Molecular identification bacteria pearl oyster

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Molecular identification of bacteria isolated from culture medium of the gold-lipped pearl oyster *Pinctada maxima* larvae

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Abstract. *Wullur S, Napitupulu H, Wantania LL, Ginting EL, Mamangkey NGF, Smolak R, Ogello E. 2020. Molecular identification of bacteria isolated from culture medium of the gold-lipped pearl oyster* Pinctada maxima *larvae. Biodiversitas 21: 5291-5297.* This study was conducted for the molecular identification of bacteria species isolated from culture medium of the gold-lipped pearl oyster, *Pinctada maxima* larvae. The pearl oysters were cultured using live-microalgae (*Isochrysis* sp) and fish waste diet (FWD) as food sources. Bacteria were isolated from the oyster larvae and identified based on the 16S rRNA gene sequence. The isolated bacteria were grown on agar plate and incubated at 37˚C for 24 to 48 hours. Representative colonies of the bacteria were selected and cultured for molecular analysis. The 16S rRNA genes of the bacteria were amplified and the sequences were matched with the NCBI GenBank database. Seven different colonies were observed based on morphological characters. Similarity test by conducting the Basic Local Alignment Search Tool (BLAST) in the NCBI GenBank database, using the 16S rRNA gene sequences showed that the seven isolates colonies possess high similarity to five bacteria species i.e. *Pseudomonas pachastrellae*, *Vibrio alginolyticus*, *Bacillus filamentosus*, *Bacillus cereus* and *Idiomarina fontislapidosi* belonging to four different genera i.e. *Bacillus*, *Staphylococcus*, *Vibrio*, and *Alteromonas*.

Keywords: 16S rRNA, bacteria, fish-waste diet, *Pinctada maxima*, gold-lipped pearl oyster

INTRODUCTION

Pearl oyster culture industry in Indonesia mostly uses oyster seeds collected from the wild. If not managed well, the wild harvesting can potentially destroy oyster population and may affect the sustainability of the national pearl industry (Manez et al. 2010). This is demonstrated by the Segara Anakan lagoon in South Central Java, Indonesia, where the flourishing pearl oysters *Pinctada maxima*/*Pinctada margaritifera* are depleting due to overharvesting (Manez, 2010). In order to maintain stock of oyster in the wild, several private hatcheries commenced the production of the larvae (*P. maxima)*, and the juveniles are restocked to the wild for further adult collection at suitable size prior to seeding for pearl production (Kvingedal et al. 2010).

In the process of culturing, however, hatcheries use live microalgae and are cultivated on-site as exclusive food nutrition for the larvae. In fact, the production cost of the live microalgae culture is about 30–50% of hatchery operational cost. Also, technical sources and skilled personnel are required for successful production of live microalgae with adequate quantity and high quality. However, the unavailability of these requirements is common problem for oyster hatchery operations (Tanyaros et al. 2016; Southgate et al. 2016). For this reason, cheap and easily handed food sources based on baker's yeast were introduced. This resulted in stunted growth and high mortality of the oyster because of nutritional deficiency of the yeasts. This resulted in the growth and high mortality of oysters as there was nutritional deficiency of yeast (Tanyaros et al. 2016). Furthermore, non-living microalgae such as powder and paste were used for larval rearing of oysters (Wassnig and Southgate, 2016). The use of non-living microalgae promotes growth and survival of reared oyster larvae, which is equivalent to live-microalgae (Southgate et al. 2016). However, individual cells of the non-living microalgal-based food are non-motile, negatively buoyant, and eventually settled onto the bottom which is the source of culture crash. Also, the powder microalgae insufficient nutrition due to drying process, while the paste product has short shelf life (Wassnig and Southgate, 2016). The prices of these two non-living microalgal-based foods are expensive, therefore they are preferred by hatcheries in developing countries (Wullur et al. 2020). This led to the development of an inexpensive and easy diet as an alternative food for microalgae, the fish waste diet (FWD) (Ogello et al. 2018; Napitupulu et al. 2019; Ogello et al. 2019, 2020; Wullur et al. 2019, 2020). The FWD was developed from the composition of fish waste and probiotic bacteria. Attempts to feed rotifers with this diet were shown to have a high level of acceptance, as they were harvested at densities of about 2000 to 3000 ind./mL every week during semi-continuous culture

