GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
*(**Published By**: Global Institute for Research & Education)*
www.gifre.org

Bacillus subtilis A9 PRODUCING LIPASE ISOLATED FROM SHRIMP PASTE

Yati Sudaryati Soeka & Maman Rahmansyah
Research Center for Biology, Indonesian Instituite of Sciences, Jl. Raya Jakarta-Bogor km 46, Cibinong Science Center, Cibinong 16911, West Java, Indonesia

Abstract

Lipase-producing-bacterium had been isolated from "Terasi (Shrimp Paste)", a traditional fermented food made from penaeid prawn. The isolates cultures produce holozone on Bypta Agar Culture because of its enzymatic activity. By using 16S rRNA amplification within the genomic DNA, the isolate identified as *Bacillus subtilis* A9. The isolate become collection belong to Indonesia Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences. Furthermore, crude enzyme extracted from isolates then tested to catalyze algal oil, and it turn into various fatty acids by using precipitated (PEM) and immobilized (IEM) crude enzyme. PEM and IEM reach optimum activity at 96 hours to change olive oil as standard substance. Ten fatty acids were achieved as due to crude enzymes esterification. The highest percentage of lauroleic acid was obtained, and followed by palmitic and stearic acid. The IEM was able to produce behenic acid in 13.6 percent quantity.

Keywords: *Bacillus subtilis* A9, fatty acid, lipase, immobilized, precipitated.

1. Introduction

Floral producing oil and microbial producing fatty acid were become valuable biomass and bioresources for various industrial purposes. Microbial extracellular enzymes had considerable commercial interest for biotechnological applications as they can be produced at low cost (Schmidt-Dannert *et al.*1996), while in the other hand, algae producing oil had chance and prospective to environmental friendly production to be practiced (Scott *et al.*, 2010).

Lipases occured widely in nature, and microbial lipases are more favorable because low cost in production, greater stability and availability than plant and animal lipases. The lipases originally achieved from fungi, molds or bacteria; and most of lipases formed extracellular (Aravindan *et al.*, 2007). Follow to some researcher work on *Bacillus* spp., the most useful lipase produce among gram-positive bacteria (Jaeger *et al.*, 1994), and it is renowned as the main sources of lipolytic enzyme (Schmidt *et al.*, 1994; Luisa *et al.*, 1997). Lipolytic enzymes are grouped into three main categories such as esterase, phospholipase and lipase (Arpigny and Jaeger, 1999). Lipase-producing bacteria had been found in diverse habitats including in soil contaminated with oil, dairies, industrial wastes, oilseeds and decaying food (Sztajer *et al.*, 1988); as well as in compost heaps, coal tips and even found in the hot springs (Wang *et al.*, 1995). A large number of *Bacillus* genera living aerobic or facultative anaerobic bacteria, and there were usually having rod-shaped and endospore-forming bacteria. Most of them are nonpathogenic, had high secretion capacity, competent in producing various biological substances for instance secreting proteins, enzymes, biofilm, biosurfactant, and antibiotics (Goto *et al.*, 2000; Pakpitcharoena *et al.*, 2008).

Lipase was an extractable microbial enzyme and plays an important role as a catalyst in floral oil conversion which is usually found in seeds and algae cell. The oil turns into free fatty acid, glycerol, ester, and glycerin. The enzyme has ability to convert oil into high purity methyl ester, and the glycerol would be easily separated from substrate (Pandey *et al.*, 1999; Sharma *et al.*, 2000). Sangeetha *et al.*, (2010) found out that enzyme production in a medium was optimum by using yeast extract as nitrogen source, tributyrin as an inducer, and Tween 80 as emulsifier. Lipase itself was found to be resistant to proteolysis, stable between pH 8.0-12.0, and reach optimum temperature at 55^{O}C. Lipase can thus be produced as a single mixture which will serve to fulfill of few requirements which occupy the enzyme as bio-catalyst. While Ji-Won Jo *et al.*, 2009 investigated the isolates of *Bacillus subtilis* to have exceedingly produced extracellular lipase through its cultivation.

In this study, the isolate which was obtained from penaeid prawn paste, a popular Indonesian traditional fermented food collected from Samarinda's traditional market, southern Borneo-Indonesia, had been investigated in both of physiological and the molecular identity. The isolate identified as *Bacillus subtilis* A9. Crude enzyme extracted from that bacterial culture then use to turn algal oil change into methyl ester. The aim of the study was to verify the crude bacterial enzymatic activity to have function in rendering algal oil into some potential fatty acids resources.

