Jurnal Teknologi

Full Paper

ISOLATION OF MODERATELY HALOPHILIC LIPASE PRODUCING BACTERIA FROM SPONGES IN PAHANG COASTAL WATER, MALAYSIA

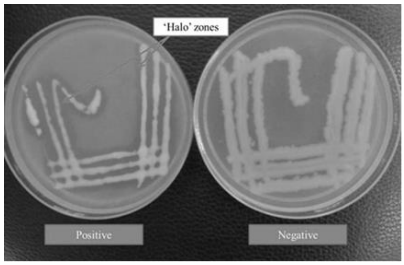
Tengku Haziyamin Tengku Abdul Hamid*, Azzmer Azzar Abdul Hamid, Nurul Husna Padzil

Department of Biotechnology, Kulliyah of Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200, Kuantan, Malaysia

Article history
Received
3 July 2015
Received in revised form
18 September 2015
Accepted
22 October 2015

*Corresponding author
haziyamin@iium.edu.my

Graphical abstract



Abstract

Sponges (*Porifera*) harbour diverse microorganisms which can be the potential source for microbial enzymes such as lipase. In this study, moderately halophilic lipase producing bacteria were isolated from sponges tissues collected near Balok, at Pahang coastal water. Out of 70 isolates that grew on tributyrin agar plate, only 7 isolates had produced clear zones surrounding their colonies. Out of these, 5 isolates appeared to be gram-positive rod; meanwhile, the other 2 isolates were gram-negative rod in morphologies. These isolates were subjected to several biochemical tests i.e., oxidase, gelatin hydrolysis, lactose fermentation, citrate and motility test, and 16S rRNA gene amplification and sequencing. The results from 16S rRNA sequencing showed that 2 isolates (NHTH 6B and NHTH 28A) were highly similar (>97%) with *Paenibacillus illinoisensis;* isolate NHTH 26A with *Stenotrophomonas pavanii;* and isolate NHTH 29A with *Enterobacter aerogenes*. Phylogenetic analysis on selected isolates (NHTH 6B, NHTH 26A, NHTH 28A and NHTH 29A) with other species from the database showed high bootstrap values of above 50%. This showed that diverse phyla of lipase producing bacteria were isolated from the sponge collected from Pahang coastal water. In the isolation of industrial important species, the presence of pathogenic group of microorganism in this sponge could indicate issues on water quality and safety in this area.

Keywords: Halophilic bacterium, lipase producer, sponges

1.0 INTRODUCTION

Sponges (Phylum: Porifera) has been the target for medicinal and bioactive compounds. It is one of the unique and interesting marine organism to be studied because of their potential industrial applications. Microbiologist have been fascinated with sponges because of their diversity, ancientness, ecological significance, production of bioactive metabolites and their interaction with microorganisms [1]. Despite sponges can be a useful source of novel compounds, most of the bioprospecting activities have been based on microbial organisms [2].

Extremophiles are diverse microorganisms that can be found in extreme conditions such as in thermal vent, Artic or Antarctic environment, deep seas or even in highly alkaline or acidic waters. These organisms produce enzymes that can withstand or stable under extreme conditions that may be suitably utilised in some industries. Since enzymes cannot withstand harsh industrial conditions, extremophiles has gained great attention due to the increasing demands for enzymes that can survive industrial conditions [3]. Halophile can be readily isolated from sponges and it can survive the high salinity environment of the ocean. Furthermore, it can produce various types of industrially important

hydrolases such as amylases, lipases and proteases [4]. Lipase (EC 3.1.1.3), a triacylglycerol hydrolase, is an example of useful industrial enzyme with various industrial applications, including biodiesel production, food flavoring, detergent industry, paper industry and cosmetic production [5]. The major commercial application of lipase is their use in laundry detergents [6].

Sponge, an aquatic organism that survive the relatively saline seawater environment, is a potential hosts for halophilic bacterium. Salt tolerant microorganism can be isolated from sponges, and they can be a useful source for 'salt tolerant' lipase. Hence, this work reported on the isolation and characterization of sponge-associated lipase producing microorganisms. This strains could be the potential source for lipases useful in various industrial applications. In an attempt to isolate industrial important sppecies, this work also reported on the presence of some lipase producing pathogenic species in the area.

