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Isolation and Identification of Lipid-Degrading Bacteria from Fish (*Sardinella longiceps*) Canning Wastewater

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***Abstract*— The aim of this study is to isolate, characterize and identify the lipase-degrading bacteria from fish canning wastewater. From this study, seven isolates of lipase-degrading bacteria were obtained. The high lipase-degrading activity was showed by isolate AG-2 and AD-1 with activity of 12.22 U/mL and 11.11 U/mL, respectively. Both isolates then characterized for the optimum temperature and pH. The result showed that the optimum temperature for growth of isolate AG-2 was 27°C, whereas isolate AD-1 was 37°C. Both of the isolates have the same pH for optimum growth, pH 8. Because the optimum temperature of isolate AD-1 has the same condition with temperature of sample, isolate AD-1 is chosen as alternative for biological wastewater treatment. Molecular identification of isolate AD-1 using 16S rRNA sequencing showed that it has 99% similarity with *Bacillus cereus* SBD1-8.**

***Keywords*— isolation; identification; characterization; lipid-degrading bacteria; fish canning wastewater; 16S rRNA**

I. Introduction

Due to the increase of population and rapid industrialization, the problems of industrial waste water requiring urgent attention. Fish canning industry is one among the polluting food industries. In Indonesia, the production of fish has increased every year, from 2010-2012 the fish production was 7679.4 (thousand tons), 8419.9 (thousand tons), and 8881.5 (thousand tons), respectively [1]. Fish canning industry plays a significant role in the national economic and social wellbeing. However, it can also leads the environmental problems because the high content of organic matter, ammonia, salts, oil and grease in their wastewater [2]. High lipid (fats and oils) concentration contained in wastewater inhibits the activity of microbes in biological wastewater treatment systems [3]. Recently, various industries have replaced the use of chemicals which are detrimental to the environment by new processes that use enzymes under less corrosive conditions. A biological wastewater treatment using lipid-degrading microorganism is needed to be environmental friendly for handling the oil contaminated waste. The application of lipase producing microorganisms to degrade oil and fat has become an interesting strategy because it eliminates the need for pretreatment processes and the lipid residues are converted into carbon dioxide and water, and cost of treatment is less [4]. This study aims is obtaining the lipid-degrading bacteria from fish canning wastewater with a high lipolytic activity for possible application in treatment of lipids-contaminated wastewater.

II. Material and Method

Wastewater samples were collected from PT. Sumberyala Samudera, Muncar, Banyuwangi, East Java. The temperature of sample was between 35°C to 37°C and the pH between 5.5 to 7.0.

A. Isolation of lipid-degrading bacteria

Samples have been plated onto Rhoda mine B-agar by streak plate method and incubated at 37°C for 24 h [5, 6]. The lipolytic strains were visualized as orange fluorescence under UV rays (λ=352 nm), but the non-lipolytic strains revealed pink colonies (Figure 1). Isolates was screened by tributyrin agar method. Isolates were grown on tributyrin agar base by streak plate method and incubated at 37°C for 24 h. The lipase producing bacteria were identified by the presence of clear hydrolytic zone (Figure 2).

B. Lipase production and extraction of crude lipase

The bacterial culture was grown in 100 mL Erlenmeyer flask containing containing Nutrient Broth with olive oil 5% inoculated with 10% (v/v) cell suspension and incubated at 37°C on shaker water bath (90 rpm) for 20 h (the end of logarithmic phase). The harvesting time of lipase carry out at the end of logarithmic phase because at this time the maximum of cells occurred, so it can be assumed that lipase also produced maximum. Crude lipase extracted by adding

buffer citrate phosphate pH 7 into medium with ratio 1:4 (v/v), followed by vortex until homogen. The solution centrifugated at 4°C, 4000 rpm for 20 min. The settled cells were separated and their enzyme-activity was measured by culture filter [7].