2. Materials and Methods

2.1. Bacterial determination

Two hundred fifty milligram of "terasi" sample dissolve in 100 ml distilled water inside 250-ml Erlenmeyer flask, and well mixed with magnetic stirrer (novice-1). Make a serial dilution (1 ml sample of novice-1 into 9 ml sterilized aquadest) in the glass tube containing aquadest to reach final deliberation up to 10^{-5} dilution (novice-2). A hundred micro liters of novice-2 material poured onto surface of the selective Bypta Agar Media (5 g beefs extract, 10 g peptone, 3 g yeast extract, 5 g NaCl, 1 g polyvinyl alcohol, 10 ml tributyrin, dissolved in 1000 ml aquadest, add with 25 g agar and autoclaved in 121^{o}C at 1 atmosphere pressure for one hour) inside the petridish, trimmed with spatula, and incubated in room condition waiting for the colonies culture to sprout on top of the media. Subsequently, choose a single colony

which has specific colony producing wide holozone growth in the media that mean indicating high specificity of lipase enzymatic activity. Single selected colony was cultured in the slant nutrient agar (NA) to be a "single isolation growth" (SIG-1), and keep as working collection.

The isolate was sequenced for genomic recognition by using Neighbor Joining method (Saitou and Nei. 1987) and followed by MEGA 5.2.2. tecnique program (Tamura *et al.*, 2011); subsequently, phylogenetic tree was calculated with bootstrap method by using 1000 replications (Efron, 1979). Each genome of DNA was amplified by PCR work with the primer sets of 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACG ACTT-3') 16S rRNA gene. All of the PCR products were confirmed by a second PCR that were designed in the DNA sequences of the first PCR product. In addition, other closely related species belonging to the species group was recognized by Gene Bank (NCBI; http://www.ncbi.nlm.nlh.gov/) blast search through the high similarity indexes to the isolate genotype. Finally, the isolate *Bacillus subtilis* A9 strongly recognized as *Bacillus subtilis* JCM 1465^T (AJ276351) (see Figure-1). The isolate was kept in Indonesia Microbial Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences, Cibinong Science Center. Collection number is InaCC B 399.

2.2. Crude lipase extraction

Microbial enzyme activity was measured follow to Paskevicius (2001) method. A tiny spot of SIG-1 isolate producing lipase was inoculated in liquid media culture (0.5% beefs extract, 0.5% peptone, 0.3% yeast extract, 0.25% NaCl, 0.1% polyvinyl alcohol, and 1.0% glycerin tributyrin), and shake for five days inside rotary shaker-bath incubator (120 rpm; 37^{O}C). The culture was prepared as the seed stock (SIG-2). Other liquid basal medium for lipase production was made as above preparation by replacing glycerin tributyrin with olive oil. Furthermore, *Bacillus subtilis* A9 culture from SIG-2 preparation (5 ml) were dropped into 500 ml liquid basal medium, and incubated for few days inside rotary shaker-bath incubator (120 rpm; 37^{O}C). Daily sampling of culture filtrate was taken from samples to determine daily enzymes' activities followed to Kwon and Rhee (1986) method by determined fatty acid content in the culture. The culture was precipitated by centrifuge (2270 *g*) for five minutes, and keeps the supernatant as SIG-3 preparation.

For further lipase extraction, 100 ml SIG-3 was placed in one liter flask and followed by enzyme precipitation work with slowly and gradually pouring 400 ml acetone into the flask mixed with magnetic stirrer and keeps the flask in cold condition by putting some ice cube surrounded along repealing process. Precipitated enzyme (PEM) was separated from the solution with centrifugation (3500 rpm) for five minutes, and dissolved with 15 ml phosphate buffer (50 mM; pH 5). Afterward, the PEM then sends to freeze-drying and freeze for two hours to become pellets, then keeps the PEM in the refrigerator for the next verification. The same preparation was made as above supply but different centrifugation processes (10 minutes in 10.000 rpm agitation). The freeze pellets then was added with 100 ml NaCl (0.85%), 5 g casein, and finally with 5 g Na-alginate to have "immobilized enzyme" (IEM); and keeps the IEM in the refrigerator for the next verification.

