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CHARACTERIZATION OF PREDICTED BACTERIAL COLD-ADAPTED LIPASE FROM SEAFOOD COLD STORAGE

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ABSTRACT

Lipases constitute as top three most important group of enzymes along with carbohydrases and proteases, and are widely used in various industries. In particular, lipase that perform high activity at low temperatures, or referred as cold adapted lipase (CLPs) considered as attractive catalyst due to its activity at low temperature. This unique feature is the main advantage of cold adapted lipase utilization because it requires a low energy source that is correlated with lower production costs and energy. In addition, reactions occur in cold temperatures may result in better product quality. The purpose of this research was to perform screening and characterization of bacterial cold adapted lipase from seafood cold storage. Among 53 isolates, Kr_16_30, TI_37_14 and Kr_16_28 showed the highest activity with 4.12 U/mL: 3.87 U/mL and 3.21 U/mL, respectively. Isolates Kr_16_30 seemed to be typical cold adapted lipase with optimum temperature at 20°C and pH 7. Isolates Kr_16_28 performed highest lipolytic activity at 30°C while TI_37_14 suspected to be similar to typical mesophilic lipase with optimum temperature at 40°C. Species identification based on sequencing of gene encoding 16s rRNA revealed that isolates Kr_16 30 and Kr_16 28 are belong to genus Pseudomonas and Bacillus, repectively.

Keywords: Bacteria, Cold-Adapted Lipase, Characterization, Fish Cold Storage

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1. INTRODUCTION

In recent decades, enzymes have received great attention in the effort to reduce chemical-based reactions in industrial process. Enzyme-mediated reactions are considered environmentally friendly. It produces less waste, require less energy, reduce the risk of pollution and potentially improve product quality Kavitha (2016) . Lipase (acyl glycerol hydrolases, EC 3.1.1.3) is one of most important enzymes with a wide range of applications in biotechnology, food, chemical, pharmaceutical, biodiesel and other industrial fields. Lipases are able to catalyze both the hydrolysis and the synthesis of esters from glycerol and long-chain fatty acids Jaeger and Eggert (2002) .

One of the main focus in enzyme development is the activity under extreme conditions Santiago et al. (2016) . Temperature seemed to be one of the most crucial environmental factors in enzymatic reactions. Higher temperatures, which is often applied to industrial processes, will increase the rate of reaction, but also accelerate the deactivation of enzymes. Heat-resistant enzymes (thermophiles) can be used to solve this problem. However, reactions at high temperatures will increase production costs Cowan (2010) .

While thermophilic enzymes play an important role in reactions involving high temperatures, exploration of enzymes that are active at low temperatures gained more interest as its ability to promote reaction under mild condition Feller (2010) . This group of lipases referred as cold adapted lipases (CLPs) or cold active lipase. These enzymes displayed great and efficient activity at low temperatures, leading to significant energy savings. For some applications, cold-adapted enzymes also maintain some fragile compounds from being damaged by higher temperatures Collins et al. (2012), Georlette et al. (2004). Therefore, they become an attractive choice to be applied in pharmaceuticals products, food, environmental bioremidiation and detergent ingredients Joseph et al. (2008) .

Microorganisms that grow in cold environments are good candidates to produce cold-adapted enzymes. They may develop special adaptations to support their metabolism and growth in those extreme condition. Compared to its exploration in extreme high or moderate temperature, the study of lipase producing bacteria in cold environment is relatively limited.

The studies on cold adapted lypolitic enzyme generally come from psychrophilic environments or psychrophilic microorganisms isolated from polar and Antarctic regions, for example from genus Moraxella Feller et al. (1991), Psychrobacter Kulakova et al. (2004), Xuezheng et al. (2010) and Pseudomonas Zhang and Zeng (2008) as well as deep sea areas and food storage at low temperatures Dieckelmann et al. (1998), Domínguez De María et al. (2005). Polar region and constantly extreme temperature places are very potential as cold lipase source. However, in many cases, the enzymes are unstable even at moderate temperatures Joseph et al. (2008) . Several cold adapted lipases obtained from the tropics area showed better thermal stability than those obtained from the alpine regions Kavitha and Shanthi (2013) .

