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Isolation and characterization of luminescent bacterium for sludge biodegradation

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Abstract

Microtox is based on the inhibition of luminescence of the bacterium *Vibrio fischeri* by the toxicants. This technique has been accepted by the USEPA (United States Environmental Protection Agency) as a biomonitoring tool for remediation of toxicants such as hydrocarbon sludge. In the present study, a luminescent bacterium was isolated from yellow striped scad (*Selaroides leptolepis*) and was tentatively identified as Vibrio sp. isolate MZ. This aerobic isolate showed high luminescence activity in a broad range of temperature from 25 to 35 °C. In addition, optimal conditions for high bioluminescence activity in range of pH 7.5 to 8.5 and 10 gl^{-1} of sodium chloride, 10 gl^{-1} of peptone and 10 gl^{-1} of sucrose as carbon source. Bench scale biodegradation 1% sludge (w/v) was set up and degradation was determined using gas chromatography with flame ionised detector (GC-FID). In this study, *Rhodococcus* sp. strain AQ5NOL2 was used to degrade the sludge. Based on the preliminary results obtained, Vibrio sp. isolate MZ was able to monitor the biodegradation of sludge. Therefore, Vibrio sp. isolate MZ has the potential to be used as a biomonitoring agent for biomonitoring of sludge biodegradation particularly in the tropical ranged environment.

Key words

Bioassay, Biodegradation, Isolation, Sludge, Tropics, *Vibrio* sp.

Introduction

Of all the contents of oil, oily sludge is most difficult to biodegrade. Although there are numerous research showing successful bioremediation based on the high biodegradation effectiveness for oil-polluted soils, these systems also have some limitations especially in degrading oily sludge (Ouyang *et al.,* 2005). Oily sludge is the most recalcitrant pollutant. In order to assess the effectiveness of biodegradation of sludge, luminescent bacteria bioassay such as Microtox can be applied as a monitoring tool (Girotti *et al.,* 2011). However, *V. fischeri* in Microtox assay needs exactly 15 °C in order to work optimally (Warne *et al.,* 1999; Johnson 2005). Other commercially available assays such as ToxAlert™ and Biotox™ can only be used in between 15 and 25 °C (Dewhurst *et al.,* 2002) and for use in tropical climate, a refrigerated water bath is thus required. This is not realistic, costly to maintain, and instrument-dependent for biomonitoring study especially in tropics (Girotti *et al.,* 2002). Malaysia as one of the tropical countries experiences a wide range of daily temperature between 26 to 34 °C (Lee and Pradhan, 2007). Thus, a bacterium having tropical-ranged optimal luminescence characteristics could be used as a biomonitoring agent without the need of a refrigerated system. In the present study, *Vibrio* sp. isolate MZ isolated from yellow striped scad (*Selaroides leptolepis*) exhibited excellent luminescent activity within a broad range of temperature covering tropical range. A bench scale hydrocarbon sludge biodegradation was conducted and monitored using *Vibrio* sp. isolate MZ to demonstrate monitoring application of this bacterium without need for a refrigerated system.

Materials and Methods

Isolation, identification and characterization of bioluminescent bacteria: Four different fish namely *i*kan cencaru or torpedo scad (*Megalaspis cordyla*), ikan lidah or tounge fish (*Cynoglossus spp.*),

ikan selar kuning or yellow striped scad (*Selaroides leptolepis*) and ikan kembong or Indian mackerel (*Rastrelliger spp.*) were brought from Parit Jawa Muar Market during February, 2011. They were cut into pieces and the meat was immersed into 1% NaCl solution overnight. Sea water agar containing per litre of distilled water, 10 g NaCl, 10 gpeptone, 10 g sucrose, 0.5 g yeast extract, and 20 g agar was prepared. Solution from the immersed meat fish were collected using a clean falcon tube. About 100 µl of the sample was pipetted on the sea water agar. The samples were spread thoroughly over the surface of the sea water plates using glass spreading rod. The samples were allowed to absorb into the agar (about 5 min). The plates were inverted and incubated at room temperature. The plates were examined after 24 h until the bacterial colonies were visible (Dunlap and Kita-tsukamoto, 2006). After that, 50 ml of sea water media in 200 ml conical flask was prepared and used to inoculate single colony of the bacteria. The culture was incubated for 12 h at room temperature (28±1 °C) on 150 rpm rotary shaker.

