International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.12 No.1, pp 121-128, 2019

Assay of Lipase and Biosurfactant Production Activity of Two Keratinolytic Bacteria*Aeromonas media* LU04 and *Enterobacter tabaci* PK09

Yayuk Putri Rahayu, Dwi Suryanto*, Erman Munir

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Jln. Bioteknologi No. 1, Medan 20155, Indonesia.

Abstract : Assay of lipase and biosurfactant productionactivity of keratinolytic bacterial isolateshas been conducted. Bacterial isolates was collection of Microbiology Laboratory,Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia. Assay on lipase activity was done qualitatively using sensitive plate assay method using rhodamine-B olive oil agar and quantitatively using copper soap colorimetric method with cuppric acetate-pyridine reagent and olive oil as substrate. Characterization of lipase was performed in different pH and temperature. Biosurfactant production assay was conducted using oil displacement method to measure oil displacement area (ODA) and measured emulsification index (EI24).The two potential bacteria *Aeromonas media* LU04 and*Enterobacter tabaci* PK09 out of four bacterial isolates showed higher lipase activity with produced more orange fluorescence around the colony with lipase activity of3.767 and 2.054 U/mL and specific activity were 0.443 and 0.346 U/mg, respectively. The lipase activity of both bacteria was optimum at pH 7 and at temperature of 40^{o}C. Biosurfactant production activity showed ODA value of 38.0 and 15.0 mm, and with emulsification index of 20 and 15%. Biosurfactant activity was in line with lipase activity. This might indicated that biosurfactant prodcution and activity was needed in lipase activity.

Key words : biosurfactant, keratinolytic bacteria, lipase,*Aeromonas media*, *Enterobacter tabaci*.

Introduction

The use of enzymes in technology for bioconversion by-products into valuable materials is an attractive strategy[1]. Lipase and biosurfactants are currently widely used in many fields of biotechnology and various industries. The lipase enzyme and biosurfactant have wide range of applications such as oil hydrolysis, sewage treatment, detergent industry, petrochemical and petroleum, pharmaceutical, biomedical, cosmetic, food industries, leather industry (tannery), and oleochemistry (the production of fatty acids and derivatives). Other advantages of lipase and biosurfactant for industrialapplications include biodegradability, cost-effective productionand low toxicity[2].

DOI= http://dx.doi.org/10.20902/IJCTR.2019.120114

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) includes hydrolytic class enzymes that catalyze the hydrolysis of long chain triacylglycerols into glycerol and free fatty acids. Lipase is a hydrolytic enzyme that acts on an aqueous organic interface, catalyzes the termination of ester bonds in triglycerides and produces glycerol and free fatty acids. Many lipases are found in nature. Lipase enzymes can be obtained from animals, plants, and microorganisms. However, the largest commercial use of lipase is sourced from microorganisms. The lipase enzyme may work on a substrate containing triglycerides that have long fatty acid chains, which have insoluble properties in water contained in fatty compounds[3].

In lipase activity, an intermediate compound called biosurfactant is required. Biosurfactant or surface active compound is a surface active compound which is an active heterogeneous molecule produced extracelluler by microorganisms. Biosurfactants have properties similar to those of surfactants, which have hydrophilic and hydrophobic groups that can reduce surface tension and interference. Biosurfactant helps lipase performance in degrading oil. Biosurfactants are interfacial between polar liquids and different hydrogen bonds such as surface oils with water or air with water, so hydrocarbons are soluble in water due to biosurfactent ability to form microemulsions capable of reducing surface tension[4].

Study on lipase is growing because of its ability to be applied to various industries. Various sources of bacterial isolates are used to determine lipase activity. Among the various types of bacteria, keratinolytic bacteria are selected. The reason for using the isolates was because of its potential to degrade keratin in feathers in which the feathers is mainly covered by lipid[5], therefore, it is assumed these isolates has lipolytic potential. In addition to lipolytic ability, it is also expected to have the ability to produce biosurfactants.

Materials and Methods

Bacterial Isolates

Keratinolytic bacterial isolatesof previous study *Enterobacter tabaci* PK09 and*Aeromonas media* LU04[6], and LU02 and LU01used in this study were a collection of Microbiology Laboratory,Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia.The isolate culture stock was rejuvenated by subculturing on nutrient agar (NA) at 37°C for 24 hours.

