International Scientific Journal
Medical and Biological Sciences
http://bioscience.scientific-journal.com

BIODIVERSITY OF LIPASE PRODUCING BACTERIA FROM PEAT SWAMP FOREST IN CHANTHABURI PROVINCE, THAILAND

Pornpimon Kanjanavas[a]*,
[a] Department of Industrial Microbiology, Faculty of Science and Technology, Huachiew Chalermprakiet University, Samutprakan 10540, Thailand
*Author to whom correspondence should be addressed; E-mail: kanjanavas@hotmail.com

Montri Yasawong[b]
[b] Department of Biochemistry, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand

Kanjana Rutnatum[c]
[c] Department of Biology, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000, Thailand

Supatra Areekit[d]
[d] Innovative Learning Center, Srinakharinwirot University, Bangkok 10110, Thailand

Kosum Chansiri [e]
[e] Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand

Abstract **: Lipase has been introduced for several industrial applications, especially the bleaching process that performed at higher temperature and under acidic conditions. Lipases isolated from different sources have a wide range of properties depending on their sources. Bacteria isolates were collected from peat swamp in Chanthaburi province's forest such as Rambhai Barni forest, Klong Sukree and Khao Ta Khrao and were screened using tributyrin agar at 37 °C. The 109 isolates of lipase-producing bacteria were isolated and 4 of them (KS7.4, KS7.7, TK8.8 and RP5.2) could produce high activity. The comparison of 16S rDNA sequences and phylogenetic tree analysis revealed that the isolate KS7.4, KS7.7, and TK8.8 were similar to *Bacillus* sp., whereas RP5.2 was similar to *Staphylococcus* sp. The maximum activities of crude enzyme using *p*-NP butyrate (C4) as substrate were observed from KS7.4, KS7.7, TK8.8, and RP5.2 whereas RP5.2 showed high activity *p*-NP palmitate (C16). The optimum pH that provided maximum lipase activity was at pH7.0 (50 mM Phosphate buffer) for KS7.4, KS7.7 and TK8.8 and pH9.0 (50 mM Tris-HCl) for RP5.2. The optimum temperature was 60°C for KS7.7, TK8.8 and RP5.2 and 45°C for KS7.4. The lipase activity was activated by addition of 10 mM Na^+, K^+ and Ca^{2+} for all crude enzymes from all strains. Ca^{2+} could activate enzyme activity from KS7.7 higher than Na^+ and K^+. In contrast, EDTA strongly inhibited enzyme activity in both KS7.4 and RP5.2 whereas Mn^{2+}, Ni^{2+} and Co^{2+} slightly inhibited the activity in all strains. The lipase enzymes from KS7.4, KS7.7 and TK 8.8 showed high specificity to palm oil and olive oil. Furthermore, lipase enzyme from KS7.7 and TK8.8 could use soy bean oil as substrate.**

Keywords: Lipase, Peat swamp forest, Microbial enzyme, Bacteria

I. INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) catalyze the hydrolysis of water-insoluble esters and triglycerides at the interface between the insoluble substrate and water. Many new bacterial lipolytic enzymes have been studied since the publication of a comprehensive review article in 1994 [1]. Apart from their natural substrates, lipases catalyze the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and non-natural esters. Due to their extracellular nature, most microbial lipases can be produced in large quantities and are quite stable under non-natural conditions such as high temperatures and nonaqueous organic solvents employed in many applications [2-4]. Nowadays, industrial enzymes, especially lipases, are commonly used in the production of a variety of products, ranging from fruit juices, baked foods and vegetable fermentation to dairy enrichment [5, 6]. Microbial lipases have been used for the production of desirable flavors in cheese and other foods, and for the interesterification of fats and oils to produce modified acylglycerols, which cannot be obtained by conventional chemical interesterification. [7] Furthermore, to date, many industries demand hydrolytic enzymes that work optimally at acidic pH. An obvious source of such enzymes is microorganisms found in acidic environments. Tropical peat-swamp forests constitute a unique ecosystem found in South East Asia where the soil is extremely acidic (pH 3.5–5.0), and thus might yield novel enzymes that are active under acidic conditions. In this study, lipase-producing bacteria were isolated from peat swamp soil. The sampling site was located in Chanthaburi province, Thailand.