C. Lipolytic assay

Lipase activity was determined by titration of the free fatty acids that was released by the enzyme action on an emulsion of 2.5 mL olive oil and 22.5 mL Arabic gum (10% w/v) [7]. The emulsion was added 15 mL $CaCl_2$ 0.075 M and 10 mL NaCl 3 M in 0.1M citrate phosphate buffer (pH=6.0). The substrate and the crude enzyme were mixed with ratio 10:1 (v/v) and incubated at 37°C for 10 min. The reaction was stopped and fatty acids were extracted by heating for 10 minutes. The amount of fatty acids liberated were estimated by titrating with 0.05M NaOH using a phenolphthalein indicator. The lipase activity was calculated using as follows:

$$\text{Lipase activity} = \frac{A \times B \times 10^3}{C \times D}\ \mu \text{ mol / ml. min (Unit)}$$

A = NaOH sample-blanko (ml)
B = Concentration of NaOH (M)
C = Volume of crude lipase (ml)
D = Time of incubation (min)

D. Identification of lipid-degrading bacteria

The characters of the organism were studied following the standard microbiological methods. Identification included morphological and biochemical test such as morphology of colony, morphology of cell, Gram staining, motility test and catalase test [8], [9], 10]. Molecular identification of selected lipid-degrading bacteria using 16S rRNA sequencing [11]

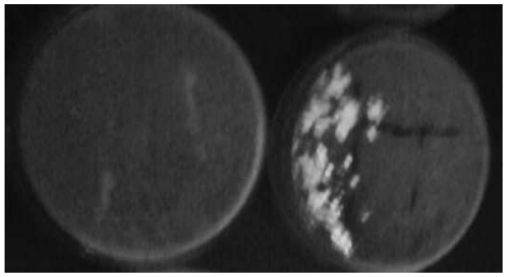
E. Characterization of lipid-degrading bacteria

Characterization of lipid-degrading bacteria included temperature and pH. The selected isolate was inoculated onto Nutrient Broth added with olive oil 5%, incubated at different temperature (22°C, 27°C, 32°C, 37°C and 42°C) and different pH (pH 5, 6, 7, 8 and 9) for 24 h. The growth of bacteria was measured using spectrophotometer λ=560 nm [12].

III. Results and Discussion

A. Isolation of lipid-degrading bacteria

Isolation of lipid-degrading bacteria using rhodamine B-agar by streak plate method and incubate at 37°C for 24 h. Twelve isolates that expected as lipid-degrading bacteria was succeeded to be isolated. The positive lipid-degrading bacteria were visualized as orange fluorescence under UV rays (λ=352 nm) (Figure 1). It occurred because the complex bond formation resulted reaction between rhodamine B cation with uranyl ion from the hydrolysis of triglycerides by lipase [5], [13].



(a) (b)

Fig. 1 Isolation of lipid-degrading bacteria using Rhodamine B agar (a) negative sample and (b) positive sample

Twelve isolates were grown onto tributyrin agar using quadrant method. Tributyrin used to purify the isolates because the colonies that grow on rhodamine B-agar could not separated perfectly. In rhodamine B-agar, it grown between globula-oil that is not mix completely with media. Positive colonies on media lipolytic tributyrin that will establish clear zone around it (Figure 2).

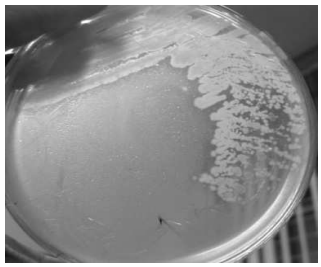


Fig. 2 Isolation of lipid-degrading bacteria using trybutyrin

B. Determination of crude enzyme activity

Lipolytic activity assays toward seven isolates was performed with a titrimetric method using 0.05 M NaOH (Table 1). The principle is made in the form of glyceride substrate emulsified and added to crude lipase. The substrate is made of olive oil in emulsion form aims to activate lipase and accelerate the hydrolysis of triglycerides by lipase to form free fatty acids [14]. Free fatty acid will be reacted with NaOH to form a pink complex due to the presence of indicators of PP (Phenolphthalein). Lipase is an extracellular enzyme excreted by microorganisms to the media during fermentation so that the extraction is done by centrifugation of the separation of the pellet and supernatant [15]. Pellet is the biomass of cells, while the supernatant containing the enzyme.