Lipolytic Activity Test

Lipase was detected using sensitive plate assay method using Rhodamin-B Olive oil Agar (ROA) plate media. Bacterial culture of 24 hours was inoculated by streakingon the surface of ROA medium. The culture was incubated at 37° C, and colony growth was observed for 8 days underultraviolet of 350 nm. Orange fluorescence formed aroundbacterial colonyindicated that the colonies produced lipase[7].

Lipase Production

Bacterial isolates were subcultured for 24hours on nutrient agar at 37°C. A 24 hours old of 0.5 ml bacterial suspension (OD_{600}=0.5) was inoculated into mineral growth medium added with olive oil (MGMO) (pepton 0.2%; $NH_4H_2PO_4$ 0.1%; NaCl 0.25%; $MgSO_4.7H_2O$ 0.04%; $CaCl_2.2H_2O$ 0.04% (w/v); olive oil 2.0% (v/v); and sterile distilled water[8]. The culture was incubated at 37°C at160 rpmfor 1 dayin shaker. Culture 5 ml was inoculated into production medium of 100 ml. Culture was incubated at 37°C at160 rpmfor 8 days. Cell growth, lipase activity, protein concentration, andbiosurfactant production activity was observed every day.

Lipase Activity Assay

Bacterial culture was harvestedevery day for 8 days. Sample was centrifuged at 13,000 rpm for 10 minutes at 4°C to obtain cell-free supernatant of crude lipase.Assay of lipase activity using colorimetric method based on hydrolysis of olive oil[9]. One ml of cell-free supernatant was added to the reaction mixture containing 1ml olive oil, 1 ml phosphate buffer 0,1 M (pH 7) and 0.02 ml $CaCl_2$ 20 mM. Solution was incubated in shaker at 37^o C at 160 rpm for 30 min. Solution with adding cell-free supernatant was used as control. After 30 minutes all reaction was stopped by adding 1 ml 6 N HCl and 5 ml isooctane by mixing it for 1 minute. Upper isooctane layer of 4 ml containing free fatty acids was transferred to a test tube and properly mixed with 1 ml copper reagent. Copper reagent was prepared according to Lowry[10]. A 5% (w/v) aqueous solution of cupric acetate (copper (II) acetete-1-hydrate/ copper (II) acetate monohydrate) was prepared and filtered. pH was adjusted to 6

using pyridine. One ml of cupric acetate-pyridine reagent was added to produce two layers. Solution was mixed vigorously for 1 minute. The mixture was allowed to stand still for about 10-20 seconds until the aqueous phase was seperated clearly from isooctane containing free fatty acidssolution. Lipase activity was determined by measuring the amount of free fatty acids released by measuring absorbance of isooctane containing free fatty acids solution at 715 nm as optical density based on the standard curves of oleic acid. Standard of free fatty acidwas prepared using 2.5-25 µmol oleic acids by dissolving with 5 ml of isooctane[9] . One unit (U) of lipase activity is defined as the amount of enzyme, which releases 1.0 µmol of free fatty acid per min under specified assay conditions[11] .

Protein Assay

Protein concentration was measured using Bradford method[12]. Standard curve of protein was made using bovine serum albumin (BSA). BSA solution was prepared in concentration of 0, 125, 250, 500, 750, 1000, 1500,and 2000 µg/ml.

Effect of pH and Temperature on Lipase Activity.

The characterization of lipase based on the effect of pH on enzyme activity was determined by incubating reaction mixture as previously described at various pHs ranging from 4 to 8 (4; 5; 6; 7 and 8) at 37^{o} C at 160 rpm for 30 min using acetate phosphate buffer (pH 4 & 5) and phosphate buffer (pH 6 to 8). To evaluate the optimal temperature for the enzyme activity, the assay was done as previously described. The assay was conducted at varying temperatures of 30;35; 40; 45and 50^{o}C at optimum pH, at 160 rpm for 30 min[11].

Oil Displacement Test.

The petri dish filled with 50 ml of distilled water followed by addition of 100 µl of olive oil to the surface of the distillate water.Furthermore, as much as 50 µl of cell-free supernatant is poured onto the surface of olive oil. The value of oil displacement is determined based on the diameter of the clear zone formed on the surface of the olive oil. For negative control use distilled water without surfactant[13].

