currently less attractive for fish farmers (Hagiwara et al. 2014; Hagiwara et al. 2017; Ogello et al. 2018; Ogello et al. 2019; Wullur et al. 2019; Ogello et al. 2020). During the late 1980s, a Japanese Chlorella industry company (Fukuoka, Japan) has developed a microalga paste Chlorella V-12® as food for rotifer. The microalgal paste is convenient since it is ready-to-use and could be stored for about 2 weeks without significant loss of its nutritional value (Hagiwara et al. 2014; Hagiwara et al. 2017). The microalgal paste is also more stable food for rotifer that enables aquaculturists to mass culture rotifer at high density but its price is high and not commonly available in hatcheries particularly in developing countries (Hagiwara et al. 2014). Recently, a cheap and ready-to-use diet for rotifer based on fishery-waste is being developed as an alternative to the microalgae-based feed (Ogello et al. 2018; Napitupulu et al. 2019; Ogello et al. 2019; Wullur et al. 2019; Ogello et al. 2020). Acceptance of rotifer on the diet was great, where population growth of rotifer fed the diet increased significantly about 2000 to 3000 ind./ml within approximately 3 to 5 days culture (Wullur et al. 2017), and its dietary value to fish larvae was comparable to rotifer fed with microalgae paste (Ogello et al. 2018; Ogello et al. 2019; Wullur et al. 2019; Ogello et al. 2020).

The fishery diet acts as a source for bacterial bloom which further used by the rotifer as their nutritional source for growth and development, but the bacterial species involved in decomposing the fishery diet is unknown until recently. To address this lack of information, a small-ribosomal subunit 16S rRNA gene was used to identify

species of bacteria at molecular level in the present study. The 16S rRNA gene is commonly used to study bacterial taxonomy as it is present in almost all bacteria species, its function over time has not changed, and its length (1.500 bp) is large enough for informatics purpose (Fuks et al. 2018; Wantania et al. 2019; Wondal et al. 2019; Akihary et al. 2020). Understanding the bacterial composition in rotifer cultures is important for the aquaculture industry.

MATERIALS AND METHODS

Procedures

Bacterial isolation and culture

Bacterial isolates in this study were obtained from culture of rotifer *Brachionus rotundiformis* fed with fishery waste diet. The procedure for the preparation of the fishery waste diet was adapted as described in Patent No. P00201609066 registered in Indonesia. Culture of rotifer was conducted using sterilized seawater at salinity 25 ppt and placed in a room at ambient temperature at around 25-28˚C. Bacterial isolation was conducted by taking 1 mL water sample from the rotifer culture (rotifer population with the density of about 100 ind./ml) and was serially diluted (for 10 to 1000-fold) in sterile seawater and plated on 2% nutrient agar (NA). The NA plates were incubated for 24-48 hours at 37˚C for bacterial colonies observations following Laboffe (2012). Representative distinct colonies of the bacteria according to their phenotype characters were isolated and grown in nutrient broth for 24-48 hours, harvested, and centrifuged (14.000 rpm for 5 minutes) for molecular analysis.

Molecular identification

Genomic DNA of the bacterial isolates were extracted using Qiaprep Miniprep Kit (Qiagen, Hlden, Germany) according to the manufacture's instruction. The bacterial 16S rRNA gene was amplified using the universal PCR primer pairs (Integrated DNA Technologies-IDT, Singapore) 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-ACCTTGTTACGACTT-3') (Fuks et al. 2018; Wantania et al. 2019; Wondal et al. 2019; Akihary et al. 2020). The amplification reaction was performed in a total volume of 25 µl, consisting of 5 µl of 5x Hotfirepool, 17 µl of ddH2O, 1 µl of Primer 8F, 1 µl of Primer 1492R, and 1 µl sample. Amplification for 35 cycles was performed in Professional Thermocycler (Biometra, Analytik Jena). The temperature profile for PCR was 95˚C for 6 min (1 cycle), 95˚C for 30 sec, 52˚C for 30 sec, and 72˚C for 30 sec (35 cycles), followed by 72˚C for 10 min at the end for final cycle. PCR products were electrophoresed on 1% agarose gel then visualized with ethidium bromide staining to check the success of PCR amplification. For DNA sequencing, the amplicons and the primers (forward and reverse) were sent to First-Base Co., Selangor, Malaysia. A BigDye® Terminator v.3.1 Cycles Sequencing Kit (Applied Biosystems, USA) was used to bi-directionally sequence the DNA, and read using an ABI PRISM® 377 automatic DNA sequencer. Sequences quality was assessed using Sequence Scanner version 2.0 Software (Applied Biosystem) and the sequence traces were trimmed, assembled and manually edited using Geneious Prime version 2020 (http://www.geneious.com, Kearse et al., 2012) prior to being subjected to 16S rRNA sequence BLAST analysis at The National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) and EzBioCloud (https://www.ezbiocloud.net/) (Yoon et al. 2017). BLAST result data were limited to identity percent values between 97-100%, and query cover between 98-100%.