Micromorphological and macromorphological features of *Vibrio* sp. isolate MZ were characterised and identified by observation on sea water agar, gram staining, and followed by 16S rRNA gene analysis. The genomic DNA was extracted using DNeasy® Blood and Tissue Kit (Qiagen, USA) according to manufacturer's instructions. The 16S rRNA gene was amplified by using genomic DNA and universal primers. The primers were synthesised by 1st BASE Sdn. Bhd. (Malaysia). The sequences of primers used were 8FPL 5'-AGT TTG ATC CTG GCT CAG -3' and primer 1492 5'-GGT TAC CTT GTT ACG ACT T -3' (Turenne *et al.* 2001). The mixture of polymerase chain was composed of 2.5 µl PCR buffer (10), 2 µl $MgCl_2$ (25 mM), 0.7 µl dNTP (10 µM) each primer solutions, 1 µl Taq Polymerase (Qiagen, USA) and 1 µl of template DNA. DNA was amplified using T-Gradient thermal cycler. After an initial denaturation at 94 °C for 4 min, the DNA was amplified during 30 cycles of 94 °C for 1 min, 58 °C for 2 min, and followed by a final extension at 72 °C for 10 min (Sambrook *et al*., 1989).The amplicons were purified using Wizard SV Gel and PCR Clean-up System following the manufacturer's instruction. The purified PCR product was then sent for the sequencing at 1st BASE Sdn. Bhd. (Malaysia). The resulting bases were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis: 20 multiple alignment of 16S rRNA gene sequences were retrieved from GenBank and aligned using ClustalW (Larkin *et al.,* 2007) through the PHYLIP output option. These gene sequences closely matched isolate MZ. To check any apparent misalignment, careful observation was done. Alignment positions with gaps were omitted from the list. By using PHYLIP, version 3.573 (J. Q. Felsenstein, PHYLIP—phylogeny inference package, version 3.573, [http://evolution.genetics.washington.edu/phylip.html]), a phylogenetic tree was created with *Rhodococcus ruber* strain AM 6as outgroup in cladogram. Then, the neighbour-joining method was used to infer the phylogenetic tree (Saitou and Nei, 1987). {Saitou, 1987 #29;Saitou, 1987 #41}Confidence levels for individual branches in tree was checked for each algorithm. The method was then analysed usingthe PHYLIP analysis with 1,000 bootstraps (Felsenstein, 1985) by the SEQBOOT program in the PHYLIP package. Ml methods using the CONSENSE program, which is a family of consensus tree methods, was used to find the topologies. From the topologies found, majority rule (50%) consensus trees were constructed and using Tree View (Page, 1996) the constructed tree was viewed.

Measurement of luminescence: Beckman Counter DTX 800 multimode detector was used to measure luminescence and reported as relative light unit. Samples were measured in DTX 96-well microplate. 200 µl of samples was collected in each well and the readings were taken in triplicates.

Statistical analysis: GraphPad Prism Software (Version 5) was used to analyse the data. This software was retrieved from www.graphpad.com. In characterisation study, values shown were mean ± SD. Student's t-test or a one-way analysis of variance with post-hoc analysis by Tukey's test was used to evaluate the statistical significance between groups. $P<0.05$ was considered statistically significant.