Harnessing enzyme-based reaction largely depends on the bacterial genetics and optimum condition to ensuring the optimum activity. This study attempted to evaluate the potential bacteria producing cold adapted lipase from seafood cold storage. This work including screening, identification and initial characteriation to determine optimal conditions for better lipolytic activity.

2. MATERIALS AND METHODS

Screening for Lipase-Producing Bacteria

Clams, fish egg and crab meat samples were obtained from three cold storage at a modern supermarket in Malang City, East Java, Indonesia. A total 1 gram of sample was crushed and added by 50 mL of Basal Salt Solution (BSM) and incubated at 25°C for 48 hours. The mixture was diluted in a serial dilution calculation using 0.9% NaCl. Aliquots of 10-5 to 10-9 were inoculated on agar media containing 5g/L tryptone, 2.5 g/L yeast extract, 9g/L agar with 1% Tween 20 or Tween 80 added as initial lipolytic screening. Incubation was carried out for 2-10 days at 16°C, 25°C and 37°C, respectively. Lipolytic activity is characterized by the appearance of white precipitation around the bacterial colonies. In order to collecting lipolytic bacteria, colonies growing with white precipitation sign were taken for further screening to validate their lipolytic activity.

Each bacterial colony was taken and separately inoculated by spotting on Sierra's Medium (10 g/L peptone, 5g/L NaCl, 20g/L agar, 0.1 g/L CaCl2, and 1% Tween 80 or Tween 20 as lase substrates). Incubation was carried out at three different temperatures (16°C, 25°C and 37°C) for 2-6 days Mukesh et al. (2012) . Lipolytic activity is characterized by the appearance of white precipitation around the bacterial colonies. Three potential bacterial isolates that showed largest

precipitation zone were selected and re-grown on agar medium using the streak plate method to obtain a single colony.

Production of Lipase Crude Extract

Potential bacterial isolates were inoculated separately into 10 mL of broth medium containing 1% tryptone, 1% sodium chloride, 0.5% yeast extract and incubated for 24 hours with 150 rpm agitation. The next day, 2% culture was re-inoculated in 50 mL of production medium containing 1% tryptone, 1% sodium chloride, 0.5% yeast extract and 1% lipase substrate. Incubation was carried out with agitation at 150 rpm for 2x24 hours. The culture was centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred into a sterile tube and referred to as the lipase crude extract.

Activity of Lipase Crude Extract

Lipase activity assay was carried out based on the method described by Stoytcheva et al. (2012) with slight modification of reaction mixture. A total of 2 mL of lipase substrate was added to reaction containing 2 mL of 0.1 M sodium phosphate buffer pH 6.5: 2.5 ml of distilled water, 0.5 ml of 0.1 M CaCl2 and 1 ml of crude extract. The reaction mixture was incubated with agitation at 150 rpm for 15 minutes. The reaction was stopped by adding 96% ethanol. Enzyme activity measured by titration method using 0.05 M NaOH, with the addition of phenolphthalein as an indicator. One unit of crude extract lipase activity was defined as amount of enzyme required to produced 1 mol of fatty acid per minute under the measurement condition.

Effect of Temperature and pH on Enzyme Activity

Characterization of lipolytic activity were carried out at different temperature and pH condition. Determination of the optimum temperature was done by testing at various temperatures of 10˚C, 20˚C, 30˚C, 40˚C, 50˚C, 60˚C and 70˚C.

Optimal pH values were measured by incubation of crude extract at various different buffers with pH range from 5 to 11. Buffer systems consist of CH_3COONa/CH_3COOH (pH5), KH_2PO4/Na_2HPO4 (pH6, 7); Tris HCl (pH 8,9) and $Na_2HPO4/NaOH$ (pH 10.11). Similar procedure is applied as the standard method. The lipolytic activity for each temperature and pH were converted into relative activity using the highest lipase activity as control (100% of relative activity).

Amplification of partial gene encoding 16s rRNA

Potential isolates were subjected to species identification based on 16s rRNA sequence. The genomic DNA was isolated using wizard genomic DNA purification kit (Promega, USA). The concentration and purity of DNA was analyzed using nanodrop 2000 (Thermofisher Scientific, USA). Amplification of the 16s rDNA sequence was carried out using PCR with specific forward primer 63F and reverse primer 1387R.