II. MATERIALS AND METHODS

1. Bacterial strains collection & screening of the lipase producing bacteria

Soil and water samples from peat swamp at Chanthaburi province's forest such as Rambhai Barni forest, Klong Sukree and Khao Ta Khrao were previously collected and screened for lipase producing bacteria using dilution techniques, the isolated colonies were tested for lipase activity on tributyrin agar plate. A colony forming clear zone was selected after 72 h incubation at 37 ^{o}C.

2. Identification of the lipase producing bacteria isolate

The single colony that produced wide clear zone from tributyrin agar was characterized and strained with Gram's straining. The single colony was cultured and DNA extracted for the PCR amplification and sequencing of HV region, A forward primer was 5' AGA GTT TGA TCC TGG CTC AG -3'. A reverse primer consisted of 5' GGC TAC CTT GTT ACG ACT T -3'. The PCR product of 1,500 bp in sizes was expected. The PCR mixture was consisted of 1X PCR buffer (GIBCO BRL®), 1.5 mM $MgCl_2$, 10mM dNTPs, 20μM of each primer and 3 unit of *Taq* DNA polymerase (GIBCO BRL®), sterile distilled water was used to make total volume to 25 μl and the reaction was performed using the Peltier Thermal cycle (MJ Research, PTC-200). The PCR condition contained one cycle of 95^{o}C for 3 min followed by the additional 30 cycles of denaturation at 95^{o}C for 1 min, annealing at 55^{o}C for 30 second and extension at 72^{o}C for 1 min. The PCR fragment was excised from the 1.2 % agarose gel at 100 volt for approximately 30 min and purified by using QIAGEN PCR purification kit (QIAGEN). The sequences of PCR product was determined using Big Dye Terminator Cycle sequencing procedure and analyzed by ABI PRISM 3100 (Perkin Elmer).

The nucleotide sequencing data was detected by the input of the data into BLAST program from http://www.ncbi.nlm.nih.gov/BLAST. The nucleotide sequences of 16S rDNA gene were compared with nucleotide sequences of various organisms that have been previously submitted in GenBank. The phylogenetic tree was generated by MEGE 6 for study the phylogenetic relationships with the neighbor-joining approach and 10,000 bootstrap replicates. [8-11]

3. Lipase activity assay

The activity of the extracellular lipase was assayed in the cell-free liquid after separation of biomass by centrifugation at 5,000 rpm for 20 min. Lipolytic activity was determined by measuring the optical density (OD) at 410 nm using *p*-nitrophenyl ester (*p*NP- ester) (SIGMA) as substrate [12]. The substrate solution consisted of solution A (0.4 g *p*NPP dissolved in 12 ml isopropanol) and solution B (0.4 g tritonX-100; 0.1 g gum arabic; 90 ml potassium phosphate buffer pH 7). Subsequently solution A was added to solution B to a final composition 1:30 (v/v). The cell free supernatant (1ml) was added to 9 ml of substrate solution and then, the mixture was mixed and incubated at 37^{o}C. After 20 min, the reaction was stopped by boiling and enzyme activity was measured by monitoring the change in absorbance at 410 nm that represented the amount of released *p*-nitrophenol (*p*NP). One unit of lipase activity is defined as the amounts of enzyme releasing 1 μmol *p*NP per minute under the assay conditions.

3.1 Substrate specificity

The *p*NP fatty acyl esters of butyric acid (C4), caprylic acid (C8), caprric acid (C10), lauric acid (C12), myristic acid (C14), palmitic acid (C16) and stearic acid (C18) (SIGMA) were used as substrates and assayed spectrophotometrically as previously described. [13, 14] The substrates were dissolved in isopropanol at a concentration of 10 mM. The results were expressed as a percentage of the reactivity obtained with *p*NP lauric acid.