RESULTS AND DISCUSSION

It is well known that rotifers consume bacteria as their nutritional source for growth and reproduction (Wullur et al. 2019). In nature, the bacteria are important food components in rotifer microbial web, while in culture, the bacteria proved to have beneficial effect on culture quality of rotifers (Loo et al. 2016) and reared fish larvae (Nevejan et al. 2018). On the other hand, there are reports which indicated bacteria as possible cause of culture crash on rotifer or larval mortality due to pathogenic outbreak (Hache and Plante 2011). Population densities of rotifer cultured using the FWD in the present study were around 100 ind./mL at which the bacterial samples were collected. The capability of rotifer to grow and reproduce in FWD without any microalgae addition as the current procedure in larvicuture industry was also reported previously (Ogello et al. 2018; Ogello et al. 2019; Wullur et al. 2019; Ogello et al. 2020). We suggested that the FWD was the source of bacteria bloom inside the culture medium and further utilized by the rotifer as their nutritional source.

The size, color, elevation, margin, and type are well-known characters in characterization of bacterial colonies (Sousa et al. 2015). By collecting samples from the culture medium of rotifer, we successfully isolated 6 different colonies of bacteria based on the characters of colony morphology. All isolates in this study showed small sized round type colonies (isolates F0032, F0033, F0034, F0035, F0036, and F0037) except isolate F0031, having large-sized colony in comparison to other isolates. The colony color of isolates F0031, F0032, F0033, and F0035 were milky white, while whitish-pink for isolates F0036 and F0037. The colonies elevation was umbonate (F0031) and convex (F0032, F0035, F0036 and F0037), while the margins were erose (F0031) and smooth (F0032, F0033, F0035, F0036 and F0037).

Bacteria can be transmitted to fish or crustacean larvae when rotifers are given as food, and the bacteria could increase growth and survival of the larvae or vice versa. According to Fialkowska et al. (2019) and Onianwah et al. (2018), the main genera identified in rotifer and fish larviculture were *Pseudomonas*, *Vibrio*, *Moraxella*, and *Flavobacterium*. In the present study, based on BLAST result using database of 16S rRNA at GenBank and EzBioCloud gene marker, we identified 5 different bacterial species (*Bacillus cereus* species complex, *Vibrio rotiferanus*, *Bacillus jeotgali*, *Staphylococcus warneri*, and *Alteromonas fortis*) belonging to 4 different genera

(*Bacillus*, *Vibrio*, *Staphylococcus*, and *Alteromonas*) which present in the culture of rotifer fed FWD (Table 1).

Two isolates (F0031 and F0033) identified were belonged to genera of *Bacillus*, a genus of gram-positive, rod-shaped bacteria, that have been used for years as probiotics, which confer health benefits to host. The isolate F0031 has similar value (i.e. score, query cover, e-value and identity; 2593, 99%, 0.0, and 99.79%, respectively) to *B. wiedmannii* (NR_152692.1), *B. proteolyticus* (NR_157735.1) and *B. cereus* (NR_074540.1; NR_113266.1; NR_115714.1) (Table 1). According to Miller et al. (2016; 2018) and Liu et al. (2017), *B. wiedmannii, B. proteolyticus* and *B. cereus* were members of *B. cereus* species complex, which comprises about 12 closely related species, and other 9 new members of the *Bacillus* species complex as proposed by Liu et al. (2017). *B. cereus* is a gram-positive, aerobic-to-facultative, spore-forming rod-shaped bacteria having wide distribution in the environment (Bottone 2010). *B. cereus* is a pathogenic bacterium that is harmful to humans and causes foodborne illness, but some strains of *B. cereus* have long been known as probiotic bacteria in rotifer culture (Md et al. 2015; Loka et al. 2016). In spite of that, *B. cereus* was also reported to have a significant role in enhancing growth performance of fish fingerlings (Chandran et al. 2014; Vargas-Albores et al. 2017; Sultani et al. 2019). Vargas-Albores et al. (2017) reported that *B. cereus* isolated from tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) revealed growth and immune enhancer features for shellfish. *B. cereus* was reported to have the capability of enhancing various immune parameters, i.e. phenoloxidase, lysozyme, respiratory burst, and bactericidal activity in *P. monodon* (Chandran et al. 2014).

