JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2009. Vol. **3**(2), p. 387- 392

Molecular Characterization of Lipase Producer from Local Marine Environment

Syarul Nataqain Baharum[1]*, Maya Erna Nathan[2] and Mohd Azinuddin Ahmad Mokhtar[1]

[1] Institute of Systems Biology Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.
[2] Faculty of Biotechnology and Life Sciences Jalan Zirkon A7/7, Seksyen 7, 40000 Shah Alam, Selangor, Malaysia.

(Received: 30 June 2009; accepted: 07 August 2009)

Lipolytic bacteria were screened from marine water sample in Pantai Morib, Selangor. Eight out of 100 isolates exhibited positive lipolytic activities via qualitative screening assay using the tributyrin agar plates. The halo formation around bacteria colonies indicates the production of lipase activity. Quantitative assay was performed and isolate S14 exhibited highest lipase production (6.404 U/ml) compared to other isolates. The Polymerase Chain Reaction (PCR) technique was employed for the 16S rDNA. The 16S rDNA fragment with 1500 bp in size was cloned and sequenced. However, there was only 1004 bp of 16S rDNA nucleotides was successfully sequenced. Isolate S14 was identified as *Bacillus* sp. based on 95% homology with the reference sequences of *Bacillus sp*. from the GenBank. Based on these results we conclude that lipase producer was successfully isolated from the local marine environment and identified as *Bacillus* sp.

Key words: Lipase, Marine bacterium, 16S rDNA, *Bacillus* sp.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are glycerol ester hydrolases that catalyze the hydrolysis of triaclyglycerols into fatty acid, partial acylglycerol and glycerol[1]. Lipases can synthesize ester, perform transesterification and other reactions[2]. They had ability to hydrolyze triacylglycerol at lipid water interface and also ability to reverse hydrolytic reaction into synthetic reaction in non-aqueous media.

There are many applications of lipase enzyme today because of its high market demand and was frequently used since they capable in catalyze various useful reactions[3]. It includes the reforming of lipid ingredients, production of milk flavor and degradation of lipids in feed. In the medical field, lipase is used as a digestive enzyme. While in chemistry, one of its major applications is the enantioselective production of various chiral compounds, such as a key intermediate for synthesis. Moreover, lipase enzyme also used for biotechnology application, such as in dairy industry, pharmaceutical industry, detergent and cosmetic industry.

* To whom all correspondence should be addressed.
Ph.: + 603-89214550; Fax: 03-89213398
E-mail: nataqain@ukm.my

Many researchers were interested to study marine microorganism. This is due to the great potential in marine studies because marine microorganisms grow in unique and extreme habitats, such as in psychrophile condition. So, it may have unique characteristics that differ from others microbial. Microorganisms are extremely abundant in marine environment that thought to achieve density up to 10^6 per ml[4] and it is believed that containing a total of approximately 3.67×10^{30} microorganisms[5] in 71% of total earth water surface. This enormous potential has made many researches interested to study marine microorganisms[6].

In addition, enzymes from marine microorganism are not fully explored by the scientists. Due to restriction in culturing by conventional means which only 1% marine microorganisms able to be cultured[6]. There are thousands of important marine microorganisms out there that never gotten into research yet. Marine microorganisms catalyze all the major biochemical transformations on this planet. Its help maintain nutrient availability, and also rich with genetic information resources, which may be useful for biotechnology[7].

Several marine organisms can be the source of lipases enzyme production. For example, marine bacteria *Psychrobacter okhotskensis sp*. can produce lipase in the psychophile environment[8]. Meanwhile *Shewanella japonica* not only produced lipases but also proteinases (gelatinases, caseinases), amylases, agarases, and alginases[9].

This study focused on the isolation and identification of lipase from *Bacillus* sp. from our local marine environment. The wild type lipase shows high lipase production and we believed it has a promising potential for further study.

MATERIAL AND METHODS

Marine Microorganisms

Water sample were collected from Pantai Morib, Selangor, Malaysia. About eight bacteria colonies that exhibited lipolytic activities were isolated out of 100 colonies bacteria in tributyrin agar plates. The bacteria were incubated at 37 °C for 24 h.

Screening of Lipolytic Producer

The screening of lipolytic production was carried out qualitatively on tributyrin agar plate, consists of marine agar and 1% (v/v) of tributyrin. About 1.0 ml of marine water samples was culture in marine broth and incubated for 24 hours at 37 °C. An aliquot of 1.0 ml of microbe solution was diluted in 9.0 ml with the dilution factor 10^{-6}. Then, 100 µl of 10^{-4}, 10^{-5} and 10^{-6} dilution factor samples were transferred into tributyrin agar plates and incubated at 37 °C for 24 h. Each sample was duplicated. Then the lipolytic producer was screened. Screening on Rhodamine B and Victoria blue was carried out for further confirmation[10].