(Ogello et al. 2018; 2019, 2020; Wullur et al. 2017, 2019). In addition, it was suspected that fish waste contains bacteria used by rotifers as their nutritional source (Wullur et al. 2020).

Pearls oysters are reported to feed on bacteria in natural environment. However, bacteria are present in the rearing water of oyster and microalgae culture in hatcheries, it provides metabolic requirements for oyster's larvae by making direct or indirect provision of organic molecules and exoenzyme required for the growth and survival of larvae (Karim et al. 2013). On the other hand, the presence of opportunistic bacteria often causes disease and massive larval mortality in oyster hatcheries (Saulnier et al. 2010). However, the use of probiotics, which provides health benefits and protection against bacterial pathogens, is essential to maintain a healthy culture environment (Karim et al. 2013). Today, the bacterial population dynamics in live cultures for *P. maxima* is not clear. Furthermore, the bacterial population identification analysis should be more important when additional diets such as FWD are used. The knowledge on bacteria present in living cultures is important for designing nutritional strategies for oyster culture. In this study, a molecular approach was used to identify the species of bacteria present in rearing water of oyster culture. Also, a small-ribosomal subunit 16S rRNA gene was sequenced to identify bacterial species.

MATERIALS AND METHODS

The larvae of pearl oyster *P. maxima* (14-days old) were cultured in 700 mL seawater (salinity 35 ppt) at a density of about 33 ind./mL for two weeks, using transparent plastic containers. Each container was equipped with aeration of 0.66 mL/min and placed in a room at 25-26°C. The larvae were fed with live-microalgae *Isochrysis* sp. (PBM) and a FWD, which was previously soaked with EM4® (EM) for 24 hours. The preparation of the FWD was adapted as described in Patent No. P00201609066 registered in Indonesia.

The bacteria were isolated from the rearing water of oyster culture at the end of rearing period, with a sample of approximately 1 mL serially diluted (10 to 1000-fold) in sterile seawater. The samples were spread on 2 % nutrient agar and incubated at 37°C for 24-48 hours to observe bacterial colonies. A distinct colony was further selected according to their phenotype characters and grown in nutrient broth for 24 - 48 hours to obtain pure bacterial strains. Bacteria were harvested and centrifuged at 14,000 rpm for 5 minutes for molecular analysis.

The Qiaprep Miniprep Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA and 16S rRNA gene from the genomic DNA of the bacteria was amplified using two primers (8F; forward, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R; reverse 5'-ACCTTGTTACGACTT-3'). The PCR reaction was prepared with a total volume of 25 μl which consist of 1 μl sample, 5 μl of 5x Hot fire pool, 1 μl of each primer and 17 μl of ddH_2O. It was then placed in a Professional Thermocycler (Biometra, Analytik Jena) for 35 cycles amplification at 95°C for 6 min (1 cycle), 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec (34 cycles), followed by 72°C for 10 min as final cycle.

The amplicons and the primer pairs were sent to First-Base Co., Selangor, Malaysia to sequence the 16S rRNA gene. Sequencing procedures were conducted bi-directionally using a BigDye® Terminator v.3.1 Cycles Sequencing Kit (Applied Biosystems, USA) and further read with an ABI PRISM® 377 automatic DNA sequencer. Its quality was accessed using the Sequence Scanner version 2.0 Software (Applied Biosystem). Subsequently, sequences were trimmed, assembled, and manually edited using Geneious Prime version 2020 (http://www.geneious.com, Kearse et al. 2012) prior to the application of BLAST (Basic Local Alignment Tools) analysis at The National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