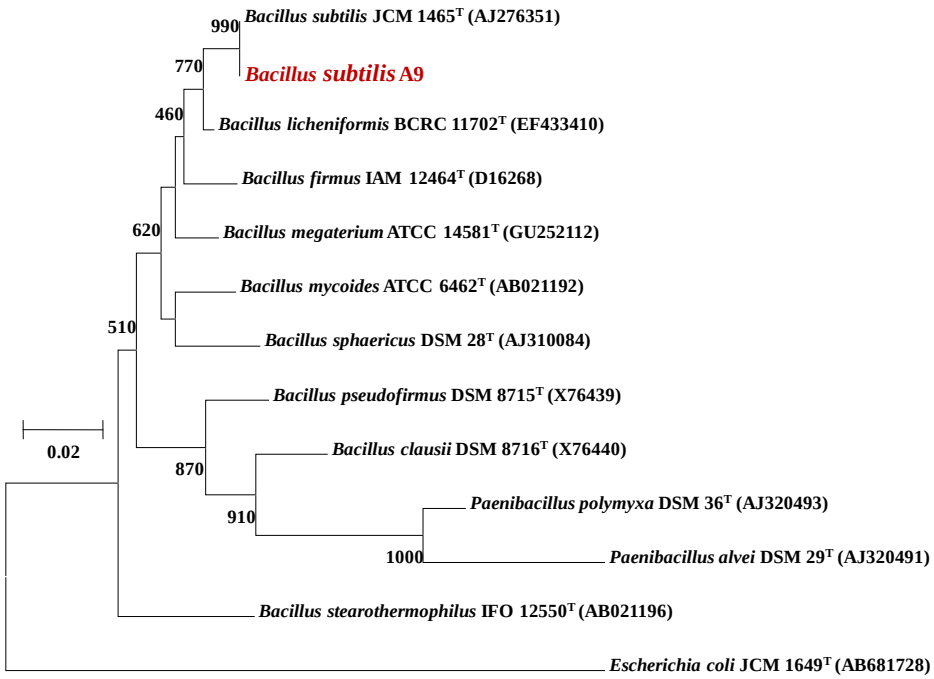

Fig. 1. Phylogenetic tree analysed by Neighbor Joining Method

PEM and IEM were measured through olive oil conversion capacity. Those of crude enzymes which are obtained from four and five days *Bacillus subtilis* A9 cultivation were used. Enzymatic activities were measured as follow: one ml of olive oil was placed in 100-ml Erlenmeyer flask, added with 0.5 ml $CaCl_2$ (0.1 M) and 4.5 ml Citrate buffer (0.05 M; pH 6), and keep in water bath (40^{O}C) for 10 minutes. Subsequently, take out the flasks and adding with PEM or IEM from stock, then mixed up inside Erlenmeyer separately in the quantity of 10 percent of the volume based solution. Put the Erlenmeyer back into water bath (40^{O}C), shakes in 120 rpm for 30 minutes, and finally stopped the enzymes activity with 10 ml of ethanol/acetone (1:1) solution. Three drops of phenoptaline indicator were plunged in the Erlenmeyer and

the solution turn into red. The solution titrated with NaOH (0.05N) solution, and it was stopped when the color disappear. A number of ml NaOH used for titration was equal to micromole of free fatty acid liberated by lipase activities.

2.3. Algal oil preparation

Single cell oil (SCO) extraction from algae was followed to Guckert and White (1988) method. *Spirulina fusiformis* which were cultured in Zarrouk media (one liter water contain 18 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1 g K_2SO_4, 1 g NaCl, 0.04 g $CaCl_2$, 0.08 g Na_2EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$), then collected and centrifuged to make pasta of biomass (PoB). Add the hexane/isopropanol (3:2 of mixed volume) solution with twice of sufficient required volume of PoB, homogenized for fifteen minutes in room condition for 12 hours. Supernatant were separated by centrifugation method at 3500 rpm for 20 minutes; move the supernatant to the other flask and dispense with 10 ml sodium sulphate (0.47 M) to have emulsion. The SCO would come out as the top of aqueous phase inside flask and it should be separated carefully. Afterward, SCO was evaporated in hot water bath (50^OC) to dry, and the residue utilized for enzyme assay.

