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Isolation, Molecular Identification and Verification of Gene Encoding Bacterial Keratinase from Crocodile (*Crocodylus porosus*) Feces

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Abstract. Keratinase is a group of protease enzymes which acts to degrade keratin. Keratin is a fibrous protein difficult to be degraded because of hydrogen and disulfide bonds. The purpose of this study was to isolate and to identify keratinase coding genes using molecular approach. The object of this study was crocodile feces from Asam Kumbang crocodile farm. Bacterial isolation was done by using feather agar (FA) and was screened in skim milk agar. Keratinolytic bacteria isolates were identified molecularly with 16S rDNA specific markers. Detection of keratinase gene was done by using keratinase-specific primers through primer3 application (version 0.4.0). The result showed that isolate FB3 are potential to produce keratinase. Isolate FB3 showed a clear zone in FA and SMA. FB3 was 100% similar to *Aeromonas hydrophila*. Amplification using *kerD* gene specific primer showed that FB3 was a novel bacteria possessing *kerD* gene with a fragment length of 750 bp. This result provided a new database of *kerD* gene.

1. Introduction

Millions of tons of chicken feather waste products are produced from poultry meat processing [1]. Generally, the method used to minimize chicken feather waste is through burning. This method has negative impacts in the environment. Besides being expensive, this method produces greenhouse gas emissions and ash disposal problems [2]. Cai *et al* [3] suggested the use of traditional methods to degrade feathers such as alkaline hydrolysis and cooking with vapor pressure. Unfortunately, these methods may damage amino acid in the chicken feathers and consume large amounts of energy [4]. One alternative to overcome feather waste without adverse environmental and ecological impacts is using biological approach in which enzymes can be used to be effective and efficient to overcome the chicken feather waste.

Some studies reported to utilise keratinase a serine enzyme or metalloprotease effectively degrading keratin [5] up to 85-99% [6,7]. Bacteria are a group of microorganisms that can grow well in keratin waste as a source of carbon and nitrogen by producing keratinase. Keratinase may be used in detergent [8], leather tanning industry [10], biofertilizer [11], and medicine and pharmacy [12]. Digestible feather meal produced from keratin is uses as feed [9]. Biogas may be produce by using keratinolytic microorganisms from keratin [13].



One group of bacteria known as keratinase producer is *Bacillus* i.e. *B. licheniformis*, *B. megaterium*, *B. subtilis*, *B. cereus*, and *B. pumilus* [14, 15, 16, 17, 18]. Other gram-positive bacteria produced keratinase such as *Microbacterium*, *Lysobacter*, *Nesterionia*, *Kocuria* [19,20,21,22], and several gram-negative bacteria such as *Stenotrophomonas*, *Vibrio*, *Chryseobacterium* and *Serratia* [23, 24, 25, 26] were also reported.

The exploration of new bacterial strains that have keratinolytic activity is still important to know their diversity and keratinolytic ability, especially in genes carried by the isolates. Many studies showed that keratinase A (*ker A*), keratinase C (*ker C*), and keratinase D (*ker D*) and F (*ker F*) have been successfully amplified from *B. licheniformis* [27], *B. subtilis* [28] and *S. maltophilia* [29], respectively. Keratinolytic bacteria originating from animal have not been widely studied yet [30]. In this study, isolation and identification of keratinolytic bacteria from crocodile feces gave an opportunity for the discovery of novel keratinolytic isolates and genes. We isolated, identified and verified genes encoding keratinase activity from crocodile feces.

2. Materials and Methods

2.1. Sampling Location

Crocodile fecal sample was collected from Asam Kumbang crocodile farm, Medan Selayang, Medan City, North Sumatra, Indonesia (E 98°37'11.24", N). The collected faecal sample was stored in 30x40 cm sterile plastic ziplock. The sample was put in an ice box until further examination in the laboratory.