Emulsification Activity.

The reaction tube was filled with 2 ml of olive oil, then added 2 ml of biosurfactant solution of cell-free supernatant. The tube was vortexed vigorously for 2 min and kept at ambient temperature for 24 hours. Emulsion index was determined as percentage of height of the emulsified layer divided by total height of solution[14].

Results and Discussion

Detectionfor Lipolytic Isolates

Lipase producing isolates can be easily identified by the formation of orange fluorescent halos around the colonies indicated lipid metabolism to free fatty acid.It was shown isolates of *A. media* LU04 and *E. tabaci* PK09 out of four showed more lypolitic activity, while LU02 and LU01 were almost showed no orange fluorescent (**Figure 1**).Rhodamine-B was used to determine the presence of free fatty acids from hydrolysis of lipid by lipase enzyme. Free fatty acids released formed a complex of Rhodamine-B cationic and fatty acid ions to form an orange fluorescence around thecolony of bacterial[7].

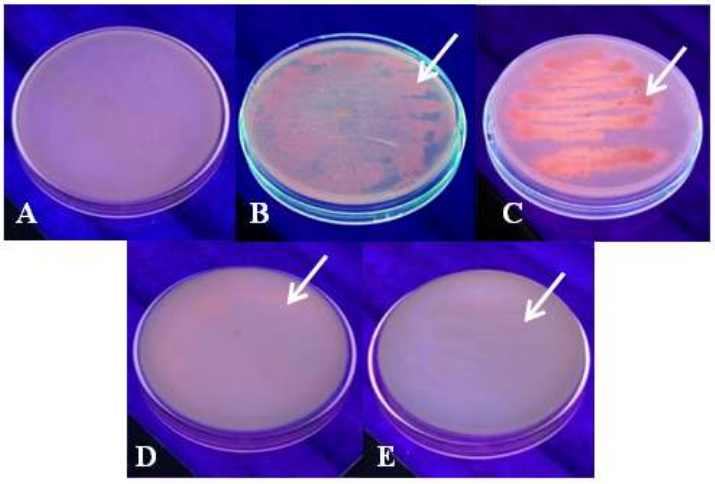

Figure 1. Lypolytic activity of keratinolytic isolates on ROA medium after 8 days of incubation under UV of 350 nm. Presence of orange fluorescence showed different levels intensity (arrowed). (A). Control (ROA medium, without bacteria) showed no orange fluorescence; (B) *E. tabaci* PK09 showed slightly orange fluorescence; (C). *A. media* LU04 showed a bright orange fluorescence; while (D). LU02 and (E). LU01 showed almost no orange fluorescence.

Lipolytic Activity Assay

Based on qualitatively assay, *A. media* LU04 and *E. tabaci*PK09 were choosen for further study. To know lipase production, all bacterial isolates were grown for 8 days and harvested everyday. Cell growth and lipolytic activity supernatant free cell were measured. Cell growth and lipolytic activity of all bacterial isolates were shown in **Figure 2**.

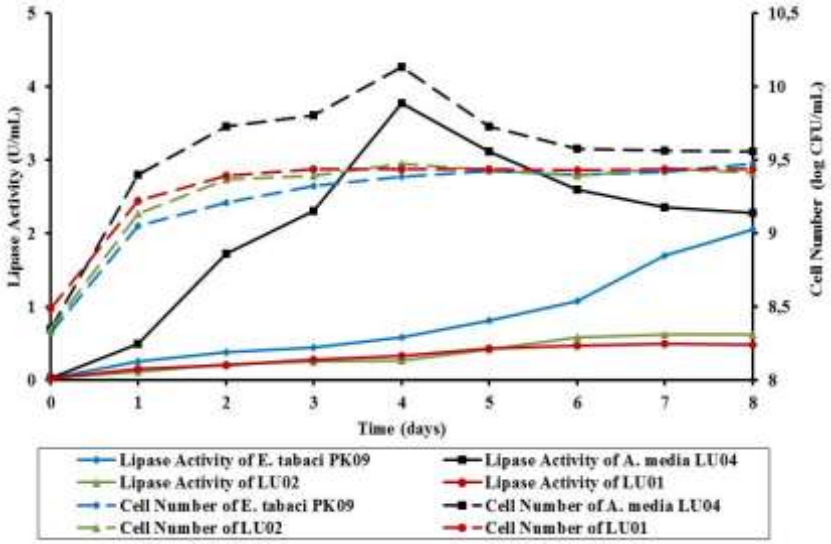

Figure 2. Cell growth and lipase activity of *E. tabaci* PK09, *A. media* LU04, LU02 and LU01.