2.4. Enzyme assay and fatty acid quantification

Enzymatic transesterification measured through the crude enzymatic activity to turn the algal oil into fatty acids. The study was assayed in 100-ml closed glass tube. In reaction mixture, filled the tube with 0.5 g olive oil and 15 ml NaOH (0.5 M solution in methanol); the reaction keep in 80^OC during 5 minutes incubation, and then cooled in the room condition. Afterward, the tubes completed with 0.5 g of PEM or IEM, and as well as 10 ml BF_3 (14 % solution in methanol) to replace PEM or IEM for chemical esterification (control). The tubes put back to water bath (85^OC) for 30 minutes incubation, and then cooled in room condition. Cooled solution then completed with 5 ml hexane, added carefully with 15 ml NaCl (saturated solution), and all mixed well with vortex. The top aqueous solvent in the tube was hexane, separated and keep into another flask, and precipitated the solution with 5 g of anhydrous Na_2SO_4.

Certain quantity of fatty acids dialysates sample as supernatant was taken from the flask, and as well as in the certain quantity (10 ul) dialysates were injected into gas chromatogram column for carbon molecule identification. Column temperature was kept in gradual set as follow: 150^OC for 30 second, accelerated with 15^OC per minute to reach 250^OC, and keep in the last temperature for 6 minutes. Temperature of injector and detector were adjusted at 245^OC and 350^OC, respectively. Fatty acids quantification was determined by measuring of free fatty acid release based on the standard curve of pure fatty acid methyl ester.

3. Result and Discussion

Lipase-producing bacteria can be purified by inoculating as a single colony onto agar plates in the media containing minimal essential nutrients for growth and enhance lipase production. To identify the selected lipase producer strain, analysis was applied to find the sequence of 16S ribosomal RNA and the partial sequence (Figure-2). Direct sequence determination of 16S rRNA gene fragments represents a highly accurate and versatile method for identification of bacteria to the species level (Akanbi *et al*., 2010). The bacteria from the formerly isolated in this work identified as *Bacillus subtilis* A9.

Since most of the microbial lipases are extracellular, the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents (Aravindan *et al.*, 2007). In the work here, separated crude enzyme extract method had brought about quantity of PEM and IEM enzymes preparation. In the mean time, evaluation through converting capacity in the standard media containing olive oil, PEM was less powerful because of increasing the specificity of IEM in this option. Both of PEM and IEM, and as well as crude enzyme from culture filtrate all had optimum enzymatic activities in four days incubation (Figure-3). The microbial inoculum level in the media is a crucial role in lipase production, and the enzyme production also associated with the growth of bacterial culture (Kumar and Kanwar, 2012). Immobilized enzyme has an advance of its stability and become reusable in catalyst function. Setiyahadi *et al*., (2011) studied for immobilizing lipase which was supported by chitin. Transesterification process increased at the first four hours incubation to reach 97 % in triglyceride conversion along three days continuous application. Sabat *et al.*, (2012) found that lipase activity belongs to *Bacillus stearothermophilus* had highest activity to turn olive oil into fatty acid compared to castrol and coconut oil conversion.

Oil of microalgae is different from most vegetable oils, and being quite rich in polyunsaturated fatty acids with four or more double bonds (Belarbi *et al*., 2000). Algal oil derived from *Spirulina fusiformis* in this study brought about ten types of carbon bonds in variation of ester as due to chemical (BF_3), and seven to eight kind methyl ester because of lipase enzymatic transesterification. That process gives different acquired the quality and quantity of fatty acid. The highest attainment was lauroleic acid and followed by palmitic and stearic acid, respectively (Table-1).

Immobilized enzymes converted algal oil into the highest quantity of lauroleic acid to reach 39.72 % content in the study. Shah (2011) had successfully study to purified lipases of *Bacillus subtilis* and stimulating with magnesium ion augmentation in the esterification process. In the other hand, algae of *Spirulina* were being easily to cultivate along puddle and pond with organic manure augmentation (Valderrama *et al*., 1987; Mitchell and Richmond, 1988). In the other opinions, Richmond and Vonshak (1986) and also Mitchell and Richmond 1987 were being consider throughout their research experiment and experience, that they had find the simple way to produce *Spirulina* mass culture within define appropriate management.