In this study, five species of bacteria (*Pseudomonas pachastrellae, Vibrio alginolyticus, Bacillus filamentosus, Bacillus cereus*, and *Idiomarina fontislapidosi*) were identified, belongs to 4 genera (*Pseudomonas, Vibrio, Bacillus*, and *Idiomarina*). These species obtained from rearing water of oyster larval culture were analyzed from seven isolates by conducting nucleotide BLAST using 16S rRNA gene at NCBI. The percent identity of the first top nearest species of 5 isolates (Table 1) was PBM21233 (99.07%), PBM31432 (99.64%), EM114133 (99.21%), EM11441 (99.01%) and EM11442 (98.87%), above the threshold value, while 2 isolates EM114233 (98.30%) and EM31042 (98.58%) were slightly below the threshold value. According to Kim et al. (2014), the threshold value to differentiate bacterial species using 16S rRNA gene has 98.65% sequence similarity/percent identity.

Three isolates namely PBM21233, EM114233, and EM11441 collected from the two feeding treatments (PBM and EM) were identified as *P. pachastrellae* (NR 040991.1) belongs to the genus of *Pseudomonas*. The members of this genus inhabit a variety of environments, including terrestrial and aquatic habitats. According to Özen and Ussery (2012), the total number of species currently included in the genus *Pseudomonas* is around 202 species, which are important in medical or biotechnological applications. Several species such as *Pseudomonas aeruginosa* (Priyaja, 2012; Giri et al. 2012; Vinoj et al. 2013), *Pseudomonas fluorescens* (Nour and El-Ghiet, 2011; Eissa et al. 2014), *Pseudomonas aestumarina*, *Pseudomonas synxantha*, *Pseudomonas chlororaphis* (Sánchez et al. 2014) have reported as a potent probiotic microorganism for marine or freshwater aquaculture. In addition, *P. aeruginosa* improved the innate immunity as well as survival of carp *Labeo rohita* against *Aeromonas hydrophila* infection (Giri et al. 2012) and enhanced survival of pearl oyster *Pinctada mazatlanica* (Macias et al. 2010). Also, *P. fluorescens* reported to improve survival of rainbow trout *Oncorhynchus mykiss* after challenging the fish with pathogen *Vibrio anguillarum*, while *P.*

aestumarina conferred protection to white shrimp *Litopenaeus vannamei* against vibriosis (Sánchez et al. 2014). The combination of *P. synxantha* and *P. aeruginosa* improved growth, survival and the immune responses of western king prawns *Penaeus latisulcatus* juveniles, while *P. chlororaphis* enhances resistance of European perch *Perca fluviatilis* against *Aeromonas sobria* infection (Sánchez et al. 2014). On the contrary, *Pseudomonas plecoglossicida* (Nakai, 2010), *Pseudomonas gessardii* (Hamza et al. 2018), *Pseudomonas alcaligenes* (Xu et al. 2015), *Pseudomonas beatica* (Lopez et al. 2012), *Pseudomonas koreensis* (Shahi and Mallik, 2014) and *Pseudomonas putida* (Mao et al. 2012) caused infection in aquaculture. Nakai (2010) and Jung et al. (2019) reported on the mass mortality of Ayu *Plecoglossus altivelis* and large yellow croaker *Pseudosciaena crocea* resulting from *P. plecoglossicida* attacks, while *P. gessardii* and *P. alcaligenes* facilitated the total mortality of freshwater crayfish *Pacifastacus leniusculus* (Korkut et al. 2018) and Chinese sturgeon *Acipenser sinensis* (Xu et al. 2015), respectively. The *P. beatica* was reported to be present in disease outbreak for flatfish *Dicologlossa cuneata* (Lopez et al. 2012), while *P. koreensis* is present in eye infections of golden mahseer *Tor putitora* (Shahi and Mallik, 2014). Furthermore, *P. putida* has been reported as an important fish pathogen of rainbow trout *Oncorhynchus mykiss*, European eel *Anguilla anguilla*, oyster toadfish *Opsanus tau*, and large yellow croaker *Pseudosciaena crocea* (Mao et al. 2012).