A. media LU04 showed to increase its lipolytic activity accordingly with its cell growth. Higher lipolityc activity of *A. media* LU04 was begun shortly after maximum cell growth, indicating optimum lipase production of the isolate, while *E. tabaci* PK09 showed to increase its lipolytic activity at late stationary phase. The maximum lipase activity observed in two isolates were *A. media* LU04 and *E. tabaci*PK09of four bacterial isolates showed the highest lipase activity of 3.767 and 2.054 U/mL, respectively.

Lipase activity increased gradually in late logarithmic phase. The limited number of carbon sources forces bacteria to use olive oil as an alternative carbon source for the production and maintenance of cell

biomass by producing more lipase production[15]. Lipases can be produced throughout the bacterial growth phase, with optimal production obtained in late logarithmic phase. The lipase production period may vary from a few hours to several days[3].

In this study the low of lipase activity in *A. media* LU04 and *E. tabaci* PK09 was caused not optimum growth media conditions needed to produce lipase. Lipase activity of crude extract of keratinolytic bacteria *Bacillus cereus* before optimization of growth culture media was obtained at 2.1 U / mL. However, after optimization of the culture medium on keratinolytic bacteria *Bacillus cereus*, lipase activity increased to 61.3 U / mL with carbon source of sucrose, and became 68.6 U / mL with nitrogen source of yeast extract[16].

Protein Assay and Lipase Specific Activity

Protein concentration of bacterial culture was observed to know its spesific lipase activity. Specific lipase activity of *A. media* LU04 and *E. tabaci*PK09 was shown in **Table 1**.

Table 1. Spesific lipase activity of *A. media* LU04 and *E. tabaci*PK09

Bacterial Isolates	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)
A.media LU04	376.7	850.5	0.443
E. tabaci PK09	205.4	594.0	0.346

The results showed that specific activity of lipase of *A. media* LU04 (0.443 U/mg) is slightly higher than *E. tabaci*PK09 (0.346 U/mg). This indicated that produced amount of protein of *A. media* LU04 is higher than *E. tabaci*PK09. High protein levels could be assumed to have a high enzyme.

Other studies showed that specific activity of lipase obtained in *Microbacterium* sp. was 4.9 U/mg for the enzyme purification, more than crude enzyme[17] . Purification of enzyme is carried out to increase the specific activity of lipase, because enzyme purified is only the desired protein obtained while other proteins are removed. So that the protein obtained is the pure protein of the lipase enzyme itself.

Although enzyme activity is not determined by protein concentration, but unit of enzyme activity used to measure specific activity. The catalytic potential is an important characteristic of enzymes is not true protein function. Even enzymes that have the same purity can have different activities. This is because some enzymes that are inactive were cannot be separated from active enzymes. Enzyme purity is usually indicated by specific activity, which is the unit of enzyme activity divided by protein content. The higher value of a specific activity is the pure of enzyme. But lower specific activity values indicate the presence of dirt or partial enzyme inactivation[18].

Characterization of Lipase indifferent pH and Temperature

Enzyme activity is influenced by environmental conditions such as pH and temperature. In general, enzyme activity is increased by increasing temperature until its reach its optimum temperature as also showed with increasing pH.pH and temperature of bacterial lipase kinetics might neutralor was alkaline[19] . Higher lipase activityshowed on *A. media* LU04 and *E. tabaci*PK09 at pH 7. The optimal pH range for lipase activity in the four bacteria is at neutral pH (pH 7 - 8)(**Figure 3**).

