The 16S rRNA analysis of proteolytic bacteria isolated from recirculating aquaculture system

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Abstract. Unused feed containing protein in the water affects the fish survivability under the recirculating aquaculture systems. Microbial communities play important roles in nutrient cycling in the aquatic ecosystems, however, bacteria that may help in protein degradation remains underexplored. This study aimed to identify the proteolytic bacteria obtained from aquaculture system based on 16S rRNA genes. Bacteria were isolated using non-selective medium and then assayed for proteolytic activity on skim milk agar. Characterizations were conducted for selected proteolytic bacteria before subjected to Sanger dideoxy DNA sequencing. The results of BLAST show that five representative isolates are closely related to Flavobacterium nitratireducens, Micrococcus aloeverae, Acinetobacter baumannii, and Exiguobacterium indicum at the level similarity of 99%. The nucleotides of collected proteolytic bacterial strains have been deposited in NCBI Genbank. Finding of those proteolytic bacteria in the recirculating aquaculture system may lead the further ecological studies about their roles in the ecosystem.

Keywords: Proteolytic bacteria, 16S rRNA gene, phylogenetic analysis, recirculated water

1. Introduction
Aquaculture produces wastes either unutilized materials or by-products. In aquaculture systems, the feed as the dominant source of waste has been reported [1]. Several factors affect the production of waste from feed including a ratio of feed size to fish size, composition of nutrient, method of feeding, procedure of production, a volume of feed per unit, and time of storage. Food metabolism products in fish or decomposed and unutilized feed are recognized as dissolved wastes in which two major substances related to phosphorus and nitrogen products [2]. Recirculating aquaculture systems applied to combine high-intensity with the low consumption of water and with minimal environmental impact.

As an impact of adding high amount of nutrient to aquaculture systems, the dissolved chemicals, gas or particulate material in the water increased [3]. As the main fish feed component, protein consist of the element phosphorus and nitrogen. Unfortunately, the body of the fish only retain less than a half of protein and nitrogen leading to the pollution in the water. Fish fecal contains maximum of 35% nitrogen and 70% protein. Thus, once those elements released into water, they can affect the survivability of fish and other aquatic organisms. As well, both total suspended solids and dissolved solids are increased due to the release of unutilized feed in water [4].

Microorganisms are commonly found in many aquatic ecosystems. Bacteria that occur either naturally or artificially added play important roles such as in the recycle nutrients recycle in aquaculture system [5]. The previous study of the abundance and diversity of bacteria in ponds
reflected the dominancy of the group of Proteobacteria, Cyanobacteria, Bacteroidetes, and Actinobacteria [6]. Strains with functional activities from the natural ecosystem are necessary for the bioremediation of organic waste in the ecosystem. Most of the aquatic bacteria are a source of enzymes having hydrolysing activity, substance such as lipases, amylases, proteases, catalases, phospholipase, and other important enzymes for industry [5]. In practical, peptide bond in the polypeptide chain of amino acids is hydrolyzed by applying proteases [7]. It was reported that several bacteria are capable of producing extracellular enzymes such as protease enzymes, amylases, and cellulases. Proteolytic Bacillus thuringiensis was successfully isolated from the sediment of extensive ponds [8].

Although several studies reported the presence of bacteria in an aquaculture system, however, information of bacteria with capability in protein degradation in the recirculating aquaculture system is limited. This study aimed to identify the proteolytic bacteria obtained from recirculating aquaculture system based on 16S rRNA genes. This study may contribute to the future application of isolated proteolytic bacteria and enhance the ecological perspective about bacteria roles in the ecosystem.

2. Material and methods

2.1. Sample collection and bacterial isolation

Sample used in this study was water from the recirculating aquaculture system. Recirculating aquaculture system consisted of three serial 150 L tanks containing fifty catfish (Clarias sp.) of each with feeding administration about 9% (b/v) body weight of fish and an additional serial tank containing bio-balls for bioremediation process of feed residue and fish fecal waste in the water. Water from bioremediation tank was connected with pipe to water reservoir tank with filter and then continued the recirculation to tanks containing the fish by the pump. Water was collected from the tank and prepared for bacterial isolation in the laboratory on August 20, 2018. Bacterial isolation was conducted on nutrient agar (Himedia) by following serial dilution. The growth of bacteria was observed after 48 h at 37ºC. A single colony was purified and determined by colony morphology. Bacterial isolates were maintained on nutrient agar medium at 4 ºC.