Table 1. Five top hits of the nearest species of the bacterial isolates after nucleotide BLAST against 16S rRNA gene in the NCBI GenBank using rRNA type strains/prokaryotic16S ribosomal RNA (bacteria and archaea) database

Isolates	Nearest species (accession number)	Score	Query cover	E-value	Identity
PBM21233	*Pseudomonas pachastrellae* (NR040991.1)	2516	100%	0.0	99.07%
	Pseudomonas oceani (NR152090.1)	2516	100%	0.0	97.79%
	Pseudomonas aestusnigri (NR126210.1)	2412	99%	0.0	97.79%
	Pseudomonas jilinensis (NR169366.1)	2289	100%	0.0	96.16%
	Pseudomonas sabulinigri (NR044415.1)	2259	100%	0.0	95.79%
PBM31432	*Vibrio alginolyticus* (NR113781.1)	2571	99%	0.0	99.64%
	Vibrio alginolyticus (NR117895.1)	2569	99%	0.0	99.64%
	Vibrio alginolyticus (NR121709.1)	2564	99%	0.0	99.57%
	Vibrio alginolyticus (NR118258.1)	2564	99%	0.0	99.57%
	Vibrio natriegens (NR117890.1)	2564	99%	0.0	99.57%
EM114133	*Bacillus filamentosus* (NR134701.1)	2521	98%	0.0	99.21%
	Bacillus endophyticus (NR025122.1)	2518	99%	0.0	98.73%
	Bacillus shackletonii (NR025373.1)	2224	99%	0.0	95.00%
	Bacillus circulans (NR112632.1)	2211	99%	0.0	94.92%
	Bacillus circulans (NR104566.1)	2207	99%	0.0	94.98%
EM114233	*Pseudomonas pachastrellae* (NR040991.1)	2468	99%	0.0	98.30%
	Pseudomonas oceani (NR152090.1)	2362	99%	0.0	97.02%
	Pseudomonas aestusnigri (NR126210.1)	2353	99%	0.0	96.88%
	Pseudomonas jilinensis (NR169366.1)	2235	99%	0.0	95.39%
	Pseudomonas sabulinigri (NR044415.1)	2202	99%	0.0	95.02%
EM11441	*Pseudomonas pachastrellae* (NR040991.1)	2527	100%	0.0	99.01%
	Pseudomonas oceani (NR152090.1)	2425	100%	0.0	99.74%
	Pseudomonas aestusnigri (NR126210.1)	2409	98%	0.0	97.99%
	Pseudomonas jilinensis (NR169366.1)	2298	100%	0.0	97.12%
	Pseudomonas sabulinigri (NR044415.1)	2266	99%	0.0	97.81%
EM11442	*Bacillus cereus* (NR115526.1)	2516	100%	0.0	98.87%
	Bacillus proteolyticus (NR157735.1)	2516	100%	0.0	98.87%
	Bacillus wiedmannii (NR152692.1)	2516	100%	0.0	98.87%
	Bacillus cereus (NR114582.1)	2516	100%	0.0	98.87%
	Bacillus cereus (NR115714.1)	2516	100%	0.0	98.87%
EM31042	*Idiomarina fontislapidosi* (NR029115.1)	2481	99%	0.0	98.58%
	Idiomarina baltica (NR027560.1)	2442	99%	0.0	98.08%
	Idiomarina aquatica (NR144584.1)	2364	99%	0.0	97.09%
	Idiomarina seosinensis (NR025826.1)	2263	98%	0.0	96.57%
	Idiomarina abyssalis (NR024891.1)	2254	99%	0.0	95.66%