Other study showed that bacterial lipase activity were optimum in a neutral pH[3] . Maximum lipase activity in *Bacillus methylotrophicus* PS3 at pH 7[11]; maximum lipase activity in *Bacillus* sp. THL027 was also obtained at pH 7[17]. Likewise, maximum activity of lipase in *Microbacterium* sp. is optimum at a neutral pH 8.5[32] . In another study lipase activity was found to be optimum at neutral pH with a range of pH 7.2 - 8.5 in the thermophilic *Bacillus*bacteria group[19].

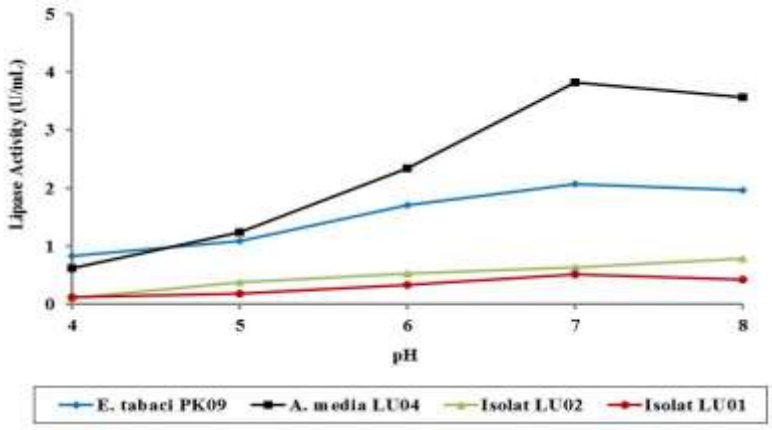

Figure 3. Effect of pH on lipase activity of *E. tabaci*PK09, *A. media* LU04, LU02 and LU01.

In this study assay on pH effect was conducted at pH of 4.0 to 8.0, while for temperature, it ranged from 30 to 50^{o}C. Higher lipase activity showed on*A. media* LU04 and *E. tabaci*PK09at 40^{o}C. The optimal temperature range for lipase activity in the four bacteria is at 40 - 45^{o}C(**Figure 4**).

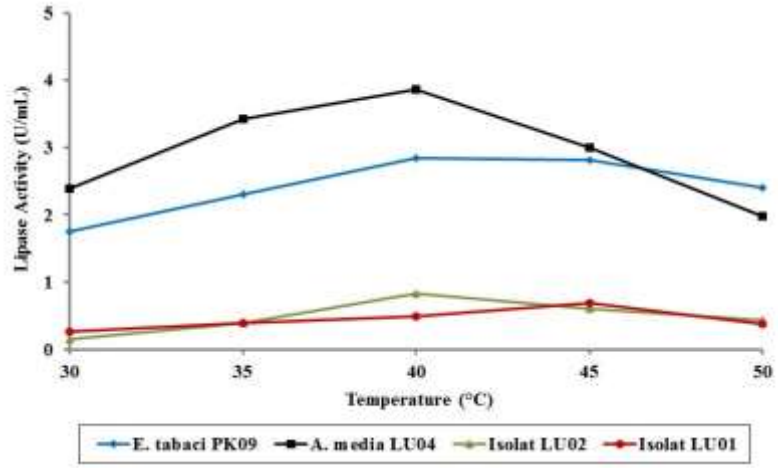

Figure 4. Effect of temperature on lipase activity of *E. tabaci*PK09, *A. media* LU04, LU02 and LU01.

Lipase bacteria have generally optimum temperatures in the range of 30 - 60°C[21] . However, other study showed that bacterial lipases were optimum in both lower and higher temperature[20] .

Biosurfactant Activity Assay

Microorganisms produce lipases and biosurfactants for the metabolism of the oil substrate[22]. The need of microorganisms for synthesizing lipases and biosurfactants occurs for metabolize water-insoluble compounds[4]. Biosurfactants are compounds produced by secondary metabolic reactions functioning for adhesion, motility, accession to carbonaceous and energy storage substrates and molecules[23].

Biosurfactant activity assay was carried out by oil displacement test and by determining emulsification activity as shown in **Figure 5**.The emulsification activity determined the productivity of biosurfactants. The emulsification index for biosurfactant is one of criteria to support the selection of potential biosurfactant producers.