2.2. Characterization for proteolytic activity

Each bacterial isolate was cultivated on skim milk agar (20 g skim milk powder, 5 g peptone, and 20 g Bacto agar per liter distilled water; pH 7.0) and incubated for 48 h at 37ºC. Bacterial strains were screened based on skim milk protein degradation as illustrated by a clear zone. Proteolytic activity was determined as the Proteolytic Index (PI) by following the formula:

\[
PI = \frac{\text{diameter of the clear zone} - \text{diameter of colony}}{\text{diameter of the colony}}
\]  

(1)

2.3. Genomic extraction and PCR amplification

Extraction of genomic DNA of selected isolates was performed using GeneAid extraction kit. Amplification of 16S rRNA was conducted with primer set of 1492r (5’-GGTTACCTTGTTACGACTT-3’) and 27f (5’-AGAGTTTGATCCTGGCTCAG-3’) [9]. Total 50 µl of PCR reaction consisted of DNA (20 ng/µl), 1 µl of 1492r primer (10 pM), 1 µl of 27f primer (10 pM), and 25 µl of GoTaq Green Master Mix (Promega). PCR condition was performed using Takara Thermal Cycler Dice (Takara Co. Inc.) on 35 cycles under condition as follows: pre-denaturation (94 ºC for 2 min), denaturation (94 ºC for 2 min), annealing (48.5 ºC for 1 min), elongation (72 ºC for 1 min), and final extension of elongation (72 ºC for 1 min). Visualization of PCR product was performed using gel electrophoresis agarose 1% and 1 kb DNA ladder. Finally, Sanger dideoxy DNA sequencing of the PCR product was performed by First BASE Laboratories (Singapore).
2.4. Phylogenetic analysis.
Phylogenetic analysis was conducted comparing the homology of the sequences obtained in this study with nucleotide databases of the GenBank (http://www.ncbi.nlm.nih.gov/BLAST) on July 25, 2019, using BLAST program. Multiple sequence alignment of five representative sequences of isolated proteolytic bacterial strain with twenty-two sequences of reference strain. Clustal X version 2.0.11 was applied to construct phylogenetic tree based on the neighbor-joining method with 1000 bootstraps.

3. Results
Total plate count of water sample collected from the recirculating tank of aquaculture system (figure 1) was about 2.2 x 10^6 CFU/ml. Based on visual observation on colony morphology, a total of ten colonies found dominant in nutrient agar plate were successfully isolated. Five of ten purified isolates (POL1, POL2, POL3, POL4, and POL5) showed the proteolytic activity. We observed that five isolates namely POL 1, POL2, POL3, POL4, and POL5 had a different level of lysis of protein that is containing in the skim milk agar medium. The highest proteolytic activity was observed in isolates POL 4 and POL 5 with 3.3 and 2.5 in the proteolytic index, respectively (figure 2).

![Figure 1](image1.png)
**Figure 1.** Water in the tank of recirculating aquaculture system containing bio balls as source of bacterial isolation (left) and representative isolate showing the high proteolytic activity (right).

![Figure 2](image2.png)
**Figure 2.** Proteolytic index of bacteria isolated from recirculating aquaculture system.
To identify five isolated proteolytic bacteria, the 16S rRNA analysis was applied. Total amplified PCR was about 1400 bp in sequence length. BLAST results demonstrated the closest relationship of isolate POL1, POL2, POL3, POL4, and POL5 was *Flavobacterium nitratireducens* N1\(^\mathrm{T}\), *Micrococcus aloeverae* AE-6\(^\mathrm{T}\), *Acinetobacter baumannii* DSM 30007\(^\mathrm{T}\), *Exiguobacterium indicum* HHS 31\(^\mathrm{T}\), and *Exiguobacterium indicum* HHS 31\(^\mathrm{T}\), respectively, at the homology over 99%. All nucleotide of those bacterial isolates has been submitted to NCBI Genbank with accession number of MK999979, MK999981, MK999981, MN220689, and MN220690 (table 1).