2.0 EXPERIMENTAL

Sponges from species *Gelliodes* sp. were collected at at Batu Pelindung, at Pahang coastal water nearby the Gebeng industrial area, 3 km north of Kuantan. Inner section of the sponge was cut into pieces using sterile scalpel and then rinsed with sterile buffered saline. About 10g of tissues were homogenized using pastel. Sample was then serially diluted with saline solution at 10^{-1}, 10^{-2} and 10^{-3} dilutions. About100 µl of dilutions were spread on marine agar in duplicate followed by incubation at 28°C, for 48 hours.

For lipase test, the single colony from the spread plate was streaked on tributyrin agar plates using inoculation loop and incubated for 72 hours at 28°C. Positive lipase-producing bacteria were determined by the formation of clear or "halo" zones surrounding the colonies on the tributyrin plate [7]. Isolates showing positive result were kept as glycerol stock for further analysis. Marine agar contained 1.0 g/L yeast extract, 5.0 g/L meat peptone, 0.1g/L Iron citrate, 19.45 g/L NaCl, 3.24 Na_2SO_4, 0.16 g/L sodium bicarbonate, 0.004 g/L sodium silicate, 0.0024 g/L NaF, 0.008 g/L Na_2HPO_4, 1.8 g/L $CaCl_2$, 8.8 g/L $MgCl_2$, 0.55 g/L KCL, 0.08 g/L KBr, 0.034 g/L SrCl, 0.0016 g/L NH_4NO_3, 0.022 g/L boric acid and 15 g/L agar. The media contained all the above components except for the agar. Tributyrin-marine agar media contained all the components used in marine agar but with the addition of 1% (v/v) tributyrin, which was homogenised before autoclaved. Selected strains were maintained on 80% (v/v) glycerol and morphological studies were carried using light microscope (Nikon) using standard gram staining protocol. Standard biochemical tests; oxidase, gelatin hydrolysis, lactose fermentation, citrate and motility tests were carried out on each selected strain.

Genomic DNA was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia) carried out according to manufacturer's manual. These were used as template for 16S ribosomal RNA gene amplification. A pair of primer (Integrated DNA Technologies, IDT Inc., USA) was used in which, the forward primer: rRNA_F_Tg_2014 F27 (5'- AGA GTT TGA TCC TGG CTC AG -3') and the reverse primer: rRNA_R_Tg_2014 R1525 (5'- AAG GAG GTG ATC CAG CCG CA – 3') [8]. The PCR reaction was carried out in 50µl volume reaction mixture containing 25µl of Taq 2x Master Mix (Vivantis, Malaysia), 1.0µl of each forward and reverse primer (0.5 µM), 3µl of DNA template (≈100ng) and 20µl of nuclease-free water making up the final volume of 50 µl. PCR was carried out using the thermocycler (Eppendorf, Germany) with the following regime: initial denaturation (94°C, 4 minutes) and 30 cycles denaturation (94°C, 1 minute), annealing (58°C, 1 minute) and extension (72°C, 1 minute). This was followed by a last step at 72°C for 7 minutes with final holding at 4°C. The amplified product were purified and subjected to sequencing (1st Base (Malaysia) Sdn. Bhd.). The sequences were analyzed using Multialign tool software, and the (BLASTN) search tool was used, available on-line at *http://www.ncbi.nlm.nih.gov*. The phylogenetic tree was also generated using Software MEGA 2.1, using the neighbour joining method in which the *Halobacterium salinarum* rRNA gene was used as an out group.

3.0 RESULTS AND DISCUSSION

3.1 Morphology and Biochemical Test

Clear zone on tributyrin agar indicated the lipolytic activitiy. Colonies that were able to show clear zone on tributyrin agar plates, as shown on Figure 1, were selected. As shown on Table 1, isolates with label NHTH 6B, NHTH 26A, NHTH 27A, NHTH 28A, NHTH 29A NHTH 32B and NHTH 40A were selected. All samples (NHTH 6B, NHTH 26A, NHTH 27A, NHTH 28A, NHTH 29A and NHTH 32B, except for NHTH 40A) were found to be oxidase negative after 24 hours incubation. In gelatin test, all isolates except NHTH 29A were positive with gelatinase activities.