Biomonitoring of sludge biodegradation: 50 ml of sea water media was inoculated with 10% (v/v) bacterial culture. Then, the culture was put on a rotary shaker at 150 rpm. After 4 hr, bacterial culture was harvested. The incubation process was done at 30 °C. After that, 15 ml of bacterial culture was centrifuged at 10000 g for 10 min and the pellet was resuspended in 15 ml of a 1% NaCl (w/v) with 50 mM Tris-HCl (pH 8.4) immediately prior to toxicity testing. After that bacterial suspension was accustomed to approximately relative light unit of one million using saline solution. In the present study, one plus minus one million relative light unit was the chosen optimal light level for toxicity test. Right after the optimal relative light unit was obtained, bacterial cells were added to 96-well white plate. After 5 min of acclimatisation in plates, bioluminescence was measured at 1000 nm using DTX 880 Multimode Detector (Beckman Coulter).

Prior to this, 1% of sludge was degraded by *Rhodococcus* sp. strain AQ5NOL2 (FJ160278). The biodegraded samples were collected at 0 and 15 days. 5 ml of each sample was mixed with equal amount of hexane. This mixture was air-dried in fume hood for a day. After that, 5 ml of 1% ethanol solution was added to the dried sample. This method was crucial to dissolve the remaining petroleum hydrocarbon. Then, this mixture was tested on the luminescent bacterium in order to check the toxicity of the biodegraded samples. The assay was conducted by mixing 20 µl of samples with 180 µl of bacterial cultures. Toxicity assay was done at room temperature (28 °C ± 1 °C). The analyses were carried continuously by scanning light emission on plate for 5, 15 and 30 min. For further analysis, the extracted residual petroleum hydrocarbon was put into vial by a gas chromatograph (GC model

Agilent Technologies 7890A), which was equipped with a capillary column (TC-1, 30 m × 320 µm × 0.25 µm; film thickness = 0.1 µm) and flame ionisation detector (FID) (JandW Scientific). All the experiments were performed in triplicate. This GC method was following method of Hasanuzzaman *et al.* (2006).

Results and Discussion

About 50 isolates were able to grow on sea water agar at room temperature (28±1 °C) and 10 isolates of single colony were chosen for brightest production of light and then they were cultured in sea water broth. Isolate from yellow striped scad was chosen because this isolate produced highest relative light unit when light production was observed using DTX800 multimode detector (Beckman Coulter) after incubation for 4 hours at 30 °C on a shaker at 150 rpm in sea water media. Isolate MZ was found to be Gram-negative and rod-shaped bacterium. Through 16S rRNA gene sequence analysis, a moderate bootstrap value (60%) link isolate MZ to *V. harveyi* strain S-16, indicating a low phylogenetic relationship (Fig. 1). This bacterium grouped with *Vibrio* species was assigned tentatively as *Vibrio* sp. isolate MZ with accession number of KC247829. This isolate, produced highest relative light unit and was chosen to be utilized in this bioassay. A number of luminescent bacteria have been isolated from marine environment and they were utilize in bioassay like *V. fisheri* V- 9579 and *V. fisheri* V- 9580 (Tsybulskii and Sazykina, 2010), *Photobacterium* sp. LuB-1(Hong *et al.,* 2010) and *Photobacterium phosphoreum* 1883 IBSO (Girotti *et al.,* 2008). However (Lin and Meighen, 2009) report that *Photohabdus luminescens* was isolated from terrestrial environment.

Nine carbon sources were tested and only two carbon sources namely sucrose and starch gave the highest relative light unit(Fig. 2a). There was no significant different ($p>0.05$) between these two sources; however, sucrose was selected as carbon source for luminescent bacteria since relative light unit resulted from sucrose was higher than starch. Sucrose was chosen as a carbon source for luminescent bacterium in the present study. Relative light unit increased linearly when the concentration of sucrose was increased. Sucrose at 10g gl^{-1} was optimum (Data not shown).

