Application of end-point PCR technique to detect bacteria encoding tyrosine decarboxylase (TDC) gene in scombridae fish

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Abstract. Tuna, little tuna, and skipjack can form tyramine from decarboxylation of tyrosine by tyrosine decarboxylase (TDC) enzyme. High tyramine level in fish could cause toxicological effects. Early detection of the tyramine-forming bacteria by using DNA-based methods is needed for seafood safety assurance because it is more sensitive, specific, and faster. This study was aimed to obtain bacterial isolates, detection of TDC and 16S rRNA genes and identifying species of bacteria that encode TDC and 16S rRNA genes. The methods of this study included several steps including bacterial cultivation, DNA isolation and amplification of TDC and 16S rRNA gene markers as well as sequencing of amplicons. Bacterial DNA isolates were successfully obtained from samples by cultivation method. The TDC gene was successfully amplified and identified as belong to Carnobacterium genus, whereas the 16S rRNA gene belong to Enterobacter tabaci, E. hormaechei, Escherichia marmotae, and Peptoniphilus genus. The DNA-based method targeted tyrosine decarboxylase gene can be applied for early detection of biogenic amine accumulation in fishery products.

Keywords: cultivation, early-detection, scombridae fish, TDC

1. Introduction

Tuna, skipjack, and little tuna of scombridae commodities play an important role in fisheries production development in Indonesia. Tuna is mostly exported to the European Union, the United States, and several Asian countries such as Thailand, Japan and China (KKP 2017). Indonesia's scombridae exports in 2018 reached 168,400 tons or 14.96% of the total export volume of fishery products. The scombridae family has a high histidine content thus in certain condition could lead into biogenic amine formation. The scombridae fish biogenic amine are produced by decarboxylase-producing bacteria converting histidine into a biogenic compound amine (Hariri et al 2018).

Biogenic amines are an organic molecule with low molecular weight having biological activity. Biogenic amines are widely found in milk, fermented soybean products, fish, and processed fish products. Biogenic amines present in fish are formed by bacteria that naturally present in fish (Landete et al 2011). Biogenic amines present in fish include histamine, tyramine, tryptamine, putrescin,
cadaverine, and spermidine (Stadnik and Dolatowski 2010). Tyramine and histamine are the most active amines among other biogenic amines, and are widely studied because of their toxicological effects (Marcobal et al 2012). Previous studies found the content of histamine, putrescin, cadaverine, and tyramine in sardines, mackerel, skipjack tuna, and tuna (Prester 2011). Consumption of foods that contain high amount of biogenic amine can cause histamine poisoning and toxicity of tyramine.

Determination of biogenic amines methods in food has been widely developed, namely analytical methods based on chromatographic and molecular. Chromatographic methods are considered as a non-early-detection method because less rapid and can only detect the type of biogenic amines after it is formed (Landete et al 2011, Shiling et al 2015). Rapid detection methods have been developed to prevent biogenic accumulation of amines in seafood, one of which is DNA-based methods (Landete et al 2011, Biji et al 2016). Tyramine is produced by decarboxylation of tyrosine by various lactic acid bacteria (BAL), including several strains of Lactococci and Enterococci. Previous studies demonstrated bacterial decarboxylase could be detected using TDC-F and TDC-R primers, and only Gram-positive bacteria produced tyramine, including Enterococcus faecalis, E. Faecium, Carnobacterium divergens as well as Lactobacillus brevis (de Las Rivas et al 2006). Enterococci is found in many digestive tracts of animals and humans. These bacteria are tolerant of heat and can live in poor environmental conditions, thus it is difficult to control Enterococci (Marcobal et al 2012).

Detection of tyramine and its content in the scombridae fish family is of importance as an indicator of food hygiene, to guarantee better food quality in the future. This study was aimed to detect bacteria producing biogenic amines based on tyrosin decarboxylase (TDC) gene and 16S rRNA gene in scombridae fish.

2. Materials and methods

2.1. Materials

The main materials in this study were tuna (Thunnus sp.), skipjack (Katsuwonus pelamis) and little tuna (Euthynnus affinis) caught in Indian Ocean that had been frozen for 1 month prior to analysis. Other materials used including DNA isolation kits (TIANamp Genomic DNA Kit, TianGen Biotech, Beijing, China), PCR 2X Taq Master Mix kit (VIVANTIS, Selangor Darul Ehsan, Malaysia), DNA Ladders 1 KB, sybrgreen, loading dye, agarose, TBE buffer, Lactose Broth (LB) media, CTAB 2%, PCI, isopropanol, CIA (chloroform, isoamilalcohol), TDC-F and TDC-R primers, 16S 27F and 1494R primers, distilled water, and 96-100% ethanol. TUBE-150-C microtubes (Extragene, Taichung, Taiwan), micropipette (ThermoFisher Scientific, Vantaa, Finland), water-bath, sonicator (BANDELIN electronic, Berlin, Germany), vortex (V1-Plus, Biosan, Warren, USA), spin down (Corning, New York, USA), microcentrifuge (Centurion Scientific 2041, Libertyville, USA), microwaves (Sharp, Osaka, Japan), PCR system (Applied Biosystem GeneAmp PCR System 9700, ThermoFisher Scientific, Vantaa, Finland), electrophoresis (HU6, SCIE-PLAS, Cambridge, England) were some equipment used in this study.