In lactose fermentation test, isolate NHTH 29A and all other gram positive isolates (NHTH 27A, 32B and 40A) formed pink coloration as lactose produced an acidic environment due to fermentation. Only isolate NHTH 26A showed lactose negative. Gram-positive isolates NHTH 28A and 6B showed no visible growth. On citrate test, NHTH 26A, NHTH 29A and NHTH 40A showed positive results while NHTH 6B, 27A, 28A and 32B showed negative results.

Table 1 Summary of morphological and biochemical tests Note; '+' indicates positive result, and '-' for negative result

Isolates	Gram Reaction	Oxidase	Gelatin Hydrolysis	Lactose Fermentation	Citrate	Motility
NHTH6B	+	-	+	-	-	+
NHTH26A	-	-	+	-	+	-
NHTH27A	+	-	+	-	-	+
NHTH28A	+	-	+	-	-	+
NHTH29A	-	-	-	+	+	+
NHTH32B	+	-	+	-	-	+
NHTH40A	+	+	+	-	+	+

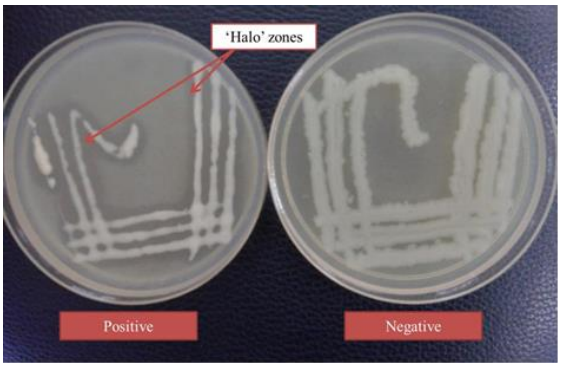


Figure 1 Positive isolates show lipase production as evidenced by the clear zone surrounding the colonies (left plate) while no clear zone was observed with negative isolates (right plate)

3.2 Ribosomal rRNA Sequencing

All 1.5 kb products (rRNA gene) were successfully amplified (Figure 2) and then sequenced, except for sample NHTH 32B which was failed to be sequenced. Sequences similarities were searched from NCBI Genebank database, and based on results shown on Table 2, the highest similarity of 99% were found for most sequences. Sample NHTH 27A and 40A however showed lower similarities (94%). Since, similarity lower than 97% threshold should be regarded as separate species [9], further identification for this two species are required. A phylogenetic tree was constructed as shown on Figure 3.

Both isolates NHTH 6B and 28A have the highest similarity with *Paenibacillus illinoisensis*. The morphological and biochemical characteristics of these isolates (see Table 1) were consistent with the report on *Paenibacillus illinoisensis.* They are gram-positive rod with motile peritrichous flagella that grow between 10 to 50°C, at pH 4.5 to 9.0. Being slightly halophilic (2% NaCl), they showed positive on gelatin test, but negative on oxidase, citrate and lactose tests. *Paenibacillus illinoisensis* was previously belonged to *Bacillus circulans* Group 6. Later study revealed that they are genetically different and distinguishable from previously described species and a new name *Paenibacillus illinoisensis* was proposed [10]. *Paenibacillus illinoisensis* isolated from the soil sample from Kanto, Japan produced cyclodextrin glucosyltransferase (CGT-ase) that stable in organic solvents with the presence of lipase enzyme. This species were also reported to have potential biotechnological use such as biological control agent in plants infection [11]. Therefore, the presence of lipase from halophilic *Paenibacillus illinoisensis* NHTH 6B and NHTH 28A could be exploited for other biotechnological application.