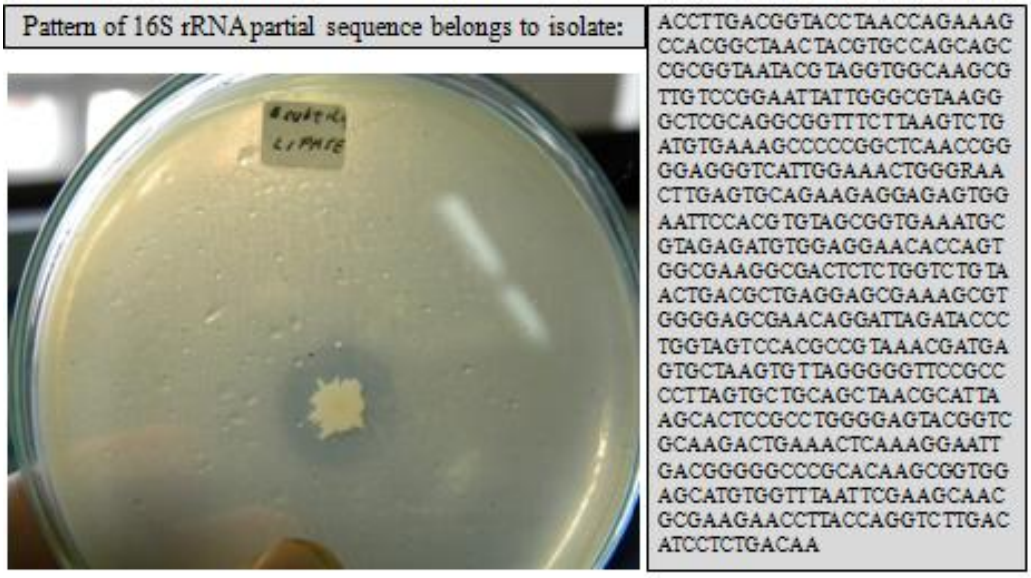

Fig. 2. Purified isolate with clear zone (left) surround culture indicating lipase activity which has produced by isolates, and reference pattern sequence (right) of isolates which is reffers to *Bacillus subtilis* A9 species

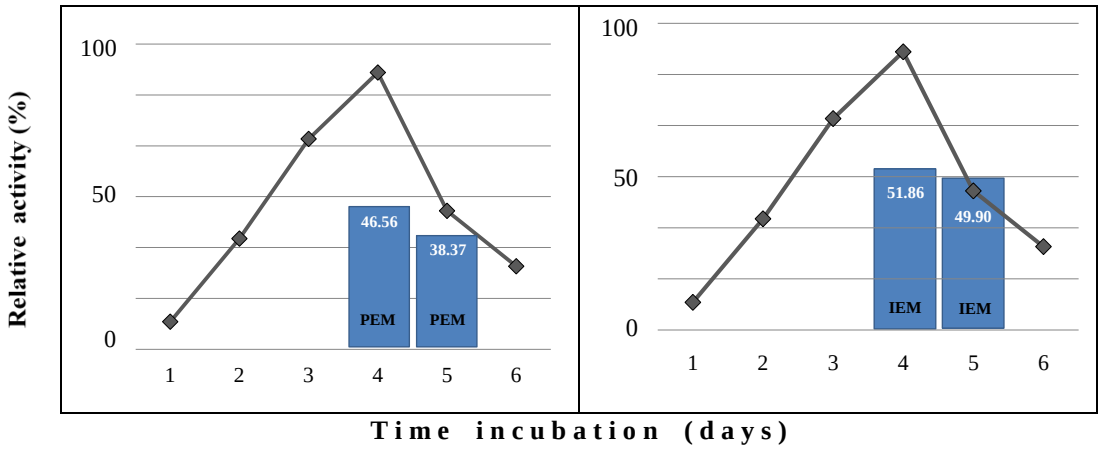

Fig. 3. Optimum activity of crude enzyme (line-chart) extracted from *Bacillus subtilis* A9 filtrate culture and being compared to precipitate (left bar-chart) and immobilized (right bar-chart) crude enzymes activities. A hundred percent activity represent to 0.30 µmol.ml^{-1} fatty acid refeal to enzymes conversion.