Referring to Fig. 2b, only peptone showed a huge potential to be nitrogen source for luminescent bacterium. Other nitrogen sources gave low luminescence. In the present study, the luminescent bacterium preferred peptone than other nitrogen sources. As reported by Dunlap and Kita-tsukamoto (2006), a commonly complete medium for luminescent bacteria is seawater, prepared with natural seawater diluted to 70 or 75% with distilled water to minimise precipitation, 5 gl^{-1} of tryptone or peptone, 3 gl^{-1} of yeast extract, and 3 ml^{-1} of glycerol, and with 1.5 gl^{-1} of agar for solid medium. Therefore, either peptone or tryptone can be used as nitrogen and energy source. However, in the present study, the luminescent bacterium *Vibrio* sp. isolate MZ preferred peptone than tryptone. By using tryptone, relative light unit emitted by bacteria was low and significantly different ($p>0.05$) in contrast with peptone. Bacteria used in other studies like *V. harveyi* strain 525 (Mariscal *et al.,* 2003), *V. fischeri* strain 4172 (Peinado, 2002) and *Photobacterium* sp. Lub-1 (Hong *et al.,* 2010) also used peptone as their nitrogen source. Besides, *P. fluorescens* strain Shk1 (Kelly *et al.,* 2004) used nutrient broth as their medium and the bacteria utilised peptone as their nitrogen source. From nitrogen sources profile, peptone was chosen for further characterisation. The effect of different concentrations of peptone on luminescent bacterial growth and luminescence were tested. Based on the data obtained (Fig. 2c), relative light unit increased according to the peptone concentration. However, 10 gl^{-1} peptone was selected in the present study because the highest concentration of peptone used in other study was 10 gl^{-1}. Other workers reported optimal peptone for maximum luminescence at 2 gl^{-1} peptone (% w/v) (Paton *et al.,* 2009), 5 gl^{-1} peptone or tryptone (% w/v) (Dunlap and Kita-tsukamoto, 2006), 3 gl^{-1} peptone (% w/v) (Bolelli *et al.,* 2006), 5 gl^{-1} peptone (% w/v) (Girotti *et al.,* 2002) and 10 gl^{-1} peptone (% w/v) (Thomulka *et al.,* 1993).

After 4 hr of incubation at room temperature, NaCl at 10 gl^{-1} supported maximum luminescence as compared to the other concentration (Fig. 2d). No growth was observed in 0% NaCl, demonstrating the prerequisite of the salt for growth. For optimum requirement for salinity, isolate MZ was able to emit highest relative light unit at 10 gl^{-1} NaCl. Beyond 10 gl^{-1} NaCl, the luminescent bacterium gave very low luminescence. In the presence of high NaCl concentration, luminescence diminished, indicating that NaCl acts as a stress factor (Nawaz and Ahmed, 2011). In contrast, Hong *et al.* (2010) report that *Photobacterium* sp. strain LuB-1 had the at ability to function 0.2 to 5% (w/v) salt concentration.

ANOVA test showed that the luminescent bacteria emitted light with highest relative light unit in phosphate buffer of pH 7.5, Tris-HCl buffer of pH 8 and 8.5 with no significant difference ($p>0.05$) between the three pH. Isolate MZ was also capable of withstanding a wide range of pH (Fig. 2e). Studies done by the Gellert (2000), Lappalainen and Broers (2004) and Kelly *et al.* (2004) reported that the luminescent bacteria grow in the medium with pH 7±0.2. As for now, there is no other study that reports on the growth of luminescent bacteria in medium pH 8.4, except for the present study. *Vibrio* sp. isolate MZ preferred slightly alkaline solution unlike other bacteria in the studies mentioned above.

Profile (Fig. 2f) shows that the relative light unit of luminescence bacteria was nearly zero at temperatures below 20 °C and higher than 40 °C. The highest relative light unit was recorded at 30 °C. Beyond 35 °C, relative light unit decreased drastically. At temperature above 35 °C, there was no relative light unit detected from the bacterium. As lux gene products does not function at this temperature (Popham and Stevens, 2006).