Table 1. Top five hits against Nucleotide BLASTed of 16S-rRNA gene of the bacterial isolates using rRNA type strains/prokaryotic16S_ribosomal RNA (bacteria and archaea) database setting in the NCBI GenBank

Isolates	Species & Accession number	Score	Query cover	E-value	Identity
F0031	*Bacillus wiedmannii* (NR_152692.1)	2593	99%	0.0	99.79%
	Bacillus proteolyticus (NR_157735.1)	2593	99%	0.0	99.79%
	Bacillus cereus (NR_074540.1)	2593	99%	0.0	99.79%
	Bacillus cereus (NR_113266.1)	2593	99%	0.0	99.79%
	Bacillus cereus (NR_115714.1)	2593	99%	0.0	99.79%
F0032	*Vibrio rotiferanus* (NR_118091.1)	2597	100%	0.0	99.65%
	Vibrio rotiferanus (NR_042081.1)	2597	100%	0.0	99.65%
	Vibrio campbellii (NR_119050.1)	2588	99%	0.0	99.58%
	Vibrio campbellii (NR_113782.1)	2577	100%	0.0	99.30%
	Vibrio alginolyticus (NR_118258.1)	2562	100%	0.0	99.23%
F0033	*Bacillus jeotgali* (NR_025060.1)	2567	99%	0.0	99.30%
	Bacillus boroniphilus (NR_041275.1)	2564	99%	0.0	99.23%
	Bacillus thioparans (NR_043762.1)	2562	99%	0.0	99.23%
	Bacillus subterraneus (NR_104749.1)	2551	99%	0.0	99.09%
	Bacillus selenatarsenatis (NR_041465.1)	2536	99%	0.0	99.22%
F0035	*Stphylococcus warneri* (NR_025922.1)	2603	100%	0.0	99.65%
	Stphylococcus pasteuri (NR_024669.1)	2579	100%	0.0	99.37%
	Stphylococcus pasteuri (NR_114435.1)	2562	99%	0.0	99.29%
	Stphylococcus epidermis (NR_036904.1)	2516	100%	0.0	98.59%
	Staphylococcus epidermis (NR_113957.1)	2512	100%	0.0	98.52%
F0036	*Alteromonas fortis* (MK007076) (EzBioCloud)	-	-	-	99.56%
	Alteromonas litorea (AY428573) (EzBioCloud)	-	-	-	99.34%
	Alteromonas tagae (NR_043977.2)	2407	100%	0.0	97.88%
	Alteromonas litorea (NR_025780.1)	2403	100%	0.0	97.88%
	Alteromonas confuentis (NR_137375.1)	2396	100%	0.0	97.71%
	Alteromonas mediterranea (NR_148755.1)	2390	100%	0.0	97.64%
	Alteromonas mediterranea (NR_148754.1)	2388	100%	0.0	97.64%
F0037	*Alteromonas fortis* (MK007076) (EzBioCloud)	-	-	-	99.40%
	Alteromonas macleodii (CP003841) (EzBioCloud)	-	-	-	99.16%
	Alteromonas macleodii (NR_114053.1)	2435	97%	0.0	98.68%
	Alteromonas macleodii (NR_037127.1)	2429	97%	0.0	98.76%
	Alteromonas mediterania (NR_148755.1)	2422	100%	0.0	97.87%
	Alteromonas litorea (NR_025780.1)	2407	100%	0.0	97.65%
	Alteromonas mediterania (NR_148753.1)	2405	100%	0.0	97.65%