In our study, POL1 showed light yellow-pigmented properties of the colony. The 16S rRNA analysis showed the closest relationship *F. nitratireducens* N1\(^\mathrm{T}\) (99.78%) (table 1) as well presented by the construction of a phylogenetic tree. As a result, isolate POL1 was located in the phylogenetic tree at the same clade with *F. nitratireducens* N1\(^\mathrm{T}\) (bootstrap 1000). It was separated clearly to other reference species of *Flavobacterium*. Bacterial isolate POL2 had the closest relationship with *Micrococcus aloeverae* AE-6\(^\mathrm{T}\) at the homology of 99.93% (table 1). Other species that share a similar sequence of 16S rRNA to POL2 were *Micrococcus yunnanensis* strain YIM65004\(^\mathrm{T}\) (99.64% in homology) and *Micrococcus luteus* strain NCTC2665\(^\mathrm{T}\) (99.56%). The constructed phylogenetic tree confirmed the closest relationship with *M. aloeverae* since bacterial isolate POL2 had a position at the same clade with *M. aloeverae* AE-6\(^\mathrm{T}\). This study clearly demonstrated in this study that POL3 has the closest relationship with *Acinetobacter baumannii* DSM 30007\(^\mathrm{T}\) (99.93% in homology). As shown in the phylogenetic tree, bacterial POL3 was in the position of the same clade with *A. baumannii*. Two other bacterial isolates POL4 and POL5 shared a similar sequence of 16S rRNA gene. Those strains had the closest relationship to *Exiguobacterium indicum* HHS 31\(^\mathrm{T}\) (both were 99.79% in homology). Although slightly different in the nucleotide sequence of POL4 and POL5 (1427 bp in length), the phylogenetic tree confirmed both bacterial isolates were in the same clade with *E. indicum* HHS 31\(^\mathrm{T}\). Those isolates were also clearly separated to *E. acrylicum* (figure 3).

### Table 1. The relationship of 16S rDNA sequences of aquaculture bacterial strains.

| Strain | Submitted Accession No of Genbank | Closest relationship of 16S rDNA [Accession Number] | Similarity (%) |
|--------|-----------------------------------|------------------------------------------------------|----------------|
| POL1   | MK999979                          | *Flavobacterium nitratireducens* N1\(^\mathrm{T}\) [NR_108520.1] | 99.78          |
| POL2   | MK999980                          | *Micrococcus aloeverae* AE-6\(^\mathrm{T}\) [NR_134088.1] | 99.93          |
| POL3   | MK999981                          | *Acinetobacter baumannii* DSM 30007\(^\mathrm{T}\) [NR_117677.1] | 99.93          |
| POL4   | MN220689                          | *Exiguobacterium indicum* HHS 31\(^\mathrm{T}\) [NR_042347.1] | 99.79          |
| POL5   | MN220690                          | *Exiguobacterium indicum* HHS 31\(^\mathrm{T}\) [NR_042347.1] | 99.79          |

### 4. Discussion

Colony morphology of *F. nitratireducens*-related bacterial isolate POL1 (99.78% in homology) demonstrating light yellow-pigmented was in line with the common recognition of genus *Flavobacterium*. The genus *Flavobacterium* has characteristics of Gram-negative, yellow-pigmented, rod-shaped bacteria [10]. In the taxonomy, the genus *Flavobacterium* is member of the family Flavobacteriaceae [11]. The presence of *F. nitratireducens* in aquatic ecosystem was not surprisingly. However, a type strain *F. nitratireducens* strain N1\(^\mathrm{T}\) that firstly reported isolated from a marine water in Bay of Bengal, India [10] may show different model of adaptation in POL1 originated from freshwater in the recirculating aquaculture system. Several sources of genus *Flavobacterium* have been reported such as marine water, freshwater, river sediments, fish tissues, soil, Antarctic lakes, glacier, earthworm gut, soils and sediments [10,11]. *Flavobacterium aquaticum* strain JC164\(^\mathrm{T}\) was
isolated from a water in the rice field in India [12]. In addition *Flavobacterium tilapiae* Ruye-71T was reported from a freshwater tilapiine cichlid fish culture pond in Taiwan [13]. A few species of this genus were reported as pathogenic. Species *F. psychrophilum* had the relationship with disease and other infections particularly for freshwater salmonid hatcheries [11]. Several abilities of adhesion and biofilm formation in the healthy fish have been observed in *Flavobacterium*. Five species of the *Flavobacterium* was previously reported to associated with the tropical freshwater [14]. A fish pathogen *F. psychrophilum* has been reported to produce an extracellular protease with an estimated molecular mass of 55 kDa [15]. To date, less information of proteolytic *F. nitratireducens* from freshwater was available as demonstrated in POL1. In contrast, a type strain *F. nitratireducens* from coastal surface seawater was originally reported to have the amylolytic property [10].