2.2. Isolation of Bacterial Strains

Bacterial isolation was conducted using methods described by Yue et al. [30] with slight modification. Bacteria was extracted from 1 gram in 9 mL NaCl (0.9% w/v) with a serial dilution of 10^{-1} to 10^{-9} . A 100 μ L of sample suspension from three dilutions (10^{-7} , 10^{-8} , 10^{-9}) was poured and spreaded on feather meal agar (FMA) containing 10 g of feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4 , 0.4 g KH_2PO_4 , and 15 g of agar in 1 liter of aquadest at pH of 7.0-7.4 in plates. The plates were incubated at $35^\circ C \pm 2$ for 48 h and periodically observed for 24 h. Bacterial colonies showing clear zones around the colonies were collected and further screened using skimmed milk agar.

2.3. Screening of Proteolytic Bacteria

Skim milk agar (SMA) (OXOID LP0031B) was used for screening isolated bacteria from previous work. Skim milk agar was mixed in sterile aquadest and was pasteurized at a temperature of $\pm 70^\circ C$ until SMA dissolved. Bacterial colonies were inoculated on SMA. Protein hydrolysis was observed when the clear zone formed around the bacterial colonies.

2.4. Genomic DNA Isolation

Bacterial genomic DNA was isolated using Mustopa and Fatimah method [31] with several modifications. Bacterial culture grew in nutrient broth were taken and put into 1.5 mL sterile tube and centrifuged at 10,000 xg for 5 min, at $4^\circ C$. Supernatant was discarded, and the bacterial cell pellet was dissolved with 740 μ L Tris-EDTA buffer (1M Tris + 0.5M EDTA, pH 8). Subsequently, 40 μ L of lysozyme (60 mg/mL) was added and was slowly homogenized and incubated at $37^\circ C$, 1 h. Soluble bacterial cells were added with 200 μ L SDS 10%, 100 μ L NaCl 5M, 80 μ L 10% CTAB and incubated at $68^\circ C$, 30 min, in which 300 μ L of chloroform was added. The mixture was centrifuged at 10,000 xg for 20 min, $25^\circ C$. The aqueous part was transferred into a new sterile microtube. Isopropanol was added with a certain volume ratio (0.6x volume aqueous which was transferred to the sterile new microtube). The tube was incubated at $-20^\circ C$ for two hours and was centrifuged at 10,000 xg for 15 min, $4^\circ C$. Pellet was collected and added with 800 μ L of ethanol 70% and centrifuged at 10,000 xg for 10 min, $4^\circ C$. Supernatant was discarded. DNA pellet was dried overnight. A 27 μ L ddH_2O , 3 μ L RNase (1 mg/mL) was added to DNA pellet. DNA solution was incubated for 0.5 h, $37^\circ C$. DNA solution was stored at $4^\circ C$ until further study.

2.5. Molecular Identification Using 16S rRNA Primers

Identification of 16S rRNA gene was done by using a pair of primers (forward-reverse), 8F primers (5'-AGA GTT TGATCATGG CTC AG-3'; positions 8 to 27 bp) and 15R (5'- AAGGAG ATC GTG CAA CCG CA-3'; positions 1541 to 1522bp) [32]. The composition of the mixture of PCR solution and PCR conditions was in accordance with what has been done by Mustopa et al [33]. Phylogenetic tree analysis was done by using the MEGA X (10) application.

2.6. Verification of *Aeromonas Hydrophila* FB3 Keratinase Gene

Isolation of keratinase gene fragment from FB3 was carried out through the amplification of keratina gene fragment using PCR with conserved primers of keratinase gene. PCR mix composition (μ L) was of 5x myTaq (2) buffer, forward primer (0.2), reverse primer (0.2), Pol DNA MyTaq (0.2), DNA template (2), ddH₂O (5.4). PCR was conducted by pre denaturation 94° C/4 m, denaturation 94° C/30 s, annealing 55° C/2 m, extension 72° C/2 m, and extension 72° C/5 m. Three pairs of keratinase primers used were originated from the keratinase N4 gene from *S. maltophilia* KER N4 (LC029453.1) and keratinase D from *S. maltophilia* YHYJ-1 (FJ765514.1) (Table 1). The primers were designed by using primary applications (version 0.4.0).