Natural oils such as coconut oil (12:0), palm oil (16:0), olive oil (18:1), grape seed oil (18:2), and soybean oil (18:3) were used as substrates for lipase assay. Determination of liberated free fatty acid (FFA) was assayed by using the modified method of Kwon and Rhee. [15]

3.2 The determination of optimum condition for lipase activity

3.2.1 Optimum pH and stability

The effect of pH on the lipase activity was determined using *p*-nitrophenyl ester (*p*NP-ester) as substrate. The substrate was prepared in 50 mM buffer phosphate buffer (pH 7-8.5), Tris-HCl (pH 9-10.5) and glycine/NaOH (pH 11-12). The lipase activity was determined at O.D. 410 nm.

3.2.2 Optimum temperature and enzyme stability

The optimum temperature for lipase activity was determined over the range of 30-65^{o}C in potassium phosphate buffer pH7. The substrate (*p*NP-ester) was prepared before the addition of enzyme. The effect of temperature stability was determined by incubating a pure enzyme for 30 min at various temperatures and potassium phosphate buffer pH 7. The lipase activity was determined at O.D. 410 nm.

3.2.3 Effect of metal ions, inhibitors and detergent

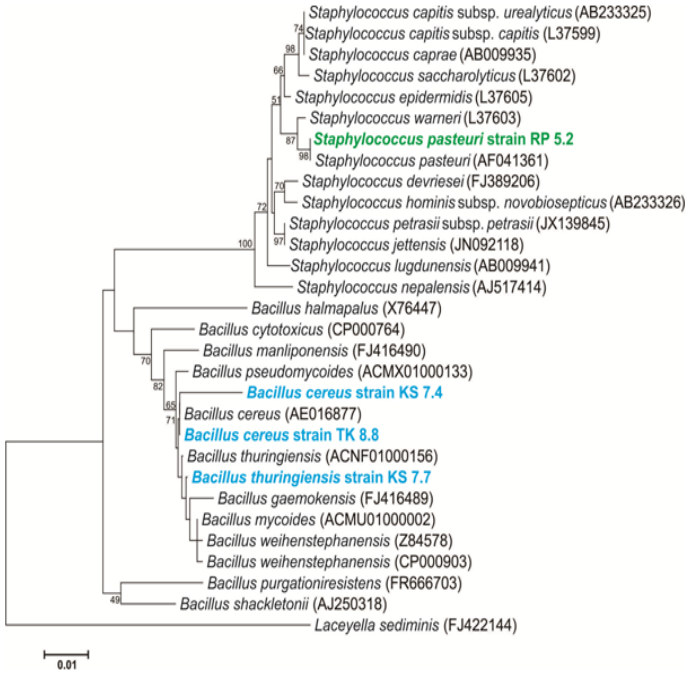
The activity of the purified lipase was determined following incubation with 1 mM of metal ions such as RbCl, NaCl, KCl, $MgCl_2.6H_2O$, $CaCl_2$, $ZnCl_2$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$,$ZnSO_4.7H_2O$, $FeSO_4.H_2O$, $Fe(II)Cl_2.7H_2O$, $LiCl_2$ and ethylenediamine tetraacetic acid (EDTA) at 25°C for 30 min. The remaining activity was determined spectrophotometrically using the standard *p*NP ester assay in the presence of an appropriate concentration of a given agent and expressed as a percentage of the activity without the agent.

III. RESULTS AND DISCUSSION

Isolation and characterization of lipase- producing bacteria from peat swamp

The one hundred and nine strains were primarily isolated by enrichment cultures in production medium. Among them, four isolates (KS7.4, KS7.7, TK8.8 and RP5.2) was chosen for a subsequent experiment due to its high activity in lipase production. The isolate KS7.4, KS7.7and TK8.8 were Gram's positive, rod-shaped and endospore-forming bacteria and RP5.2 was Gram's positive cocci-shape. The molecular classification of the phylogenetic tree based on 16S rDNA HV region exhibited the similarity of KS7.4 and TK8.8 with *Bacillus cereus* AE016877, KS 7.7 was similar with *Bacillus thuringiensis* ACNF01000156, and RP5.2 was *Staphyloccocus pasteuri* AF041361 respectively. (Fig. 1)