So far there are no reports about the role of *P. pachastrellae* in aquaculture. It was first isolated from marine sponge specimen and assigned as a new species in 2005 (Kennedy et al. 2014). *P. pachastrellae* was further identified in the polluted sand samples after a marine oil spill (Lamendella et al. 2014), as well as deep seawater (depth of 1390 m) (Wang and Sun, 2016), and also in soil samples collected from an oily environment in close proximity to the seashore (Kaskatepe et al. 2017). Apart from having highest sequences identities with *P. pachastrellae*, the isolates PBM21233, EM114233 and EM11441 share high sequences identities with *Pseudomonas oceani* (97.79, 97.02, and 99.74% identities, respectively (Table 1)). Wang et al. (2020) placed the two closes relate *Pseudomonas* species in the group of *Pseudomonas pertucinogena* lineage. The *P. oceani* inhabiting deep seawaters (Wang and Sun, 2016) and oily polluted environment (Li et al. 2020) which is similar with *P. pachastrellae*. This study showed for the first time the presence of *P. pachastrellae* in the rearing water of the gold-lipped pearl *P. maxima* larvae which was found in both PBM and EM treatments.

Isolate PBM31432 collected from PBM treatment showed percent identity value above the threshold which referred to *Vibrio alginolyticus* (NR113781.1), a species of genus *Vibrio*. The species in this genus inhabit the aquatic environment. They are judged to be responsible for vibriosis, implicated in economic losses to the aquaculture industry, and is also frequently associated with disease-causing microorganism in humans (Ashrafudoulla et al. 2020). In addition, *V. alginolyticus* is implicated in severe soft tissue infections, sepsis, and other extraintestinal infections in human, through water exposure or the consumption of contaminated seafood (Slifka et al. 2017). This species together with *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio owensii* and *Vibrio campbelli* were predominantly associated as a causative agent of vibriosis on farmed aquatic animals (Ina et al. 2019). Specifically, *V. alginolyticus* were more frequently reported in the prevalence of many farmed fish species including olive flounder *Paralichthys olivaceus*, black rockfish *Sebastes schlegeli*, red sea bream *Pagrus major*, and sea bass *Lateolabrax japonicus*, kelp grouper *Epinephelus bruneus*, Orange-spotted grouper *Epinephelus coioides* (Oh et al. 2011; Harikrishnan et al. 2012; Wang et al. 2014). The *V. alginolyticus* was also reported in the infection of farmed shrimp (i.e. white shrimp *Litopenaeus vannamei*, shrimp *Marsupenaeus japonicus* (Kitikew et al. 2013; Zhu et al. 2016), abalone (i.e. small abalone *Haliotis diversicolor*, red abalone *Haliotis rufescens* (Wu et al. 2011) and farmed pearl oyster (i.e. Akoya pearl oyster *Pinctada fucata*, *Pinctada martensii*, *Pinctada maxima* (Wang et al. 2012; 2016; Adzigbli et al. 2020)). The *V. alginolyticus* in this study was only identified in PBM and not in EM treatments. Based on the consideration as a pathogen in the competitive processes, this species is suggests to be possibly displaced by beneficial bacteria in EM.