Table 1. Specific Primers of Keratinase Encoding Gene

Name of Primer	Sequence	length	Tm	GC%
PKerN41_F	CCAGTGGCACCTGCTCAATA	20	60.04	55.00
PKerN41_R	TCCTGGAAGTGGTTGATCGC	20	60.04	55.00
PKer N42_F	ATGAAGCGACACACGCAACGCTCCG	25	79.6	60
PKerN42_R	CACCCACAGCGCCGAACCGGTGGAC	25	83.8	72
PKerN43_F	CCGGTTCGGCGCTGTGGGTGTCCGG	25	86.4	76
PKerN43_R	TCAGTGCCCCAGAACGCGGATCTTG	25	78.8	60
PKerD1_F	CTGTGACGGAACCTACCAGG	20	59.75	60
PKerD1_R	CGTCCCTGGGCAATGTAGAC	20	60.46	60

3. Results

3.1. Isolation and Bacterial Screening

One isolate FB3 showed to have hydrolytic activity in media enriched with chicken feathers. The hydrolysis zone was seen in the medium after 48 h of incubation (Fig. 2A). Proteolytic activity of FB3 was reconfirmed by it in SMA. Result showed that FB3 strain produced hydrolysis zone in SMA (Fig 2b).

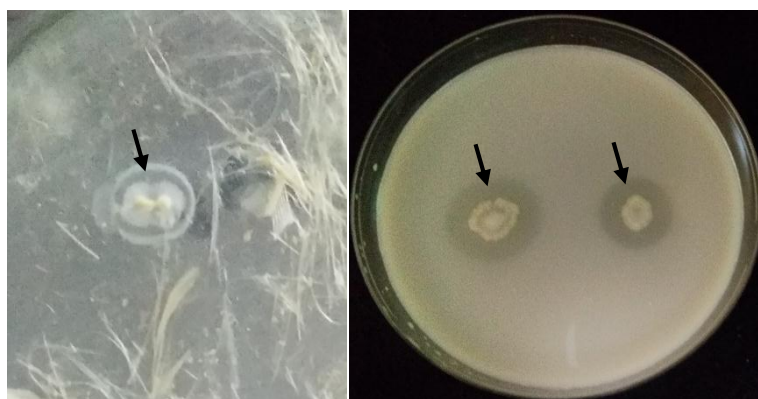


Figure 1. Screening of feather degrading and proteolytic bacterial isolate FB3. (A) FB3 on enriched chicken feather agar, and (B) on SMA incubated at 35°C±2 (48 h). (The hydrolysis zone is marked with a black arrow)

3.2. Molecular Identification of 16S rRNA

DNA isolation of FB3 was successfully carried out with purification of 1.910. FB3 was identified by using 16S rRNA molecular markers. The amplification showed a DNA fragment of 1500 bp (Fig. 3). DNA chromatogram sequencing was confirmed by using NCBI data bases. Phylogenetic tree was constructed by using MEGA X (10). It was shown that FB was *Aeromonas hydrophila* with 100% homology to available sequences (Fig. 4).