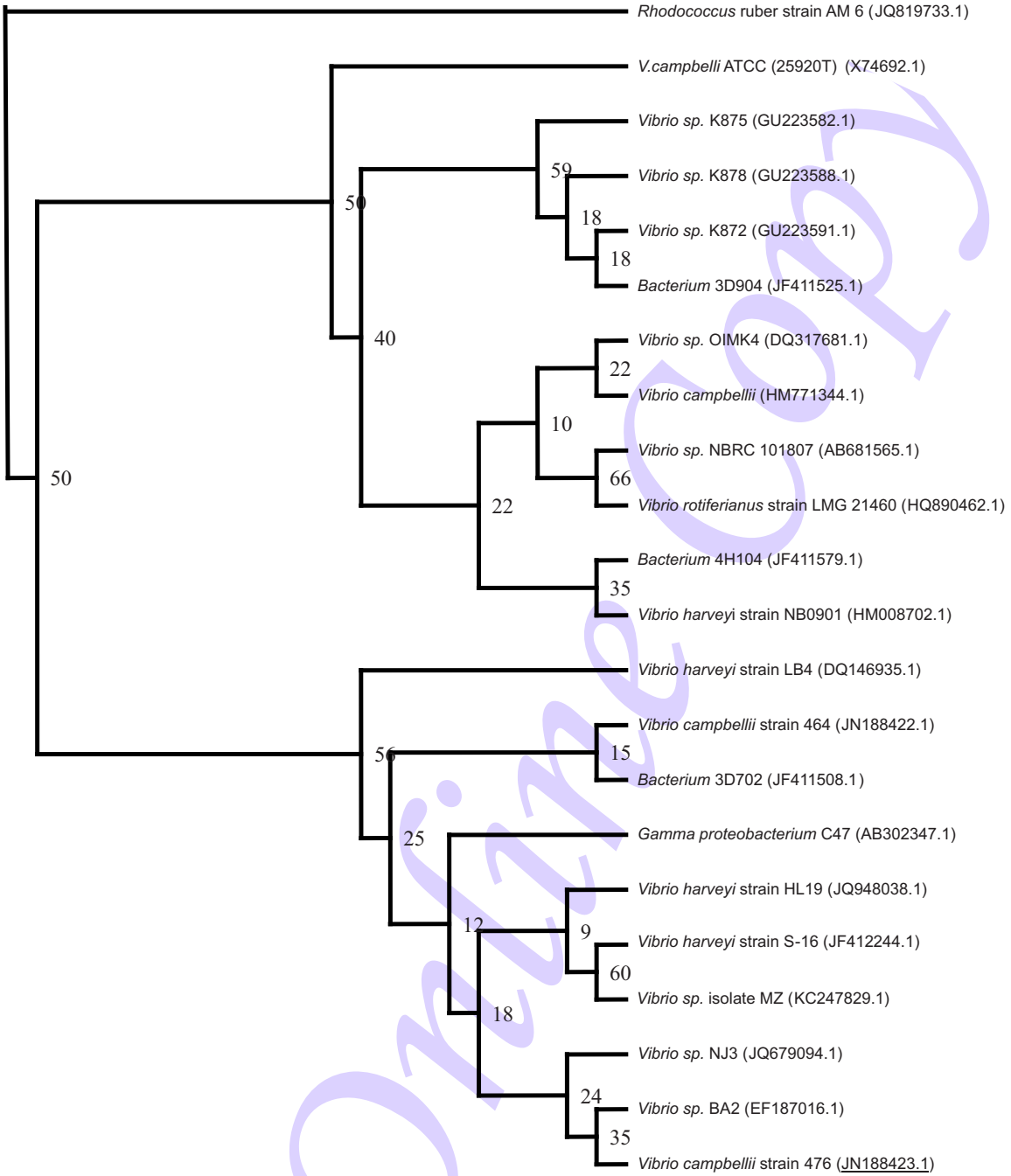

Fig. 1: Phylogram (neighbour-joining method) showing the genetic relationship between *Vibrio* sp. isolate MZ and other related references microorganisms based on the 16S rRNA gene sequence analysis from the GenBank database. *Rhodococcus* sp. is the outgroup. Species names were followed by the accession numbers of 16S rRNA gene. The internal labels at the branching points refer to bootstrap value. Scale bar represents 100 nucleotides substitution