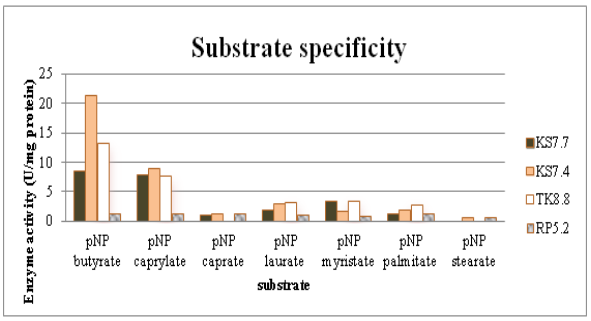
Figure 1 The phylogenetic tree of 16S rDNA sequences of KS7.4, KS7.7, TK8.8, and RP5.2 isolates.



The Comparison of substrate specificity, pH and temperature optimum

The four isolates displayed maximal catalytic activity towards *p*-NP butyrate (C4) as substrate were observed from KS7.4, KS7.7, TK8.8, and RP5.2 whereas RP5.2 showed high activity *p*-NP palmitate (C16). (Fig. 2) The lipase from 4 isolate showed a good lipase activity toward short chain fatty acid esters indicated that they were esterase that hydrolyzes exclusively short chain fatty acyl esters. The lipase enzymes from KS7.4, KS7.7 and TK 8.8 showed high specificity to palm oil (16:0) and olive oil (18:1). Furthermore, the enzyme from KS7.7 and TK8.8 could use soy bean oil (18:3) as substrate in contrast; lipase from RP5.2 hydrolyzed olive oil better than palm oil. The lipase from *Bacillus* sp. RN2 could hydrolyze coconut oil, olive oil, soy bean oil and palm oil, respectively [16]. The optimum pH that provided maximum lipase activity was at pH7.0 (50 mM Phosphate buffer) for KS7.4, KS7.7 and TK8.8 and pH9.0 (50 mM Tris-HCl) for RP5.2. The optimum temperature was 60°C for KS7.7, TK8.8 and RP5.2 and 45°C for KS7.4.

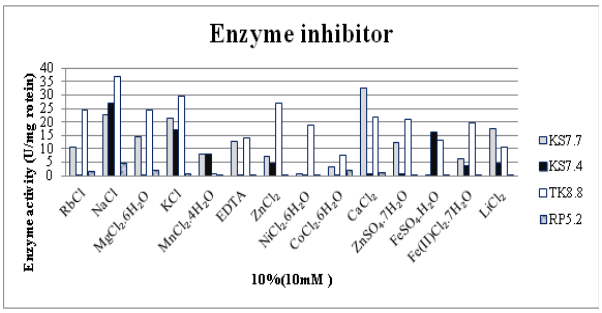
Figure 2 The substrate specificity of lipase activity from four isolates and analyzed with pH 7 and 37 °C.



Effect of metal ions and inhibitors on enzyme activity

The effect of different metal ions and inhibitors on isolate activity was determined using *p*NPB as substrate . Lipase enzyme activity was not altered significantly by Na^+, Mg^{2+} and EDTA whereas it was slightly enhance by NH_4^+ and K^+. The lipase activity was activated by addition of 10 mM Na^+, K^+ and Ca^{2+} for all crude enzymes from all strains. Ca^{2+} could activate enzyme activity from KS7.7 higher than Na^+ and K^+ that according with lipase from *B. subtilis* 168 [17], *B. thermoleovorans* ID1 [18] , *P. aeruginosa* EF2 [19] and *Staphylococcus aureus* 226 [20]. In contrast, EDTA strongly inhibited enzyme activity in both KS7.4 and RP5.2 whereas Mn^{2+}, Ni^{2+} and Co^{2+} slightly inhibited the activity in all strains. (Fig. 3)