2.2. Methods

2.2.1. Sample preparation and bacterial cultivation method. The frozen samples (tuna, skipjack and little tuna) was prepared and the fish flesh was taken (10 grams) and mashed using a blender. Bacteria was cultivated in 50 mL Lactose Broth (LB) media. Media containing fish sample were incubated at 37°C for 16 hours. The media was subsequently centrifuged at 13,000 rpm for 3 minutes. The supernatant was removed, and the pellet was obtained for further analysis. All sample in this study were labelled as follow: (BN) tuna without bacterial cultivation, (CN) skipjack without bacterial cultivation, (TN) little tuna without bacterial cultivation (BE), tuna with bacterial cultivation, (CE) skipjack with bacterial cultivation, (TE) little tuna with bacterial cultivation.
2.2.2. **DNA Isolation.** DNA extraction was carried out using CTAB 2% with phenol-chloroform (Sambrook and Russell 2001) and commercial kit Tiangen DNA isolation kit. DNA isolation is considered an important step before amplification procedures. The isolation results were visualized in agarose gel and DNA concentration was measured using nano drop spectrophotometry.

2.2.3. **Amplification of TDC and 16S rRNA genes.** The TDC gene was amplified using TDC-F and TDC-R specific primers with a base length of 825 bp (de Las Rivas et al 2006), while 16S rRNA genes used forward (27F) and reverse (1494R) primers with base length 1,500 bp (Rochelle et al 1992). All samples were mixed into a PCR reaction with annealing temperatures of 53°C for TDC primers and 48°C for 16S rRNA primers. The amplicons were sequenced using Sanger sequencing procedure. All sequences data obtained in this study were then processed using MEGA 6.0 (Molecular Evolutionary Genetic Analysis) application (Tamura et al 2013).

3. **Results and discussion**

3.1 **Bacterial DNA isolation and amplification**

The DNA purity for bacterial isolates from sample BE, TE and CE obtained using TIANamp Genomic DNA kit was ranging from 2.04-2.05, while using CTAB 2% ranging between 1.86-1.91. The values of A260/A280 for non-cultivated (BN, TN, CN) DNA isolated using the TIANamp Genomic DNA kit were between 2.09 and 2.16, while using CTAB 2% ranging from 1.72-1.97. Moreover, the DNA concentration of bacterial isolates using TIANamp Genomic DNA kit was between 13.1-58.5 ng/µL, while the results when applied CTAB 2% method had higher values ranged between 352.5-3366.7 ng/µL. The results of DNA isolate concentrations measurement were summarized in table 1.

| Samples   | Ratio (A260/A280) | DNA concentration (ng/µL) |
|-----------|------------------|--------------------------|
| BN        | 2.11             | 32.7                     |
| BE        | 2.04             | 55.5                     |
| TN        | 2.16             | 19.8                     |
| TE        | 2.04             | 49.3                     |
| CN        | 2.09             | 13.1                     |
| CE        | 2.05             | 58.5                     |
| CTAB 2% phenol chloroform | 1.97 | 3310.3 |
| BN        | 1.89             | 1141.3                   |
| BE        | 1.72             | 3366.7                   |
| TN        | 1.91             | 1280.2                   |
| CN        | 1.83             | 1471.4                   |
| CE        | 1.86             | 352.5                    |

Note: (BN) tuna without bacterial cultivation, (CN) skipjack without bacterial cultivation, (TN) little tuna without bacterial cultivation, (BE) tuna with bacterial cultivation, (CE) skipjack with bacterial cultivation, (TE) little tuna with bacterial cultivation.

TDC gene bacteria isolated from tuna flesh (BE) was successfully amplified using primer pair of TDC-F and TDC-R at annealing temperature of 53°C (figure 1). The success of PCR amplification can be influenced by the suitable primer pairs and the annealing temperature. Research conducted by de Las Rivas et al (2006) showed PCR amplification successfully performed using TDC-F and TDC-R primers at 53°C annealing temperature with a DNA molecular weight target of 825 bp.
Amplification of DNA isolates from cultivated sample (BE, CE, and TE) using 16S rRNA primers produced single DNA band at 1,500 bp (figure 1). The 16S gene is a highly conserved gene. Each organism that has a certain kinship can be aligned, so it is easier to determine the differences in sequences of these organisms. The 16S rRNA gene is frequently used to study taxonomy and phylogenetics because it can be found in almost all bacteria. Conserved areas in the 16S gene are used as a universal primer resulting an amplicon at 1,500 bp (Cai et al 2003).

![Electropherogram of DNA amplification using: a) TDC gene, (b) 16S rRNA gene](image)

**Figure 1.** Electropherogram of DNA amplification using: a) TDC gene, (b) 16S rRNA gene; (M) marker, (BN) tuna without bacterial cultivation, (CN) skipjack without bacterial cultivation, (TN) little tuna without bacterial cultivation (BE), tuna with bacterial cultivation, (CE) skipjack with bacterial cultivation, (TE) little tuna with bacterial cultivation, (K+) positive and (K-) negative control.