PCR mixture contained 1 µL DNA genome, 5 µL GoTaq Green Master Mix (Promega, USA), 1 µL of each primer and nuclease free water up to 10 uL final reaction. Verification was done by electrophoresis in 1% agarose gel (w/v). The PCR product was purified and injected into 3130 DNA analyzer machine (Applied Biosystems, Foster City, USA) following the official running protocol. After analyzing, NCBI-BLAST were performed to find the closest match of the contiguous sequence (https://www.ncbi.nlm.nih.gov/).

3. RESULTS AND DISCUSSIONS

Lipase enzymes are naturally present in almost all living organism, including microorganism (such as bacteria and yeasts), plants and animals Gupta et al. (2004) . Lipase enzymes from microorganisms have important value in the industrial world because its convenience and production efficiency. Various studies focused on the exploration and use of lipases that are active at extreme temperatures, due to industrial processes that are mostly carried out at high temperatures. However, the use of high temperatures may not be very beneficial in terms of the energy and production cost. In addition, high temperature may affect the quality of fragile components in food or pharmaceuticals.

Therefore, the exploration of lipases that are active at cold temperatures or commonly referred to as cold-adapted lipases or cold-active lipases gained a lot of attention due to their activity under mild condition. Thus, significant study has been devoted to performing screening and characterization of cold adapted lipase from bacterial and fungal origins.

This present work aimed to obtain and determine the characteristics of lipase enzymes from clams, fish eggs and crab meats samples in cold storage. Total bacteria were grown on non-selective Plate Count Agar medium. Tween 20 and Tween 80 were added into the medium for initial screening and make it easier to selecting the candidate of lipase producing colony for further screening. Incubation was carried out at three different temperatures, which is 16°C, 25°C and 37°C.

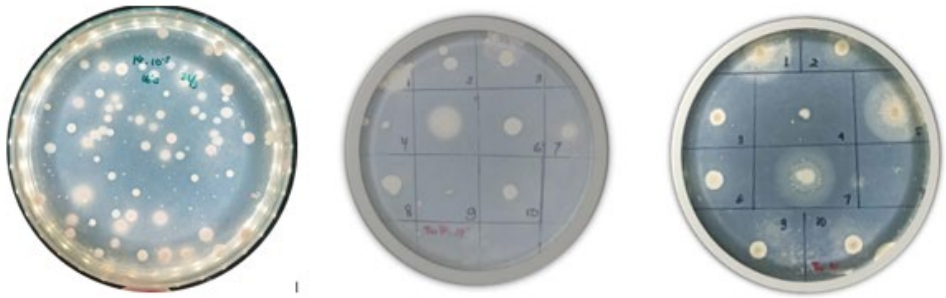

Figure 1 The growth of total bacteria on PCA medium added with Tween 80 (A), the lipolytic activity at Sierra's Medium containing Tween 20 (B) and Tween 80 (C). The white presipitation around the colony indicating lipolytic activity

The incubation time required for bacterial growth turn out to be significantly different for each temperature od incubation, around 18 hours (37°C), 24-48 hours (25°C) and 7 days (16°C) incubation temperatures, respectively. Clams, fish eggs and crab meat samples from modern market in Malang city, contained bacteria with varying amounts from 6,27x105 up to 1.23x107. Bacterial colonies with white presipitation were selected and re- inoculated separately on Sierra's Medium as validation of lipolytic avtivity Figure 1.

Table 1 Total isolated bacteria and the persentage of lipase producing bacteri from each sample and temperature of incubation

SampleID, temperature	Isolated bacteria	Lipolytic bacteria
Clam, 16°C	32	15 (46.88%)
Clam, 25°C	10	5 (50%)
Clam, 37°C	14	8 (57.14%)

Fish egg, 16°C	5	1 (20%)
Fish egg, 25°C	9	2 (22.22%)
Fish egg, 37°C	12	9 (75%)
Crab meat, 16°C	9	3 (33.33%)
Crab meat, 25°C	10	6 (60%)
Crab meat, 37°C	7	4 (57.14)

Lipolytic activity assay using Tween 20 or 80 based on the principle of calcium salt precipitation. Hydrolysis of tween releases fatty acids which bind to calcium derived from $CaCl_2.2H_2O$ in the medium. This complex will form insoluble crystals, appear as white precipitation around the colony (Figure 2 B, C), as indication that the bacterial colony produces extracellular lipase Rai et al. (2014).