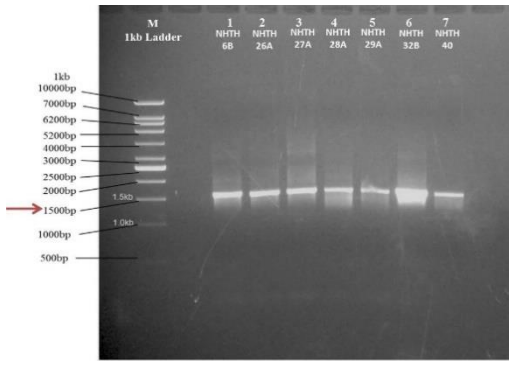


Figure 2 PCR products of 16S ribosomal RNA gene amplification electrophoresed on 1.0% (w/v) agarose gel Lane 1 to 7 contains sample from isolates. Lane M contains 1 kb ladder marker (Vivantis, Malaysia). Arrow indicates the PCR products expected size of approximately 1500bp

Isolate NHTH 26A showed the highest similarity to *Stenotrophomonas pavanii*. This is a gram-negative rod, non-spore former and nitrogen-fixing bacterium isolated from Brazillian sugar cane. This strain was slightly halophilic (0.7 to 3% NaCl) with growth temperature range of 20-37°C, with pH 5 to 12 [12]. The species appeared positive on citrate and gelatin, but negative for oxidase and lactose tests. Consistent biochemical tests with the reported *Stenotrophomonas pavanii* was observed except for lipase production. However, a closely related species *Stenotrophomonas maltophilia* was reported to produce lipase [13]. Despite of this similarity, *Stenotrophomonas maltophilia* was negative for gelatinase test [14]. Further characterization on isolate NHTH 26A could confirm if it could be a new species.

Isolate NHTH 29A showed the highest similarity with *Enterobacter aerogenes* (99%). This is a Gram-negative motile rod, and a non-spore former [15]. It is ubiquitous and often found in marine, fresh water, sewage, soil and plants. They grow optimally between 20°C to 37°C, and showed positive on

lactose and citrate tests, but negative for oxidase or gelatin tests (see Table 1). *Ent. aerogenes* is a coliform found in marine environment polluted by domestic sewage and reported to present in large number in Tolo Harbour, Hong Kong [16]. The survival of *Ent. aerogenes* in seawater was affected by the underlying bottom sediments with higher concentration of organic nutrients. The possibility of the sampling site being polluted cannot be excluded. Moreover, the industrial or other activities near Gebeng area could be the source for discharge into the area. Both isolates NHTH 27A and NHTH 40A showed similarity with *Pseudomonas spp*. as supported also by biochemical tests. Since the low sequence similarity from 16S rRNA sequencing, both isolates should be subjected to further characterisation to re-evaluate their species identity.

Table 2 List of hit with the highest sequence similarity to the lipase-producing isolates from Sponges based on 16S ribosomal RNA sequence using BLASTN similarity searches

Isolates	Query Length (Base pairs)	Organism	Max. Identity (%)	Accession Number (Hit sequence)
NHTH 6B	1478	*Paenibacillus illinoisens* strain NBRC 15959	99	NR_113828.1
NHTH 26A	1470	*Stenotrophomonas pavanii* strain LMG 25348	99	NR_118008.1
NHTH 27A	1200	*Pseudomonas hibiscicola* strain ATCC 19867	94	NR_024709.1
NHTH 28A	1476	*Paenibacillus illinoisensis* strain NBRC 15959	99	NR_113828.1
NHTH 29A	1469	*Enterobacter aerogenes* strain KCTC 2190	99	NR_102493.1
NHTH 40A	808	*Pseudomonas aeruginosa* sp	94	NR_118644.1