Figure 3 The effect of metal ions and inhibitors on enzyme activity



VI. CONCLUSION

Production of an extracellular lipase have been reported previously. In this study, the lipase producing bacterial strains were isolated from peat swamp in Chanthaburi province, Thailand. Upon analysis for lipolytic activity on tributyrin agar plate, the isolates that could produce high activity were selected for identification and enzyme characterization. Some isolates showed high activity in alkaline condition and worked with high temperature also such as RP5.2 also showed activity with *p*-NP palmitate (C16) with optimum pH 9(50mM Tris-HCl) that different from other and the optimum temperature was 60°C, respectively. The phylogenetic tree showed RP5.2 was similar with *Staphyloccocus pasteuri* AF041361.

ACKNOWLEDGEMENTS

We are especially thankful to a grant from Rambhai Barni Rajabhat University year 2011

REFERENCES

[1] Okahata Y, Mori T. Lipid-coated Enzymes as Efficient Catalysts in Organic media. Trends Biotechnol 1997;15:50-54.

[2] Jaeger KE, Eggert T. Lipase for biotechnology. Protein technologies and comercial enzymes 2002;13:390-397.

[3] Kazlauskas RJ, Weber HK. Improving hydrolases for organic synthesis. Curr Opin Chem Biol 1998;2(1):121-126.

[4] Schmid RD, Verger R. Lipases: interfacial enzymes with attractive applications. Angew Chem Int Ed Engl 1998;37:1603-1633.

[5] Jaeger KE, Reetz MT. Microbial lipases form versatile tools for biotechnology. Trends Biotechnol 1998;16(9):396-403.

[6] Schmid RD, Verger R. Lipases: interfacial enzymes with attractive applications. Angew Chem Int Ed Engl 1998;37:1603-1633.

[7] Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol VT. The realm of microbial lipases in biotechnology. Biotechnol Appl Biochem 1999; 29:119-131.

[8] Saitou N. and Nei M.The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 1987;4:406-425.

[9] Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985;39:783-791.

[10] Tamura K., Nei M., and Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 2004;101:11030-11035.

[11] Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 2013;30: 2725-2729.

[12] Vorderwulbecke T, Kieslich K, Erdmann H. Comparison of lipases by different assays. Enzyme Microb Technol 1992;14:631-639.

[13] Pencreac'h, G. and Baratti, J. C. Hydrolysis of p-nitrophenyl palmitate in n-heptane by the Pseudomonas cepacia lipase: a simple test for the determination of lipase activity in organic media. Enzyme Microbial. Technol 1996; 18: 417-422.

[14] Iacazio, G., Perissol, C. and Faure, B. A new tannase substrate for spectrophotometric assay. J. Microbiol. Methods 2000; 42: 209-214.

[15] Kwon, D.Y. and Rhee, J.S. A Simple and Rapid colorimetric method for determination of free fatty acids for lipase assay. J Am Oil Chem Soc 1986; 63: 89-92.

[16] Kanjanavas P, Khuchareontaworn S, Khawsak P, Pakpitcharoen A, Pothivejkul K, Santiwatanakul S, Matsui K, Kajiwara T, Chansiri K. Purification and Characterization of Organic Solvent and Detergent Tolerant Lipase from Thermotolerant *Bacillus* sp. RN2 Int. J. Mol. Sci. 2010; 11(10):3783-3792.

[17] Lesuisse E, Schanck K, Colson C. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur J Biochem 1993;216:155–160.

[18] Lee O-W, Koh Y-S, Kim K-J, Kim B-C, Choi H-J, Kim D-S,Suhartono MT, Pyun Y-R. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1.FEMS Microbiol Lett 1999;179:393–400.

[19] Gilbert EJ, Cornish A, Jones CW. Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. J Gen Microbiol 1991;137:2223–2229.

[20] Muraoka T, Ando T, Okuda H. Purification and properties of a novel lipase from *Staphylococcus aureus* 226. J Biochem 1982;92:1933–1939.