Table 2 Lipolytic activity of three most potential bacteria

Isolate	Lipase Activity
Kr_16_30	4.12 (U/mL)
TI_37_14	3.87 (U/mL)
Kr_16_28	3.21 (U/mL)

Bacteria isolated from fish egg at 16°C incubation temperature showed the highest percentage of lipolytic bacteria (75%). Among all bacterial, isolates Kr_16_30, TI_37_14 and Kr_16_28 showed the largest precipitation zone Table 2. Thus, the three isolates were considered potential and used for further analysis.

The culture of three potential bacteria were prepared using broth medium containing Tween 80. The cell free supernatants are measured to determine the extracellular lipase activity. Isolate Kr_16_30 turn out to be the most potential lipase producer with the activity values presented in Table 2. The production of lipase enzymes by psychotropic organisms varies among the species, which is also influenced by the optimum temperature, pH and specificity of the enzyme.

After incubation at various temperatures, the optimum temperature of isolate Kr_16_30 was observed at 20°C, with lipolytic activity slightly higher than its activity measured at 16°C Figure 2. At higher temperature, enzyme activity decreased to less than 80% at 30°C and about 50% activity remaining at 50°C.

Isolates TI_37_14 and Kr_16_28 had optimum activity at 40°C and 30°C, respectively. Isolate Kr_16_28 appeared to have a relatively stable lipolytic activity in the temperature range 20°C to 40°C with more than 80% of the maximum activity Figure 2. Lipase activity of all isolates are quite low at 10°C. Only isolate Kr_16_28 was able to maintain its activity at more than half of the optimum temperature. Therefore, the lipase produced by Kr_16_30 seemed closer to typical cold adapted lipase, while lipolytic activity of isolates TI_37_14 is similar to most mesophilic bacterial lipase, in the respect of the optimum temperature condition.