3.2. Bioinformatics of bacterial isolate TDC and 16S rRNA

The sequences were analyzed by comparing data to GenBank at NCBI (http://blast.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool). The results of species identification can be seen in table 2. BLAST analysis showed the TDC genes in tuna (BE) and control cultivation (KE) samples were 80.78% and 80.66% identical to that of *Carnobacterium divergens*, respectively.
Table 2. Species identification with BLAST analysis.

| Samples | Analytical results                      | Homology value | E value | Accession numbers |
|---------|----------------------------------------|----------------|---------|------------------|
| BE TDC  | *Carnobacterium divergens*              | 80.78%         | 5e-158  | CP016843.1       |
| KE TDC  | *Carnobacterium divergens*              | 80.66%         | 2e-151  | CP016843.1       |
| BE 16S  | *Enterobacter tabaci*                   | 91.05%         | 0       | NR_146667.2      |
| TE 16S  | *Enterobacter hormaechei*               | 97.15%         | 0       | NR_126208.1      |
| CE 16S  | *Escherichia marmotae*                  | 98.33%         | 0       | NR_136472.1      |
| KE 16S  | *Peptoniphilus stercorisuis*            | 75.57%         | 4e-130  | NR_134130.1      |

Meanwhile the 16S genes in tuna (BE), little tuna (TE), skipjack (CE), and control (KE) samples were identified as *Enterobacter tabaci*, *Enterobacter hormaechei*, *Escherichia marmotae*, and genus *Peptoniphilus*. The homology values of each sample were 91.05%, 97.15%, 98.33% and 75.57%, respectively.

*C. divergens* belongs to the group of Lactic Acid Bacteria (BAL) which produces tyramine (Leisner et al 2007). This bacterium is able to survive in freezing conditions for a long period (Katayama et al 2007). *C. divergens* is found in seafood and able to grow to high concentrations in fresh and preserved products. The bacteria also dominate the microbial community in frozen products of salmon, shrimp and tuna. The results of the study were in accordance with previous studies that detect bacteria by amplification using TDC-F primers and TDC-R showing that only Gram-positive bacteria produce tyramine such as *E. faecalis*, *E. Faecium*, *C. divergens*, and *Lactobacillus brevis* (de Las Las Rivas et al 2006).

The 16S rRNA identification results showed the bacteria isolated from tuna (BE) and little tuna (TE) belong to the family Enterobacteriaceae in the form of *E. tabaci* and *E. hormaechei* species. *E. tabaci* is motile gram-negative bacteria with the colonies are yellow, convex, and have smooth circular margins. The bacteria grow at 4°C-45°C and show positive results in the tyrosine test (Duan et al 2015). Meanwhile, *E. hormaechei* is motile gram-negative bacteria, catalase positive, fermentative, and positive in the tyrosine test. These bacteria exhibit general characteristics of the family Enterobacteriaceae and genus Enterobacter (Hoffmann et al 2005). Enterobacteriaceae are regarded as microorganisms with high decarboxylase activity. *Enterobacter* is abundant in fish or processed fish products (Fadhaloua-Zid et al 2012).

The 16S rRNA gene in little tuna (CE) sample was identified belong to *Escherichia marmotae*, bacteria species belong to the family Enterobacteriaceae of the genus *Escherichia*. The bacteria is non-motile gram-negative bacteria, grow at optimal temperature 25°C-37°C, anaerobic with positive result on tyrosine test (Liu et al 2015). Meanwhile, the positive control sample (KE) identification result showed *Peptoniphilus stercorisuis* species. The bacterium is gram-positive bacteria, non-motile, anaerobic, round in shape, and positive in the tyrosine test. Optimal temperature for growth condition of *P. stercorisuis* is at 30°C (Johnson et al 2014).

4. Conclusion

TDC gene was better detected from cultivated bacteria than from non-cultivated. The TDC gene amplification in samples of cultivated tuna (BE) and positive control (KE) obtained single band with size of 825 bp. Meanwhile, the 16S rRNA gene in cultivate bacteria from tuna, little tuna, skipjack,
and positive control (BE, TE, CE, and KE) was successfully amplified resulting single band with a size of 1.500 bp. Samples were also successfully identified as C. divergens in BE and KE TDC samples, while in BE, TE, CE, and KE 16S were identified as species E. tabaci, E. hormaechei, E. marmotae, and P. stercorisuis. Future studies should be conducted to screen in the process of bacterial cultivation in order to obtain a single target-specific colony. It is necessary to detect tyrosine, so that the amount of tyrosine contained in an ingredient can be determined for comparison between chemical and molecular methods.

Acknowledgement

The authors gratefully acknowledge the research funding from Indonesian Ministry of Research, Technology and Higher Education (RISTEKDIKTI) “Hibah INSINAS Mitra” for Dr. Mala Nurilmala and Team with contract ID 12/INS-1/PPK/E4/2019.

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