Therefore, luminescent bacterium was able to grow at temperature ranging from 25 °C to 35 °C. The abilities to survive and emit light at these temperatures are considered as an advantage especially, for biomonitoring system in the tropics specifically in Malaysia. The present study showed that isolate MZ was able to produce significant luminescence at slightly high

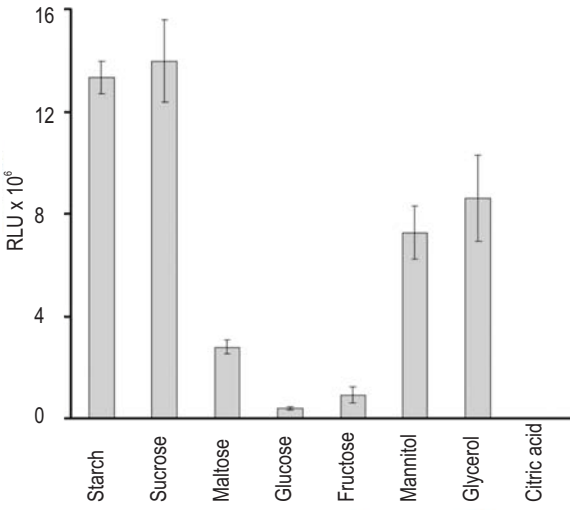

Fig. 2a: Effects of carbon sources on relative light unit production of *Vibrio* sp. isolate MZ. The cultures were grown in sea water broth and incubated for 4 hrs at 30 °C on orbital shaker (150 rpm). Relative light unit was determined by DTX800 multimode detector (Beckman Coulter). In the figure, each point represents mean of triplicate ± SE

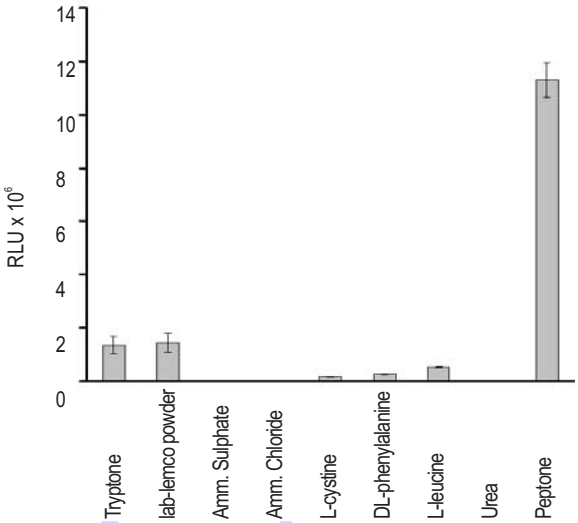

Fig. 2b: Effects of nitrogen sources on relative light unit production of *Vibrio* sp. isolate MZ. The cultures were grown in sea water broth and incubated for 4 hs at 30 °C on orbital shaker (150 rpm). Relative light unit was determined by DTX800 multimode detector (Beckman Coulter). In the figure, each point represents mean of triplicate ± SE

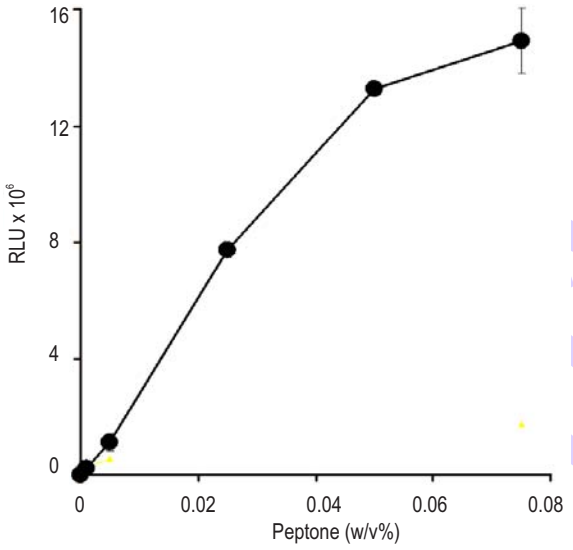

Fig. 2c: Effects of peptone on relative light unit production of *Vibrio* sp. isolate MZ. The cultures were grown in sea water broth containing different concentrations of peptone and incubated for 4 hrs at 30 °C on orbital shaker (150 rpm). Relative light unit was determined by DTX800 multimode detector (Beckman Coulter). In the figure, each point represents the mean of triplicate ± SE