Assay for lipase activity

Determination of liberated free fatty acid (FFA) was measured using Kwon and Rhee (1986) method as also described by Baharum *et al.,*(2003), Rahman *et al.*, (2005) and Rahman *et al.,* (2006). Olive oil was used as a substrate. The reaction mixture comprised of 1.0 ml enzyme, 2.5 ml of olive oil emulsion and 0.02 ml of 20 mmol $CaCl_2$ was incubated for 30 min, 200 rpm at 37 °C. The reaction was stopped by addition of 1.0 ml of 6M HCl and 5.0 ml of isooctane. The upper layer (4 ml) was pipetted out into test tube and 1.0 ml of cupric acetate pyridine was added. The free fatty acid dissolved in isooctane was determined by measuring the absorbency of isooctane solution at 715 nm. Absorbances reading were taken for triplicate on each sample. Lipase activity was determined by measuring the amount of FFA from the standard curves of oleic acid. One unit of lipase activity (U) is defined as the rate of enzyme, which liberated 1 µmole of free fatty acid in one minute at standard assay condition.

Statistical analysis

For statistical analysis, standard deviations for each of the experimental results were calculated using Excel Spreadsheets, with Microsoft Excel software[11].

16S rDNA sequence identification and phylogenetic tree analysis

Genomic DNA extraction was used as a template to perform PCR amplification for 16S rDNA identification with a set of universal primers that highly conserved among prokaryotes and could amplify 1500bp as described by Rahman

irradiation. Meanwhile, the lipase producers that cultured on the Victorian agar blue was appeared blue in color around it colonies due to change of the blue indicator via the fatty acid formation (Figure 1c).

Gram-staining

The positive colonies were isolated and subsequently stained in order to determine bacteria gram's classses. Out of 8 isolates selected, 5 isolates appears red or pink in color which means gram-negative and 3 isolates appears blue or purple in color which means gram-positive bacteria. Changing in color of gram-positive bacteria was because of it retained the crystal violet, whereas gram-negative bacteria lost their crystal violet and become colorless. The safranin solution act as a counterstained by coloring the gram-negative bacteria into pink or red in color and leaves the gram-positive bacteria in dark purple or blue in color[13].

Assays of lipases activity

The result as shown in Table 1 was obtained from the standard curve of FFA equation. The lipase production was assayed according to Kwon and Rhee (1986) method. Based on this result, isolate S14 exhibited highest lipase production (6.404 U/ml) if compare with other isolates. We believed that this lipase produced significantly high activity as compared to other wild type lipases [11,14,15,16].

16S rDNA identification and phylogenetic tree analysis

16S ribosomal DNA was employed for identication of isolate no S14. The 16S rDNA

```
CGCTGGCGGCGTGCCTAATACTTGCAAGTCGAGCGAACAGAGAAGGAGTTTGCTCCTTTG  72

ACTTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTTATAGTTTGGGATAACT  132

CCGGGAAACCGGGGCTAATACCGAATAATCTGTTTCACCTCATGGTGAAATATTGAAAGA  192

CGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACG  252

GCTCACCAAGGCGACAATGCGTACCCAACCTGAGAGGGTGATCGGCCACCCTGGGACTGA  312

AACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGC  372

CTGATGGAGCAACGCCGCGTGAGTGAAAAAGGATTTCGGTTCGTAAAACTCTGTTGTAAG  432

GGAAAAACAAGTACAGTAGTAACTGGTTGTACCTTGACGGTACCTTATTAAAAAGCCACG  492

GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATT  552

GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCG  612

TGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGATAGTGGATTTCCAGGTG  672

TAGCGGTGAAATGCGTAGGAGATTTGGAGGAACACCAGTGGGCGAAGGCGACTATCTGGT  732

CTGTAACTGACACTGAGGCGCGAA-GCGTGGGGGAGCAA-CAGGATTAGATACCCTGGTA  790

GTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGG-TTTCCGCCC-T-A-TGCTGCAG  846

CTA-CGCAT-A-GCACTCCGC-TGGGGA-TACG-TCGCA-GACTGA--CTCAA-GGA-T-  894

GACGGG                                                         900
```

Fig. 2. The 16S rDNA nucleotide sequence from isolate no.14 with accession no EU 311720

constructed on the basis of comparison of the 16S rDNA sequence of this sample with 16S DNA sequence of *Bacillus* sp. that available in the GenBank database. It evidenced a high degree of homology (95%) with the majority of *Bacillus sp* including stain B-23288, B-23286, B-23282, B-23284, BD-111, LYB, LLD and other sp of *Bacillus* such *B. fusiformis*, CO64 and *B. sphaericus* strain PLC-5. The result indicated that S14 (SAMPLE2007) was slightly distances from other *Bacillus* sp.