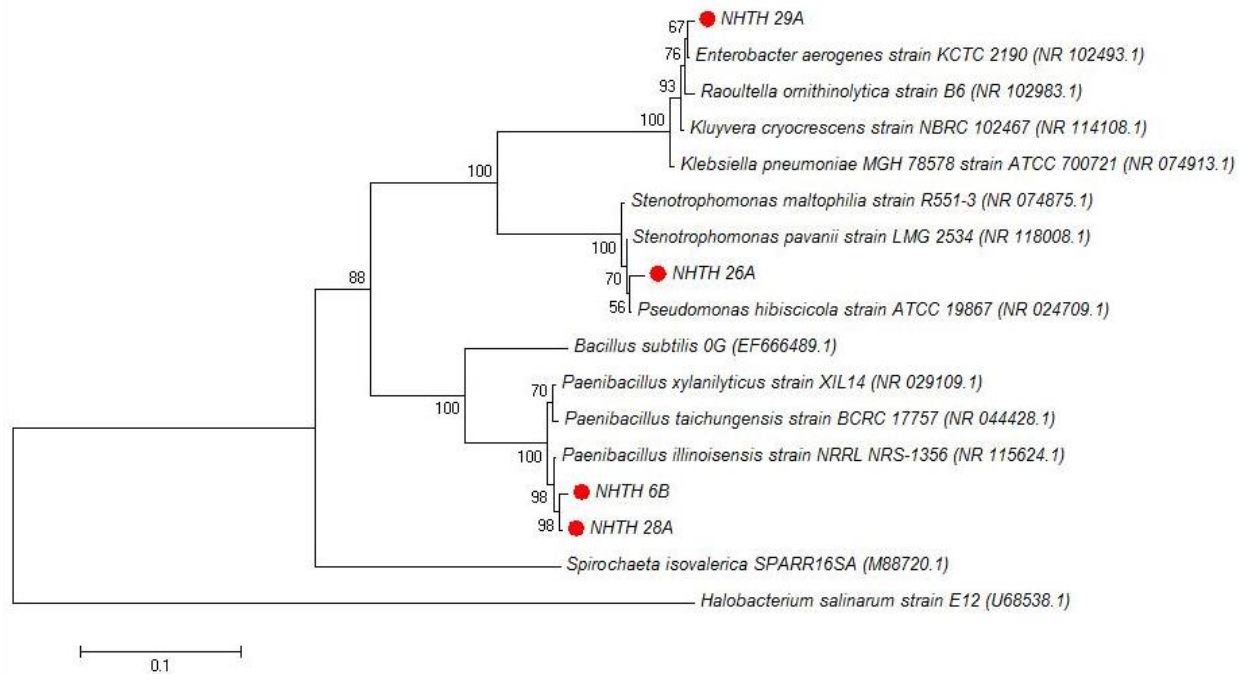


Figure 3 Neighbor-joining phylogenetic tree of 16S rRNA gene from isolates NHTH 6B, NHTH 26A, NHTH 28A and NHTH 29A. The tree shows how these isolates radiate with other closely related sequences. The sequence of *Halobacterium salinarum* was used as an outgroup

4.0 CONCLUSIONS

The presence of moderately halophilic lipase producing bacteria from the sponge tissues was confirmed by the formation of the clear zones on tributyrin agar plates. The characterization of 7 lipase-producing halophilic
bacteria were determined based on morphological, biochemical studies and supported by molecular

identification. Five isolates (NHTH 6B, NHTH 27A, NHTH 28A, NHTH 32B and NHTH 40A) were gram-positive; while two isolates (NHTH 26A and NHTH 29A) were gram-negative. Sequencing of 16S ribosomal RNA gene showed that out of all 7 isolates, 2 isolates (NHTH 6B and NHTH 28A) have the highest similarity (>99%) with *Paenibacillus illinoisensis*. These two isolates could be the potential sources for lipase that have great potential in biotechnological application. Meanwhile, isolate NHTH 26A was highly similar with *Stenotrophomonas pavanii*; and isolate NHTH 29A was with *Enterobacter aerogenes*. In contrast, NHTH 27A and NHTH 40A showed similarity to *Pseudomonas* spp. (> 94%). In this work, moderately lipase producing halophilic bacterium isolated from a sponges (*Gelloides* sp.) are of from diverse genera such as *Paenibacillus*, *Stenotrophomonas* and *Enterobacter*. In view that some of these lipase producing species are also belonging to some pathogenic organism, as well as the presence of water contaminating bacteria such as coliform, the degree of water contamination and safety could be another area of concern, particularly at Batu Pelindung, Balok, Pahang.

Acknowledgement

We are grateful for the IIUM for financial the support given in initiating this work.