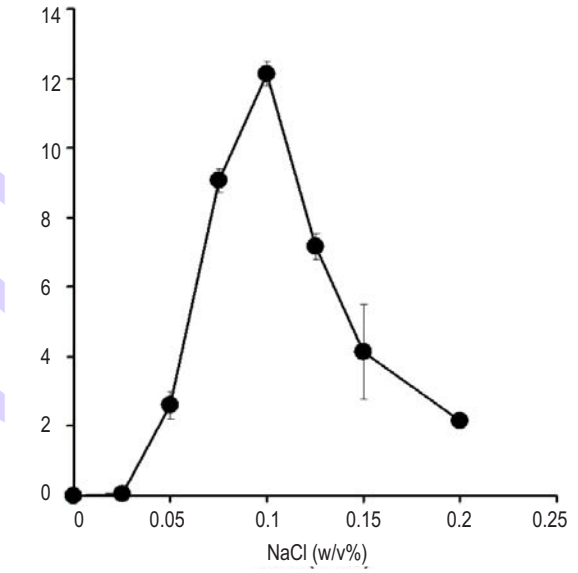

Fig. 2d: Effects of NaCl on relative light unit production of *Vibrio* sp. isolate MZ. The cultures were grown in sea water broth containing different concentrations of NaCl and incubated for 4 hrs at 30 °C on orbital shaker (150 rpm). Relative light unit was determined by DTX800 multimode detector (Beckman Coulter). In the figure, each point represents the mean of triplicate ± SE

temperature environment. This isolate did not need a stringent temperature conditions; thus, no thermostat was needed. Due to which, the test was simpler and more economical as compared to Microtox, which is a bioassay that uses bioluminescent bacterium (*V. fischeri*). This temperature factor is so essential and critical particularly in analysing a lot of samples at one time. In addition, tropical-range isolates are suitable in the field due to less temperature constraint like ambient in Malaysia. However, other

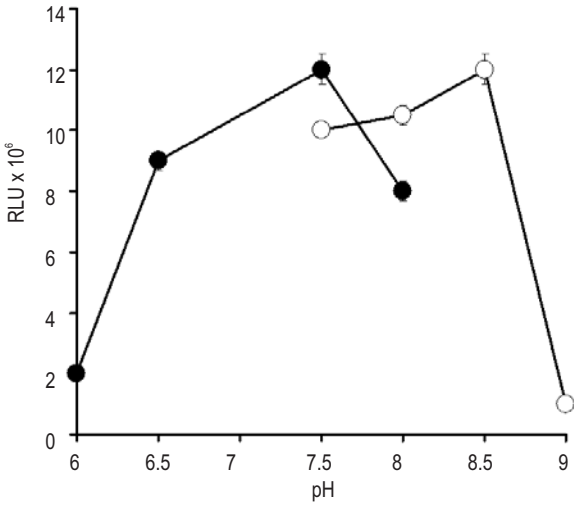

Fig. 2e: Effects of pH on relative light unit production of *Vibrio* sp. isolate MZ. The cultures were grown in sea water broth and incubated for 4 hrs at 30 °C on orbital shaker (150 rpm). Relative light unit was determined by DTX800 multimode detector (Beckman Coulter). In the figure, each point represents the mean of triplicate ± SE