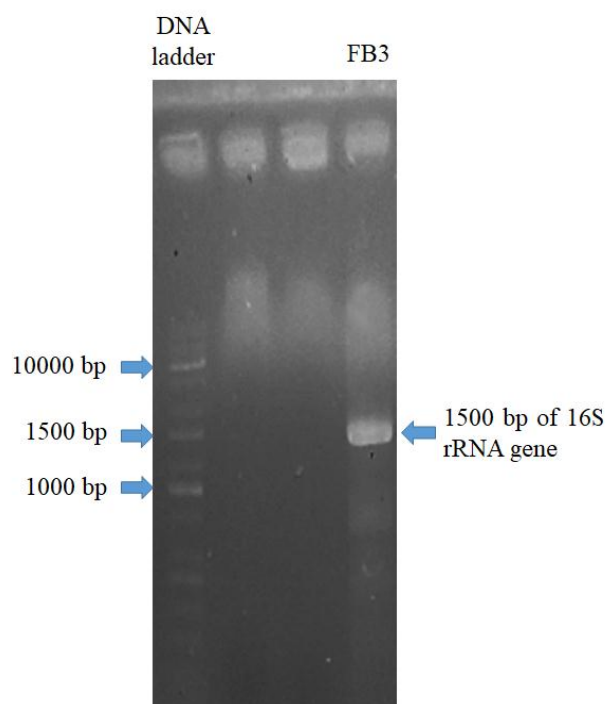


Figure 2. Amplification of 16S rRNA of FB3 produced a 1500 bp of PCR product

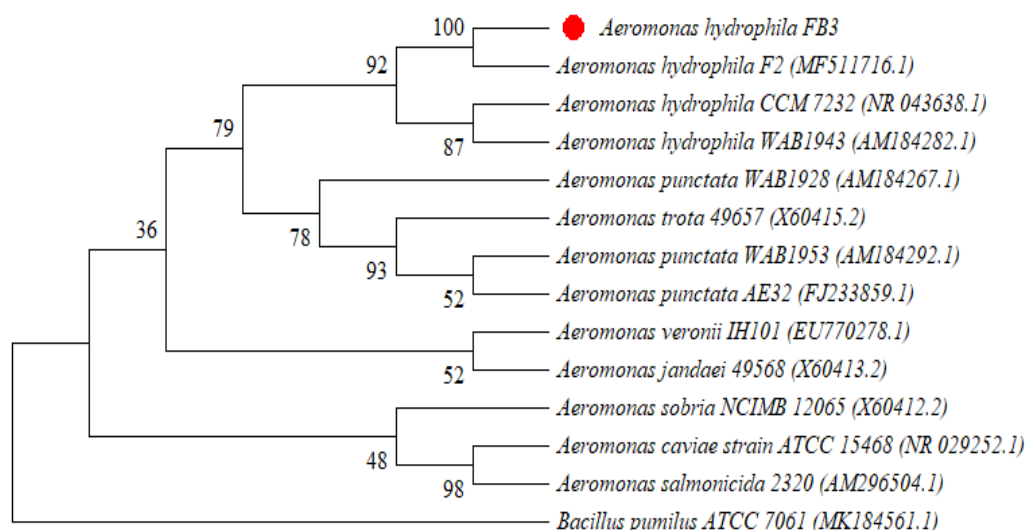


Figure 3. Phylogenetic relationship of keratinolytic FB3 isolate from crocodile feces with other *Aeromonas* spp. having keratinolytic activity (right). Constructed with neighbor-joining method in MEGA X; the bootstrap consensus tree inferred from 1000 replicates

3.3. Verification of *Aeromonas hydrophila* FB3 Keratinase Gene

Verification of keratinase coding gene with PCR by using PKerD1_F/R primer produced a 750 bp band (Fig. 4). This verification showed that DNA genome of FB3 carrying *kerD* gene.