Two isolates, EM114133 and EM11442, which were collected from EM feeding treatment showed percent identity value above threshold referring to species of *Bacillus filamentosus* (NR134701.1) and *Bacillus cereus* (NR 115526.1) respectively. Species of bacteria in this genus are often referred to as probiotic microorganisms, as they provide health benefits to the host. According to Cutting (2011), genus *Bacillus* was used for decades as probiotic medicine or dietary supplements for human or veterinary use. In aquaculture, they play an important role in modulating hepatic indexes, gene expression, antioxidant and digestive enzymes. They also enhance water quality, disease resistance and immunity to promote better growth and survival of the reared fish or larvae (Kuebutornye et al. 2019). Particularly, *B. cereus* has generally been recognized as a human pathogen due to their role in food poisoning. In aquaculture, however, *B. cereus* has a significant role in enhancing immune responses, growth, or disease resistance to cultured fish (i.e. rainbow trout *Oncorhynchus mykiss*, silver catfish *Rhamdia quelen*, catfish *Clarias gariepinus*, Asian sea bass *Lates calcarifer*, Crucian Carp *Carassius auratus gibelio*, tambaqui *Colossoma macropomum*, Pengze crucian carp *Carassius auratus* var. *Pengze*, grouper *Epinephelus lanceolatus* and *Epinephelus fuscoguttatus* (Orsod et al. 2012; Souza et al. 2012; Gisbert et al. 2013; Hapsari, 2016; He et al. 2017; Ling et al. 2019; Yang et al. 2019), cultured shrimp (i.e. giant tiger prawn *Penaeus monodon*, pacific white shrimp *Litopenaeus vannamei*, pink shrimp *Farfantepenaeus brasiliensis*, whiteleg shrimp *Penaeus vannamei* (De Souza et al. 2012; Chandran et al. 2014; Hao et al. 2014; Khademzade et al. 2020)), and other aquatic organisms (i.e. sea cucumber *Apostichopus japonicus*, hooded oyster *Saccostrea cucullata*, mud crab *Scylla paramamosain* (Umayaparvathi et al. 2013; Wu et al. 2014 Zhao et al. 2016). However, there have been no reports of the presence of *B. filamentosus* in aquaculture. It was first isolated from a marine sediment in India and was assigned as a new species in 2015 (Sonalkar et al. 2015). Further, the *B. filamentosus* was reported as a plant growth promoting bacteria on wheat *Triticum aestivum* grown in a saline environment (Khalifa et al. 2020). Interestingly, two species of *Bacillus* i.e. *B. cereus* and *B. filamentosus* in this study were only found in EM treatment, which shows an opportunity for using probiotic supplements to supply good bacteria as probiotics in oyster larvae culture.

Isolate EM31042 from EM feeding treatment showed percent identity value lower than the threshold referring to species *Idiomarina fontislapidosi* (NR 029115.1), belongs to the genus *Idiomarina*. This genus is a member of the family *Idiomarinaceae* proposed by Innova et al.(2000), to accommodate true marine bacterial species in the group of *Alteromonas*-like γ-*Proteobacteria*. The genus *Idiomarina* currently consists of about 27 species and are typically euryhalophiles thriving in wide salinity ranges of marine environment (Liu et al. 2018). These include coastal and oceanic waters and sediments (Zhang et al. 2012; Hameed et al. 2016), saline-alkaline soil (Leon et al. 2015), inland hypersaline wetlands (Zhong et al. 2014), solar salt-making works (Lee et al. 2015) and deep sea sediment (Du et al. 2015). In aquaculture, bacteria in this genus live in a

recirculating aquaculture system with other heterotrophic bacteria (Michaud et al. 2009; Garcia et al. 2019; Teitge et al. 2020). They are also found as microbiome in fish gut (Yukgehnaish et al. 2020), in brine shrimp *Artemia franciscana* (Riddle et al. 2013), in a microalgal mass culture for finfish hatchery (Sandhya et al. 2017). However, their role in aquaculture, whether as a pathogen or probiotic is not well known.

In conclusion, this study shows that the rearing water of oyster larval culture contains at least 5 different species of bacteria, which are *P. pachastrellae* (PBM21233, EM114233, and EM11441), *V. alginolyticus* (PBM31432), *B. filamentosus* (EM114133), *B. cereus* (EM11442) and *I. fontislapidosi* (EM31042) belonging to the genus of *Pseudomonas, Vibrio, Bacilus and Idiomarina*. The species of bacteria in the treatment of PBM were *P. pachastrellae* and *V. alginolyticus*, while those in the treatment of EM were *P. pachastrellae*, *B. filamentosus*, *B. cereus* and *I. fontislapidosi*. In addition, EM treatment is suspected to involve bacterial species in the process of diet decomposition and subsequently became a food source or decomposed organic material as nutrition for the oyster larvae

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