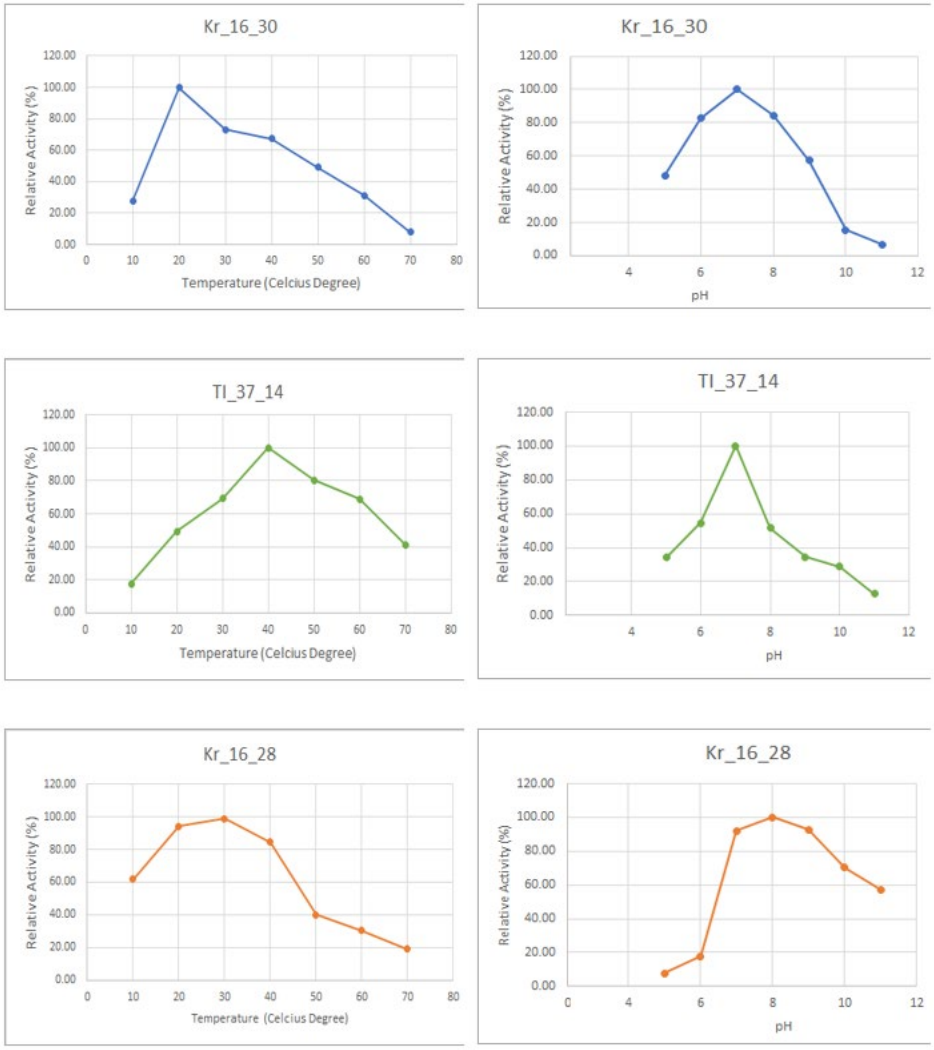

Figure 2 Lipolytic activity of lipase crude extract from potential bacteria under various temperature (left) and pH (right)

The pH value of 7 was determined as the optimum condition of isolates Kr_16_30 and TI_37_28 Figure 2. Isolate Kr_16_30 had fairly stable activity in the pH range of 6 to 8, while a sharp decrease was observed in isolate TI_37_28 in same pH value. Under alkaline treatment with pH value of 11, the activities of isolates Kr_16_30 and TI_37_14 were measured to be 6.65% and 12.92%, respectively.

Changes in pH value affected the lipolytic activity of isolate Kr_16_28 with a different pattern. The optimum pH was observed at pH 8, with more than 90% activity at pH 9. The lipolytic activity of this isolate was sensitive to acidic conditions and was relatively maintained at alkaline temperatures with a remaining activity of 61.68% activity at pH 11. The acidity of the media greatly affects various enzymatic processes and the transport of compounds across the cell membranes Kuddus and Ramteke (2008).

Table 3 Species identification of potential bacteria producing cold adapted lipase

Sample ID	Identified Bacteria by BLAST	Query Cover	E value	Percent Identity
Kr_16_30	*Pseudomonas aeruginosa*	100%	0	97.21%

	unculturable bacterium clone ncd2191b	100%	0	97.21%
	Pseudomonas Aeruginosa strain BGS1	100%	0	97.10%
Kr_16_28	*Bacillus velezensis strain* IMJ11	100%	0	99.90%
	Bacillus siamensis strain ICMP 20282	100%	0	99.90%
	Bacillus amyloliquefaciens strain GuBC-1	100%	0	99.90%

In this study, the species identification of isolates TI_37_28 was not performed because its optimal activity was observed at temperature higher than 30°C. The species identification of Kr_16 30 and Kr_16_28 performed by 16s rRNA sequencing. BLAST results of amplified DNA sequences provided information that the two isolates were member of phylum Proteobacteria and Firmicutes. The fragment sequence of isolates Kr_16_30 described the highest similarity with *Pseudomonas aeruginosa* (97.21% similarity, 100% query cover), unculturable bacterium clone ncd2191b (97.21% similarity, 100% query cover), and *P. Aeruginosa* strain BGS1 (97.10% similarity, 100% query cover). The isolate Kr_16_28 is part of the Genus Bacillus, with the highest similarity to *Bacillus velezensis* strain IMJ11 (99.90% similarity, 100% query cover), *Bacillus siamensis* strain ICMP 20282 (99.90% similarity, 100% query cover) and *Bacillus amyloliquefaciens* strain GuBC-1 (99.90% similarity, 100% query cover).

4. CONCLUSIONS AND RECOMMENDATIONS

A total of 53 lipolytic bacteria ware screened from clam, fish egg and crab meat samples collected from fish cold storage. Isolates Kr_16_30, TI_37_14 and Kr_16_28 showed the highest activity with 4.12 U/mL: 3.87 U/mL and 3.21 U/mL, respectively. Isolates Kr_16_30 seemed to be typical cold adapted lipase with optimum temperature at 20°C and pH 7. Isolates Kr_16_28 performed highest lipolytic activity at 30°C while TI_37_14 suspected to be similar as most mesophilic lipase with optimum temperature at 40°C. Species identification based on sequencing of 16s rRNA revealed that isolates Kr_16 30 and Kr_16 28 are belong to genus Pseudomonas and Bacillus, respectively.

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