Table 1. Algal oil transesterification turn into methyl ester as due to chemical (boron-trifluoride) and compared with lipase enzymes of precipitated (PEM) and immobilized (IEM) extraction from *Bacillus subtilis* culture

Methyl Ester (ME)			Transesterification of algal oil which is converted by:					
Carbon bonds	IUPAC	Trivial names	Boron-trifluoride		PEM		IEM	
			RT*	% ME	RT	% ME	RT	% ME
C 8	Octanoic acid	Caprylic acid	1.05	6.33	1.02	0.68	0.92	7.64
C 10	Decanoic acid	Capric acid	1.61	5.95	1.54	0.63	1.36	7.14
C 12-0	Dodecanoic acid	Lauric acid	2.12	0.02	2.02	0.003	1.78	0.02
C 12-1	9-dodecenoic acid	Lauroleic acid	2.98	34.92	2.82	3.97	2.57	39.72
C 14	Myristic acid	Tetradecanoic acid	3.96	0.03	3.76	-	3.42	0.03
C 16-0	Hexadecanoic acid	Palmitic acid	5.40	21.46	5.19	2.09	4.86	20,51
C 16-1	9-hexadece-noic acid	Palmitoleic acid	6.93	0.04	-	-	-	-
C 18	Octadecanoic acid	Stearic acid	8.90	14.11	8.70	1.17	8.35	11.30
C 22-0	Docosanoic acid	Behenic acid	11.18	0.02	11.07	-	13.47	13.64
C 22-1	*cis*-13-docosenoic acid	Erucic acid	14.16	17.12	13.94	1.44	-	-

*retention time (minutes)

4. Conclution

In this research, crude lipase activity belongs to *Bacillus subtilis* A9 isolated from shrimp paste had successfully characterized, and keeps in culture collection as InaCC B 399. The crude enzyme resulted from the isolates could be useful as sources of biological active compounds, and considerable to further diverse applications. The bacterium status was being affirmed by the enzymatic system due to various metabolic activities and valuable compounds production within algal oil conversion that make an offer for generating biological resources availability to achieve certain fatty acid production throughout improvement of lipase enzymatic processes.

Acknowledgements

The molecular work had been carried out in Indonesia Culture Collection (InaCC) laboratory which we are being grateful for. Great appreciation also is known to the colleagues in Microbial Division for valuable suggestion and criticism.

References

Akanbi T., Kamaruzaman A.L., Abu Bakar F., Sheikh Abdul Hamid N., Radu S., Abdul Manap M.Y. and Saari N. (2010). Highly thermostable extracellular lipase-producing Bacillus strain isolated from a Malaysian hot spring and identified using 16S rRNA gene sequencing. *International Food Research Journal*, 17:45-53.

Aravindan R., Anbumathi P. and Viruthagiri T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology*, 6:141-158.

Arpigny J.L. and Jaeger K.E. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochem. J.*, 343:177-183.

Belarbi E-H., Grima E.M. and Chisti Y.A. (2000). A process for high yield and scalable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. *Enzyme Microb. Technol*., 26:516–29.

Efron B. (1979). Bootstrap methods: another look at the jackknife. *The annals of Statistics*, 7: 1-26.

Goto K., Omura T., Hara Y. and Sadaie Y. (2000). Application of the partial 16S rDNA sequences as an index for rapid identification of species in the genus Bacillus. *J. GenAppl Microbiol*., 46:1–8.

Guckert J.B. and White D.C. (1988). Evaluation of a hexane/isopropanol lipid solvent system for analysis of bacterial phospholipids and application to chloroform-soluble nuclepore (polycarbonate) membranes with retained bacteria. *Journal of Microbiological Methods*, 8:131–137.

Jaeger K.E., Ransac S., Dijkstra B.W., Colson C., van Heuvel M. and Misset O. (1994). Bacterial Lipases. *FEMS Microbiology Review*, 15:29-63.

Ji-Won Jo, Sung-Ho Hur, Yong-Soo Han, and Ji-Yeon Kim. (2009). Isolation of Lipase producing Bacillus subtilis and some characteristics of the Enzyme. *J. Appl. Biol. Chem*., 52(3):151-156.

Kumar A. and S.S. Kanwar. (2012). Lipase production in solid-state fermentation (SSF): Recent developments and biotechnological application. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*, 6(1):13-27.

Kwon D.Y. and Rhee J.S. (1986). A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *Journal of the American Oil Chemists' Society*, 63(1):89-92.