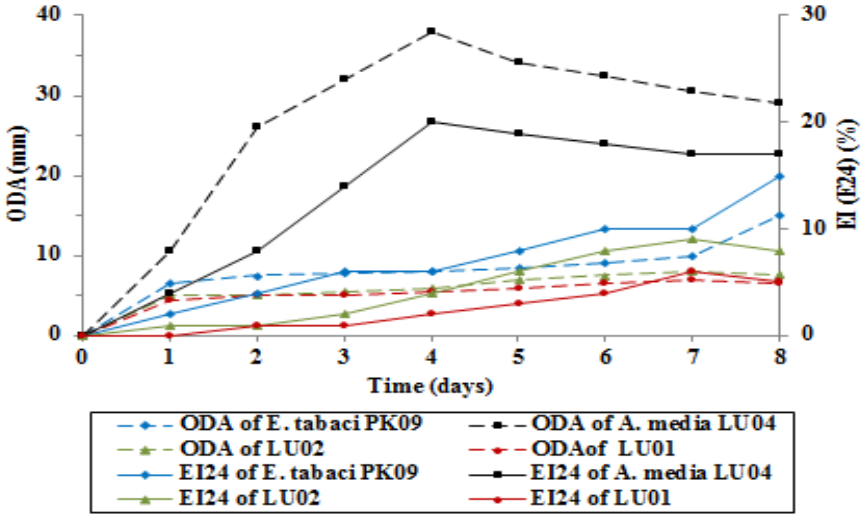

Figure 5. Oil Displacement Area (ODA) and Emulsification Index (EI24) of isolate *E. tabaci* PK09, *A. media* LU04, LU02 and LU01.

In this study, the highest activity of biosurfactant production in *A. media* LU04 and *E. tabaci*PK09 (ODA = 38.0 and 15.0 mm; E24 = 20 and 15%, respectively) was compared with other bacterial isolates (LU02 and LU01). The activity of biosurfactant production was in line with activity of lipase enzyme of the isolates. Other study showed that on the oil displacement test test, obtained a clear zone diameter of 24 mm and an emulsification index of 76% of *Bacillus subtilis* A1. The presence of activity from oil analysis and emulsification indicating the presence of biosurfactants available in the cell-free supernatant[14].

Biosurfactant production activity increases lipase activity. Lipase activity was obtained 90% with surfactant of Tween 20, Tween 80 and Triton X-100. Surfactant of Tween 20 increases lipase activity from *Pseudomonas aeruginosa* SRT 9 as studied by Borkar *et al.*[24].

Conclusions

All the keratinolytic bacteria in this study had lipase and biosurfactant activity, with two bacteria of *A. media* LU04 and *E. tabaci* PK09 showed to have more activity of lypolitic and biosurfactant compared to that of LU01 and LU02. Lipase activity were more active in optimum pH 7 and temperature of 40^{o}C for *A. media* LU04 and *E. tabaci* PK09, respectively. Increased biosurfactant activity is in line with lipase activity because biosurfactant activity is needed in lipase activity.

Acknowledgment

Special thanks are addressed to University of Sumatera Utara for partially supporting this research.

References

1. BrandelliA., Sala L.and Kalil S.J., Microbial Enzymes for Bioconversion of Poultry Waste Into Added-Value Products. *Food Research International*,2015, 73:3-12.
2. Zarinviarsagh M., Ebrahimipour G.and Sadeghi H., Lipase and Biosurfactant from *Ochrobactrum intermedium* strain MZV101 Isolated by Washing Powder for Detergent Application. *Lipids in Health and Disease*., 2017, 16:177.
3. Gupta R., Gupta N.and Rathi P., Bacterial Lipases: An Overview of Production, Purification and Biochemical Properties. *Appl. Microbiol. Biotechnol.*, 2004, 64(6):763-781.
4. DesaiJ.D.and Banat I.M., Microbial Production of Surfactant and Commercial Potential. *J. Microbiology and Molecular Review.*, 1997, 61:47-64.
5. Considine M.J., New Enzyme Technologies For Poultry By-Products. *Proc. Aust. Poult. Sci. Sym.*, 2000,12:163-165.