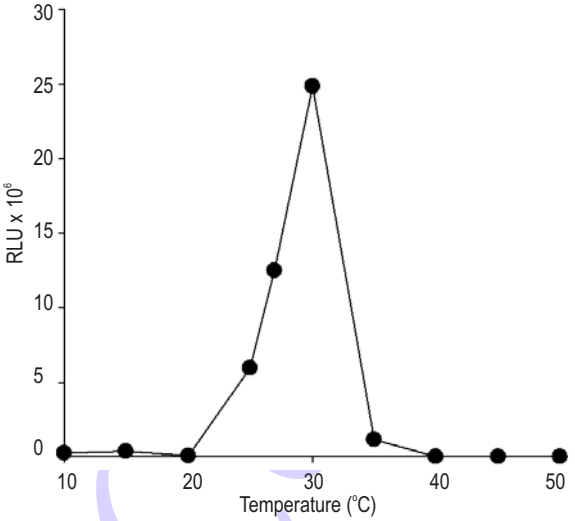

Fig. 2f: Effects of pH on relative light unit production of *Vibrio* sp. isolate MZ. The cultures were grown in sea water broth and incubated for 4 hrs at 30 °C on orbital shaker (150 rpm). Relative light unit was determined by DTX800 multimode detector (Beckman Coulter). In the figure, each point represents the mean of triplicate ± SE

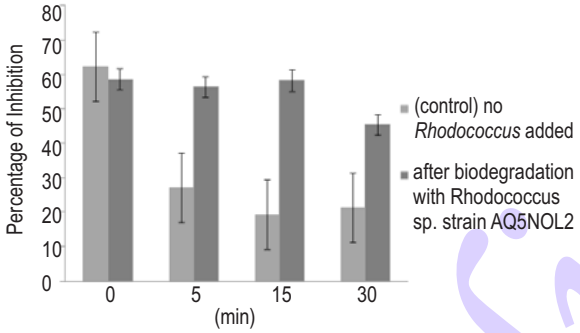

Fig. 3: Biotoxicity of the degradation sludge by-products after degradation compared to control sample (no degradation).

bioluminescent bacteria have been reported and have lower temperature for efficient bioassays than isolate MZ. Gellert (2000) and Girotti *et al.* (2002) report that *V. fischeri* (DSMZ 7151/ NRRLB-11177) and *V. logei* used in their study can only survive in the temperature of 20 °C.

In the present study, *Rhodococcus* sp. strain AQ5NOL2 was used to degrade 1% of oily sludge as genus *Rhodococcus* is considered a special group of microorganism which is able to biodegrade compounds that cannot be transformed by almost all other microbes (Solyanikova and Golovleva, 2011). Referring to Fig. 3, it was found that the sludge samples were more toxic to luminescent bacterium after degradation. However, the degraded samples were still toxic to the luminescent bacterium. This could be due to heavy metals and unknown compounds that remained in the sludge. According to Girotti *et al.* (2008), the short-chain molecules produced from remediation process are more toxic to the luminescent bacteria. As these short chain molecules and heavy metals cannot be degraded further, their toxicity persists. Successful results on biodegradation product from hydrocarbon contaminant showed less toxic response to Microtox. This condition appeared to be found in complete bioremediation studies in soils in bioaugmentation or biostimulation experiments. However, these studies showed reduction in toxicity as measured using the Microtox assay after a long period of remediation often over 6 months (Mueller *et al.,* 2003). Girotti *et al.* (2011) use *V. fischeri* (NRRL B-111777), *V. logei* and *P. phosphoreum* 1883 IBSO to monitor bioremediation of petroleum hydrocarbons and chlorinated aromatic hydrocarbons polluted soil. Subsequently, after 45 days of bioremediation toxicity values were found to be increased. Thus, the present study proved that the biodegraded product of xenobiotics can be more toxic than original compounds as reported in earlier (Milanova *et al.,* 1998). In future, remediation work should be monitored for six months or preferably for one year to give a better result and the popularity of using luminescent bacterial assay to monitor remediation should be explained (Mueller *et al.,* 2003).Based on the results obtained, *Vibrio* sp. isolate MZ was able to monitor the biodegradation of 1% sludge. Therefore, *Vibrio* sp. isolate MZ had potential to be used as a biomonitoring agent for sludge biodegradation, particularly in tropical environment.

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