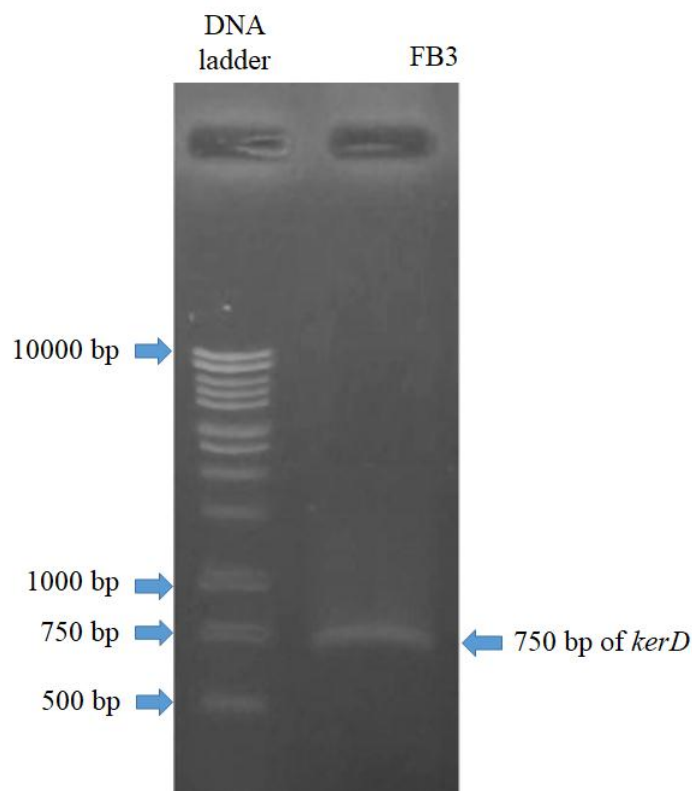


Figure 4. PCR amplification of *kerD* gene from keratinolytic FB3

4. Discussion

Bacterial isolation with keratinase activity was successfully carried out. One isolate FB3 was tested for protease activity in enriched feather and skimmed milk agar. Molecular identification of 16S rRNA gene showed that the FB3 was identified as *A. hydrophila*. Previous studies reported that *A. hydrophila* showed protease activity, *A. hydrophila* Ni 39 was reported to have protease activity in azocasein substrates [34]. Elgendy et al. [35] reported that *A. hydrophila* from polluted waters had protease activity after incubation in media containing 1.5% skim milk. Study by Izuchukwu [36] also reported that *A. hydrophila* HX 201006-3 was able to hydrolyze gelatin substrate through zymogram analysis.

Protease studies from bacteria are highly promising to be applied in industry. Keratinase of one protease (E.C. 3.4.21/24/99.11) grouped in metallo or serine protease degrade keratin a resistant degradable protein. Keratin was fibrous and insoluble substrate. This protein structure was abundant in nature as main component structure of vertebrate skin, nails, hair, wool, and horns [37]. Keratinolytic activity from genera *Aeromonas* has not been widely studied yet. Initial report by Bach *et al.* [38] showed that *A. hydrophila* K12 isolated from soil can degrade keratin in feathers. In this study, we isolated keratinolytic FB3 which is an *A. hydrophila* from crocodile feces. Screening of crocodile fecal sample in enriched feather agar showed keratinase activity of this isolates after 48 h of incubation. FB3 also showed hydrolysis activity in reconfirmed test of protease on skim milk agar by producing clear zone around its colony.

To confirm keratinolytic activity carried by *ker* genes, three specific primer pairs of bacterial keratinase genes were used (Table 1). It was shown that a primer that successfully amplified keratinase gene of FB3 was the PKerD1_F/R. This specific keratinase primer was designed from *kerD* gene derived from *Stenotrophomonas maltophilia* YHYJ-1 to produce $\pm$ 750 bp band. This study was the

first report to show that *A. hydrophila* have *kerD* gene. Previous studies only reported that *S. maltophilia* carried *kerD* gene. Successful verification of keratinase gene from *A. hydrophila* FB3 was the novelty in this study. Considering that it has never been reported before, it is important to carry out further biochemical characterization and purification of this keratinase including isolation of keratinase-specific *A. hydrophila* FB3 gene. Keratinase gene from *A. hydrophila* FB3 may be used as potential gene source in industrial applications.

5. Conclusion

Keratinolytic bacteria FB3 was successfully isolated from crocodile feces. Molecular identification of 16S rRNA showed that FB3 was *A. hydrophila* with 100%. Initial verification of keratinase gene of FB3 was successfully amplified by the specific primer PKerD1_F/R keratinase D gene. This was the first report that *A. hydrophila* carrying *kerD* gene.

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