6. Bungsu A., Suryanto D. and Hannum S., Isolation and Identification of Keratinolytic Bacteria Isolated from Several Sources of Keratin and Characterization of Their Enzymes. Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Jln. Bioteknologi No. 1, Medan 20155, Indonesia.E-mail: dwisuryanto@usu.ac.id, 2018*).
7. Kouker G. and Jaeger K.E., Specific and Sensitive Plate Assay for Bacterial Lipases. *Appl. Environ. Microbiol*., 1987, 53(1):211-213.
8. Mobarak-Qamsari E., Kasra-Kermanshahi R. and Moosavi-nejad Z., Isolation and Identification of a Novel, Lipase-Producing Bacterium, *Pseudomnas aeruginosa* KM110. *Iran. J. Microbiol*., 2011, 3(2) : 92-98.
9. KwonD.Y. and RheeJ.S., A Simple and Rapid Colorimetric Method for Determination of Free Fatty Acids for Lipase Assay. *JAOCS.*, 1986, 63(1): 89-92.
10. Lowry R.R. and Tinsley I.J., Rapid Colorimetric Determination of Free Fatty Acids. *Journal of The American Oil Chemists's Society*., 1976, 53:470-472.
11. Sharma P., SharmaN., Pathania S. and Handa S., Purification and Characterization of Lipase by *Bacillus methylotrophicus* PS3 Under Submerged Fermentation and Its Application in Detergent Industry. *Journal of Genetic Engineering and Biotechnology*, 2017. http://dx.doi.org/10.1016/j.jgeb.2017.06.007.
12. Bradford M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, 1976, 72:248-254.
13. Thavasi R., Sharma S., and Jayalakshmi S., Evaluation of Screening Methods for The Isolation of Biosurfactant Producing Marine Bacteria. *J. Pet. Environ. Biotechnol*, 2011, S1:001. Doi: 10.4172/2157-7463.S1-001.
14. Parthipan P., Preetham E., Machuca L.L., Rahman P.K.S.M., Murugan K. and Rajasekar A., Biosurfactant and Degradative Enzymes Mediated Crude Oil Degradation by Bacterium *Bacillus subtilis* A1. *Front. Microbiol*.,2017, 8:193. Doi: 10.3389/fmicb.2017.00193.
15. Lima V.M.G., Krieger N., Sarquis M.I.M., Mitchell D.A., Ramos L.P. and Fontana J.D., Effect of Nitrogen and Carbon Sources on Lipase Production by *Penicillium aurantiogriseum*. *Food Technol Biotechnol*, 2003, 41:105-110.
16. Uma-maheswari N. and Balasundari A., Optimization and Biodegradation of Feather Waste. *Int J Recent Sci Res.*, 2016,7(9):13302-13306.
17. Tripathi R., Singh J., Bharti R.K. and Thakur I.S., Isolation, Purification and Characterization of Lipase from *Microbacterium* sp. and Its Application in Biodiesel Production. *Energy Procedia*., 2014, 54:518-529.
18. Bisswanger H., Review: Enzyme assays. *Perspectives in Science*, 2014, 1: 41-55.
19. Schmidt-Dannert C., SztajerH., Stocklein W., Menge U. and Schmid R.D., Screening purification and properties of a thermophilic lipase from *Bacillus thermocatenulatus*. *Biochim Biophys Acta*., 1994, 1214:43–53.
20. Dharmsthiti S.and Luchai S., Production, purification and characterization of thermophilic lipase from *Bacillus* sp. THL027. *FEMS Microbiol Lett*., 1999, 179:241-246.
21. Litthauer D., Ginster A. and SkeinE.V.E.,*Pseudomonas luteola* lipase: a new member of the 320-residue *Pseudomonas* lipase family. *Enzyme Microb Technol*., 2002, 30:209-215.
22. Colla L.M., Rizzardi J., Pinto M.H., Reinehr C.O., Bertolin T.E. and Costa J.A.V., Simultaneous Production of Lipases and Biosurfactants by Submerged and Solid-state Bioprocesses. *Bioresource Technology* 2010, 101:8308-8314.
23. Van Hamme J.D., Singh A. and Ward O.P., Physiological aspects – part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnol. Adv.*, 2006, 24:604-620.
24. Borkar P.S., Bodade R.G., Rao S.R. and Khobragade C.N., Purification and Characterization of Extracellular Lipase from A New Strain-*Pseudomonas aeruginosa* SRT 9, Braz. *J. Microbiol*. 2009, 40:358-366.

*****