Luisa M.R., Schmidt C., Wahl S., Sprauer A. and Schmid R. (1997). Thermo-alkalophilic lipase of Bacillus thermocatenulatus. Large scale production, purification and properties: aggregation behavior and its effect on activity. *Journal of Biotechnology*, 56: 89-102.

Mitchell S.A. and Richmond A. (1987). The use of rotifers for the maintenance of monoalgal mass cultures of Spirulina. *Biotech. and Bioeng*., 30:164-168.

Mitchell S.A. and Richmond A. (1988). Optimization of a growth medium for Spirulina based on cattle waste. *Biological Wastes*, 25:41-50.

Pakpitcharoena A., Potivejkulb K., Kanjanavasa P., Areekita S. and Chansiria K. (2008). Biodiversity of thermotolerant Bacillus sp. producing biosurfactants, biocatalysts, and antimicrobial agents. *Science Asia*, 34:424–431.

Pandey A., Benjamin S., Soccol C.R., Nigam P., Krieger N. and Soccol V.T. (1999). The realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem.*, 29:119-131.

Paskevicius A. (2001). Lipase Activity of Yeast and Yeasts-like fungi Functioning Under Natural Conditions. Institute of Botany. Available: http://images.katalogas.It/mealeidylla/bio4/ B16.pdf.

Richmond A. and Vonshak A. (1986). Management of Spirulina Mass Culture, p. 222-223. In W.R. Barclay and R.P. McIntosh (ed.), *Algal Biomass Technologies*. J. Cramer, Berlin Stuttgart.

Sabat S., Murthy V.K., Pavithra M., Mayur P. and Chandavar A. (2012). Production and characterization of extracellular lipase from Bacillus stearothermophilus MTCC 37 under different fermentation conditions. *International Journal of Engineering Research and Applications.*, 2(3):1775-1781.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4: 406-425.

Sangeetha R., Geetha A. and Arulpandi I. (2010). Concomitant production of protease and lipase by Bacillus licheniformis VSG1: Production, purification and characterization. Brazilian Journal of Microbiology. 41:179-185.

Schmidt C., Sztajer H., Stocklein W., Menge U. and Schimd J. (1994). Screening, purification and properties of a thermophilic lipase from Bacillus thermocatenulatus. *Biochimica et Biophysica Acta.*, 12: 43-53.

Schmidt-Dannert C., R´ua M.L., Atomi H. and Schmid R.D. (1996). Thermo-alcalophilic lipase of Bacillus thermocatenulatus I. Molecular cloning, nucleotide sequence, purification and some properties. *Biochimica et Biophysica Acta.*, 1301(1-2):105–114.

Scott S.A., Davey M.P., Dennis J.S., Horst I., Howe C.J., Lea-Smith D.J. and Smith A.G. (2010). Biodiesel from algae: challenges and prospects. *Current Opinion in Biotechnology*, 21(3):277-86.

Setyahadi S., Machsum A.L. and Mokodongan R.S. (2011). *Kitin sebagai Penopang untuk Amobilisasi Lipase pada Proses Trans-esterifikasi Trigliserida.* Prosiding Seminar Nasional Teknik Kimia "Kejuangan". Pengembangan Teknologi Kimia untuk Pengolahan Sumber Daya Alam Indonesia. Yogyakarta, 22 Februari 2011. Indonesia.

Shah K.R. (2011). Purification and characterization of lipase from Bacillus subtilis Pa2. *Journal of Biochemical Technology*, 3(3):292-295.

Sharma R., Chisti Y. and Banerjee U.C. (2000). Production, purification, characterization, and applications of lipases. *Biotechnol Adv*., 19: 627–62.

Sztajer H., Maliszewska I. and Wieczorek J. (1988). Production of exogenous lipase by bacteria, fungi and actinomycetes. *Enzyme and Microbial Technology*., 10: 492-497.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods**.** *Molecular Biology and Evolution*, 28: 2731-2739.

Valderrama G., Cardenas A. and Markovits A. (1987). On the economics of Spirulina production in Chile with details on dragboard mixing in shallow ponds. *Hydrobiologi,*. 151/152:71-74.

Wang Y., Srivastava K.C., Shen G-J. and Wang H.Y. (1995). Thermostable alkaline lipase from a newly isolated thermophilic Bacillus, strain A30-1 (ATCC 53841). *Journal of Fermentation and Bioengineering*. 5:433-438.