TABLE I
RELATIVE LIPASE ACTIVITY OF LIPID-DEGRADING BACTERIA

Isolate	Relative lipase activity (U/mL)
AA-1	10.00
AB-1	9.44
AC-1	10.00
AD-1	11.11
AE-2	7.78
AF-2	4.44
AG-2	12.22

Table 1 shown that the highest lipase activity is isolate AG-2 (12.22 U/mL) and the lowest lipase activity is isolate AF-2 (4.4 U/mL). The difference of lipase activity is because of the difference kinds of bacteria. The crude lipase that extracted from different bacteria has different activity [16].

C. Temperature and pH characterization of lipid-degrading bacteria

Temperature characterization aims to determine the optimum growth temperature of lipid-degrading bacteria. The selected isolate was inoculated onto Nutrient Broth added by olive oil 5% (v/v) and incubated at 22, 27, 32, 37 and 42^{o}C. Range of the temperature was chosen because that is a growth temperature of mesophilic bacteria and that temperature same with the condition of sample.

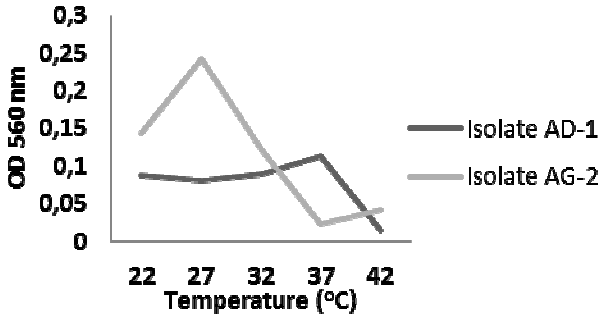


Fig. 3 Temperature characterization of lipid-degrading bacteria

Figure 3 shown that the two isolates were selected have different optimum temperature between isolate AD-1 and isolate AG-2, 37°C and 27^{o}C. Optimum temperature of lipolytic bacteria is 30^{o}C [17]. However, several lipolytic bacteria has different characteristic such as *Pseudomonas aeruginosa* which has optimum temperature and maximum temperature 37^{o}C and 42^{o}C, respectively [18] and *Corynebacterium* sp has the optimum temperature 37^{o}C [19].

pH characterization carried out by growing the two isolates that have a high lipolytic activity at pH 5, 6, 7, 8, and 9. Selection of pH range is based on the sample pH and the pH optimum of lipase. The results shown (Figure 4) that the characterization of isolates AD-1 and AG-2 has a pH optimum growth at pH 8. This possible occurred because under pH 8, cell component that predominantly consisted of protein has denatured, so the metabolism disturbed. Usually, lipolytic bacteria can growth at optimum pH 7, depend on the type of substrate [19]. Lipase can be produced optimally by *Bacillus stearothermophilus* at pH 8 and their produced decreasing lipase if pH increased [20].

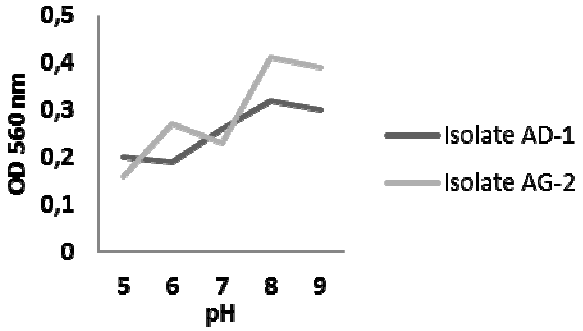


Fig. 4 pH characterization of lipid-degrading bacteria

D. Identification of lipid-degrading bacteria

The characters of the organism were studied following the standard of microbiological methods. Identification included morphological and biochemical test such as morphology of colony, cell, Gram staining, motility test and catalase test (Table 2).