![Phylogenetic tree of 16S rRNA bacterial isolates from water in the tank of aquaculture system.](image)

**Figure 3.** Phylogenetic tree of 16S rRNA bacterial isolates from water in the tank of aquaculture system. The Kimura 2 parameter model with 1000 bootstrap was applied to estimate the distance. The bar represents a 2% estimated sequence divergence. *Nitrosomonas aestuarii* Nm36T was used as an out-group.

The presence of *M. aloeverae*-related bacterial isolate POL2 in the aquaculture system was interesting since a type strain *M. aloeverae* AE-6T was originally recognized as an endophyte. As description, *M. aloeverae* was a Gram positive, spherical, small, non-motile, non-endospore-forming bacterium [16]. This strain *M. aloeverae*AE-6T was isolated from *Aloe barbadensis* (*Aloe vera*) [16]. Member of the genus *Micrococcus* widely distributed and has been isolated from a variety of habitats. In the previous report, *M. aloeverae* was also isolated from dried milk [17]. The ability of
bacterial isolate POL2 to hydrolyze protein was in agreement with the previous study that demonstrates the protease production by *M. aloeverae* [18].

The presence of strain (POL3) having the closest relationship to *A. baumannii* in the aquaculture system was interesting. It was interesting phenomenon since this species was originally isolated from human[19]. Species *A. baumannii* was notorious with some records in the pathogenic activity [20–22]. This species was recognized as the multidrug-resistant bacterium that is difficult to control and treat [23]. Widely distributed of this species may correlate with efficiently utilized nitrogen sources and was tolerant to several condition such as antimicrobial, osmotic, pH, and stress [24]. In the previous study, several members of the genus of *Acinetobacter* were detected in water from the source to the tap [25]. Successful recovery of species *A. baumannii* from the sludge and water of the wastewater treatment plant has been reported using CHROM agar *Acinetobacter* plates [26]. In the aquatic ecosystem, *Acinetobacter johnsonii* and *Acinetobacter lwoffii* was reported as an emerging fish pathogens [27]. Species *A. baumannii* was reported as protein producer. A novel protease (CpaA) from the clinical isolates of *A. baumannii* has been described and might correlate to the regulation of blood coagulation [28]. Another serine protease PKF of *A. baumannii* was also described and suggested to play a role in serum resistance and suppression of biofilm formation [29]. The proteolytic activity as recognized *A. baumannii*-related isolate POL3 was in agreement to the previously reported, however specific type of protease remains unclear and need further investigation.

Two isolates (POL4 and POL5) from the water of recirculating aquaculture system had relationship to *E. indicum*. Actually, *E. indicum* was isolated for the first time from melted water from the glacier of the Himalayan mountain, India. A type strain of this species was psychrophilic bacterium. Cells were recognized as aerobic, Gram-positive, motile and rod-shaped. The colony showed yellowish-orange in color [30] which is similar to the observed colony morphology of both bacterial isolates POL4 and POL5. This species was not only found in the glacier area but also was successfully isolated from soil. Strain *E. indicum* TBG-PICH-001 was reported to demonstrate the proteolytic activity [31]. The first publication of protease production by *Exiguobacterium* appeared in 2007. Strain *Exiguobacterium* sp. SKPB5 from soils in cold environments demonstrated the protease production in which the enzyme has a broad temperatures range (5-40°C) [32]. In the anaerobic bioreactor, protease-producing *Exiguobacterium* sp. YS1 has been applied to increase the solubilization of waste-activated sludge [33]. The draft genome of *Exiguobacterium* sp. AB2 isolated from hyperalkaline spring in Manleluag, Pangasinan, Philippines was described in size of 2.85 Mbp [34]. Several members of genus *Exiguobacterium* are capable of producing enzymes that are useful for bioremediation and degradation of toxic substances in the environment [35]. Although many reports of protease-producing *Exiguobacterium* are available, our current study of POL4 and POL5 demonstrating the highest proteolytic activity from recirculating aquaculture system was firstly reported.

5. Conclusion

Five strains of proteolytic bacteria were successfully isolated from the aquaculture system. Based on 16S rRNA analysis those five proteolytic bacterial isolates had closest similarity to *Flavobacterium nitratireducens, Micrococcus aloeverae, Acinetobacter baumannii*, and *Exiguobacterium indicum* at the level similarity of 99%. The highest proteolytic activity was demonstrated in *E. indicum* POL4. To our knowledge this study firstly reported the presence of proteolytic *E. indicum* in the water of the aquaculture system.

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