References

[1] Taylor, M. W., Hill, R. T., & Hentschel, U. 2011. Meeting Report: 1st international symposium on sponge microbiology. *Marine Biotechnology*. New York, N.Y. 13(6): 1057-1061.

[2] Íñiguez-Martínez, A. M., Guerra-Rivas, G., Ayala-Sánchez, N. E. and Soria-Mercado, I. E. 2013. Bioactive Compounds from Marine Actinomycetes, in Marine Microbiology: Bioactive Compounds and Biotechnological Applications (ed S.-K. Kim). Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

[3] Van den Burg, B. 2003. Extremophiles as a Source for Novel Enzymes. *Current Opinion in Microbiology*. 6(3): 213-218.

[4] Kumar, S., Karan, R., Kapoor, S., Singh, S. P. & Khare, S. K. 2012. Screening and Isolation of Halophilic Bacteria Producing Industrially Important Enzymes. *Brazillian Journal of Microbiology*. 38: 1595-1603.

[5] Andulema, B. and Gessesse, A. 2012. Microbial Lipases and Their Industrial Applications. *Journal of Biotechnology*. 11: 100-118.

[6] Sharma Rohit Sharma, Yusuf Chisti, Uttam Chand Banerjee. 2001. Production, Purification, Characterization, and Applications Of Lipases. *Biotechnology Advances*. 19: 627-662

[7] Chaturvedi, M., Singh, M., Chugh, M., Rishi and Kumar R. 2010. Isolation of Lipase Producing Bacteria from Oil Contaminated Soil for the Production of Lipase by Solid State Fermentation using Coconut Oil Cake. *International Journal of Biotechnology and Biochemistry*. 6(4): 585-594.

[8] Lane, D. J. 1991. 16S/23S rRNA Sequencing. *Nucleic Acids Techniques in Bacterial Systematics*. John Wiley & Sons, Chichester. 115-147.

[9] Stackebrandt, E. & Goebel, B. M. 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S Rrna Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic Bacteriology*. 44: 846-849.

[10] Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K., & Komagata, K. 1997 Emended Description of Paenibacillus Amylolyticus and Description of Paenibacillus illinoisensis sp. nov. and Paenibacillus chibensis sp. nov. *International Journal of Systematic Bacteriology*. 47(2): 299-306.

[11] Jung, W. J., Jin, Y. L., Kim, Y. C, Kim, K. Y., Park, R. D., Kim, T. H. 2004. Inoculation of Paenibacillus illinoisensis Alleviates Root Mortality, Activates of Lignification-Related Enzymes, and Induction of the Isozymes in Pepper Plants Infected by Phytophthora capsici. *Biological Control*. 30(3): 645-652.

[12] Ramos, P. L., Van Trappen, S., Thompson, F. L., Rocha, R. C. S., Barbosa, H. R., De Vos, P., & Moreira-Filho, C. a. 2011. Screening for Endophytic Nitrogen-Fixing Bacteria in Brazilian Sugar Cane Varieties Used in Organic Farming and Description of Stenotrophomonas pavanii sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. 61(4): 926-31.

[13] Hasan-Beikdashti, M., Forootanfar, H., Safiarian, M. S., Ameri, a., Ghahremani, M. H., Khoshayand, M. R., & Faramarzi, M. A. 2012. Optimization of Culture Conditions for Production of Lipase by a Newly Isolated Bacterium Stenotrophomonas Maltophilia. *Journal of the Taiwan Institute of Chemical Engineers*. 43(5): 670-677.

[14] Brooke, J. S. 2012. Stenotrophomonas maltophilia: An Emerging Global Opportunistic Pathogen. *Clinical Microbiology Reviews*. 25(1): 2-41.

[15] Gupta, V. K., and Utkhede, R. S. 1986. Factors Affecting the Production of Antifungal Compounds by Enterobacter Aerogenes and Bacillus subtilis, Antagonists of Phytophthora cactorum. *Journal of Phytopathology*. 117: 9-16.

[16] Chan, K. Y., S. H. Wong, and C. Y. Mak. 1979. Effects of Bottom Sediments on the Survival of Enterobacter Aerogenes in Seawater. *Marine Pollution Bulletin*. 10: 205-210.