CONCLUSION

We have sucessfully isolated lipase producer bacterium from marine environment. This bacterium showed significantly high homology to *Bacillus sp*. Based on this result, we conclude that lipase producer in thus study is *Bacillus sp*. Further on going study will be based on isolation of lipase gene by using a molecular biology tools approach and also to characterize this lipase based on its properties.

ACKNOWLEDGMENTS

This work was supported by research grant no. 3090106001-MOSTI from Ministry of Science, Technology and Innovation, Malaysia.

REFERENCES

1. Rahman, R.N.Z.R.A., Baharum, S.N., Basri, M. and Salleh, A.B. 2005. High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5. *Analytic. Biochem.,* 2005; **341**(2): 267-274.
2. Ogino, H., Miyamoto, K. and Ishikawa H. Organic solvent-tolerant bacterium which secretes organic solvent-stable lipolytic enzyme. *Appl. Environ. Microbiol.*, 1995; **61**(12): 4258-4262.
3. Miyawaki, O. and Nakamura, K. Enzyme reaction in supercritical fluids. In: *Lipid Biotechnology*, Marcel Dekker Inc. USA, 2002
4. Azam, F. Oceanography: Microbial control of cceanic carbon flux: The plot thickens. *Sci.*, 1998; **280**: 694-696.
5. Whitman, W.B., Coleman, D.C. and Wiebe, W.J. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci.,* 1998; **95**(2): 6578-6583.
6. Kennedy, J., Marchesi, J.R. & Dobson, A.D.W. Review: Marine metagenomics: strategies for the discovery of novel enzymes with biotechnological applications from marine enviroments. *Microb. Cell Fact*., 2008; **7:** 27-35.
7. Fukuda, F., Ogawa, H., Nagata, T. and Koike, I. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environment. *Appl. Environ. Microbiol.*, 1998; **64**(9): 3352-3358.
8. Yumoto, I., Hirota, K., Sogabe, Y., Nodasaka, Y., Yokota, Y. and Hoshino, T. *Psychrobacter okhotskensis* sp. nov., a lipase-producing facultative psychrophile isolated from the coast of the Okhotsk Sea. *Int. J. of Sys. Evol. Microbiol*.2003; **53**(6): 1985-1989.
9. Ivanova, E.P., Sawabe, T., Zhukova, N.V., Gorshkova, N.M., Nedashkovskaya, O.I., Hayashi, K., Frolova, G.M., Sergeev, A.F., Pavel, K.G., Mikhailov, V.V. and Nicolau, D.V. Occurrence and diversity of mesophilic *Shewanella* strains isolated from the North-West Pacific Ocean. *System. Appl. Microbiol*., 2003; **26**: 293–301.
10. Kouker, G. and Jaeger, K.E. Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol*., 1987; **53**(1): 211-213.
11. Rahman, R.N.Z.A, Baharum, S. N., Basri, M. and Salleh A. B. S5 Lipase: An Organic Solvent Tolerant Enzyme. *J. Microbiol.*, 2006; **44**(6): 583-590.
12. Leonel, J.O. and Jorge, O.S. The functional property of *Bacillus* for shrimp feeds. *F. Microbiol.,* 2006; **23**(6): 519–525.
13. Prescott, L.M., Harley, J.P. and Klein, D.A. (ed): *Microbiology*, New York: McGraw Hill., 2005.
14. Baharum, S.N., Salleh, A.B., Razak C.N.A., Rahman, R.N.Z.A., Basri, M. and Rahman, M.B.A. Organic solvent tolerant lipase by *Pseudomonas* sp. strain S5: stability of enzyme in organic solvent and physical factors affecting its production. *Ann. Microbiol*., 2003; **53**(1): 75-83.
15. Eltaweel., M.A., Rahman, R.N.Z.R.A, Salleh A.B. and Basri M. An organic solvent-stable lipase from *Bacillus* sp. strain 42. *Ann. Microbiol*., 2005; **55**: 187-192.
16. Shariff, F.M., Leow, T.C., Murked, A.D., Salleh, A.B., Basri, M. and Rahman, R.N.Z.R.A. Production of L2 lipase by *Bacillus* sp. Strain L2: nutritional and physical factors. *J. Basic Microbiol*., 2007; **47**: 406-412.