Findings by He et al. (2017) suggested that the use of *B. cereus* in combination with *Clostridium butyricum* and *Lactobacillus acidophilus* can be superior to the growth and production of digestive enzymes of hybrid grouper (*Epinephelus lanceolatus* and *E. fuscoguttatus*). On the same hand, Divya et al. (2015) found that *B. cereus*, in combination with *B. subtilis*, *B. licheniformis* and *B. coagulans* are suitable for bioremediation of organic detritus although they did not seem to be naturally present in sufficient population densities in either the water column or sediment. Extracellular products of *B. cereus* together with *B. subtilis* strongly inhibited the growth of pathogenic bacteria *Aeromonas hydrophila* and *Vibrio alginolyticus* isolated from diseased fish (Murillo and Villamil 2011). Isolate F0033 showed highest similarity with *Bacillus jeotgali* (NR_025060.1), *B. boroniphilus* (NR_041275.1), *B. thioparans* (NR_043762.1), *B. subterraneus* (NR_104749.1) and *B. selenatarsenatis* (NR_041465.1) (Table 1). *B. jeotgali* is a probiotic bacterium, firstly isolated from a Korean traditional fermented seafood (Jeotgal) made from salted marine organisms such as fish, molluscs and crustaceans (Yao et al. 2019). There were no prior reports so far on the presence of this probiotic bacterium in rotifer culture, but the bacterium was reported to have beneficial effect on survival and development of *Litopenaeus vannamei* larvae (Xue et al. 2016).

The isolate F0032 showed highest similarity to *Vibrio rotiferianus* (NR_118091.1; NR_042081.1; NR_119050.1), *V. campbellii* (NR_113782.1; NR_118258.1) and *V. alginolyticus* (NR_118258.1). Ke et al. (2017) grouped the three bacterial species in the *harveyi* clade of the genus *Vibrio* together with *V. harveyi, V. parahaemolyticus, V. mytili, V. natriegens* and *V. azureus*. According to Chowdhury et al. (2011), the *V. rotiferianus* had ≤ 70% DNA hybridization similarity to its most closely related species, *V. campbellii* and *V. harveyi*, although, they shared 99% identity in 16S rRNA gene sequences. The *V. rotiferianus* is a gram-negative bacterium, was firstly assigned as a new species in 2003 and was named after its isolation source, i.e. from rotifer cultures (Ke et al. 2017). Although *V. rotiferianus* was found in rotifer culture, however, there were no reports on its pathogenicity to fish larvae through transmission from rotifer. The *Vibrio* genus is often reported as inhabitants at low density of marine fish larvae culture even though there were no symptoms of vibriosis appearing (Hannan et al. 2019; Istiqomah et al. 2020). On the other hand, Sahandi et al. (2019) reported the significant use of yeast or probiotics bacteria in reducing growth of *Vibrio*.

Isolate F0035 showed highest similarity to *Staphylococcus warneri* (NR_025922.1), *S. pasteuri* (NR_024669.1; NR_114435.1), *S. epidermis* (NR_036904.1; NR_113957.1). These three species are member of the bacterial genus *Staphylococcus*, the gram-positive bacteria commonly found as part of the skin flora on humans and animals. *S. warneri* was reported as a resident in skin of rainbow trout and was not pathogenic or has a very low pathogenicity to the marine fish even when injected at very high concentrations (Musharrafieh et al. 2014). So far, there is no report on the presence of *S. warneri* in the culture of rotifer and its transmission from rotifer to fish larvae.

Isolate F0036 and F0037 showed lower similarity to several species of *Alteromonas* retrieved from GenBank. However, using EzBioCloud, F0036 and F0037 were identified as *Alteromonas fortis* with similarity of 99.56% and 99.40%, respectively. Threshold of 98.65% similarity for bacterial delineation using 16S rRNA gene has been proposed previously by Kim et al. (2014) to differentiate two bacterial species. *Alteromonas* is a genus of *Proteobacteria* found in sea water, either in the open ocean or in the coast. Its cells are curved rods with a single polar flagellum (Barbeyron et al. 2019). Genus *Alteromonas* have been well-documented previously as probiotic in aquaculture (Kesarcodi et al. 2010). The genus was reported to increase the survival of Pacific oyster, *Crassostrea gigas*, when administered in water (Irianto and Austin 2002), inhibit pathogenic bacteria *Vobrio harveyii* and promote the growth and survival of shrimp larvae (Haryanti et al. 2017). *A. fortis* is a non-flagellated bacterium specialized in the degradation of iota-carrageenan (Barbeyron et al. 2019). There were no reports so far on the presence of *A. fortis* in culture of rotifer and fish larvae.

Altogether, we show here that culture medium of rotifer fed FWD containing 5 different bacterial species i.e. *B. cereus* sp. complex (F0031), *V. rotiferanus* (F0032), *B. jeotgali* (F0033), *S. warneri* (F0035), and *A. fortis* (F0036; F0037) in the genus of *Bacillus, Vibrio, Staphylococcus* and *Alteromonas*. The bacterial species may involve in decomposing the FWD and further become food source for rotifer.

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