TABLE II
MORPHOLOGY OF COLONY, CELL, GRAM STAINING, MOTILITY TEST AND CATALASE TEST

Isolate	Morphology of colony				Cells Form	Gram Staining	Motility Test	Catalase Test
	Color	Shape	Elevation	Edges				
AA-1	Milk White	Round	Flat	Wavy	Basil	-	-	+
AB-1	White	Round	Raised	Wavy	Basil	+	-	-
AC-1	White	Round	Flat	Wavy	Coccus	-	-	+
AD-1	Yellow	Round	Raised	Wavy	Basil	+	-	-
AE-2	Milk White	Irregular	Flat	Wavy	Basil	-	-	-
AF-2	Yellow	Irregular	Raised	Wavy	Coccus	+	-	+
AG-2	Milk White	Irregular	Flat	Wavy	Basil	+	-	+

E. Molecular identification using 16S rRNA sequencing

Microbial characterization result that isolate AD-1 more potential to be applicated in biological wastewater treatment than isolate AG-2. Isolate AD-1 has optimum temperature to growth, 37^{o}C, equal to sample temperature and it has quite high lipase activity approaching isolate AG-2, 11.11 U/mL. Molecular technique have enabled to investigate microorganism more deeply and sensitively than conventional testing. To identify the isolated bacteria the molecular identification was performed in this study toward isolate AD-1. 16S rRNA sequencing is used for molecular identification because its presence in almost all bacteria, often existing as a multigene family or operons, the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution) and the 16S rRNA gene (1,500 bp) is large enough for informatics [11]. Molecular identification using 16S rRNA is molecular identification technique to study phylogenetic and taxonomy bacteria. Sequencing analysis was done by using PCR process to amplify DNA genome. Determination of sequence nucleotide of 16S rRNA was performed by sequencing analyzed using ABI. Finally, the sequences were aligned and compared with other 16SrRNA genes in the Gen Bank (Figure 5) by using the NCBI Basic Local Alignment Search Tools (BLAST) [21].

16S rRNA sequence shows that isolate AD-1 had highest homology (99%) with *Bacillus cereus* SBD1-8.

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GCTCAGGATGAACGCTGGCGGCGTGCCTAATACAT
GCAAGTCGAG  64
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Sbjct 3    TGTTTGA--TC-
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TGCATGGTTGTCGTCAGCTCGTGTC 1079

Query 1084 GT-AGA 1088
|| |||
Sbjct 1080 GTGAGA 1085

Fig. 5 Sequence of isolate AD-1 (Sbjct) and *Bacillus cereus* SBDI-8 (Query)

Genetic relationship inter-organism can be known from phylogenetic tree. Phylogenetic tree of isolate AD-1 (Figure 6) showed the comparison of isolate AD-1 with 12 bacteria strain expected had sequence equal to isolate AD-1. Genetic relationship of isolate AD-1 is closer with *Bacillus cereus* SBD1-8.

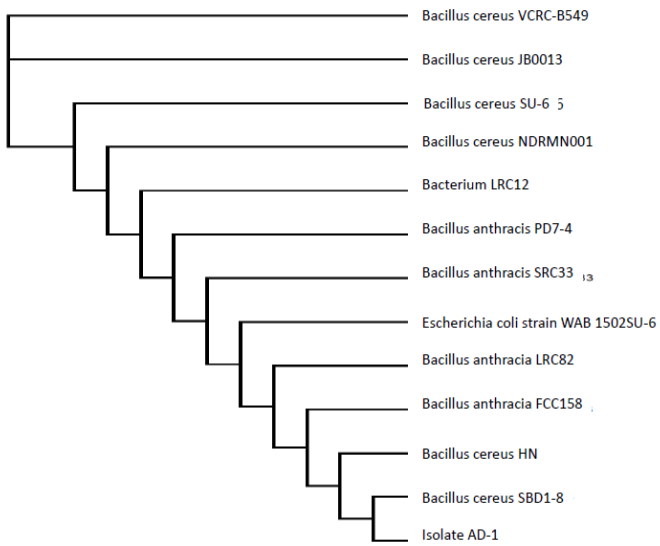

Fig. 6 Phylogenetic tree predicted from sequence aligment of isolate AD-1

IV. Conclusions

This study shown that isolation of lipid-degrading bacteria from fish canning wastewater succeeded. One isolate namely isolate AD-1 has potential application for biological wastewater treatment. Isolate AD-1 has lipase activity 11.11 U/ml and it has 99% similarity with *Bacillus cereus* SBD1-8.

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