Biodegradation of Wastewater Oil Pollutants, Identification and Characterization: A Case Study – Galing River, Kuantan Pahang, Malaysia

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Abstract—**This research is aims to access the potential of natural occurring bacteria for pollutant degradation. The study was carried out in Galing River, Kuantan, Pahang. There were four sampling sites that were chosen along the river and the samples were analyzed to identify specific bacteria that useful for pollution control. The identification shows that different bacteria isolates were identified, mention as a positive (+) or negative (—). Screening for lipolytic activity by using Tween 20, Rhodamine B and phenol red was done. The results revealed that, the bacterial consortium in Galing River can be utilized as biodegradation agent. Screening using Tween 20 showed two areas were positive has lipolytic activity. However, screening using Rhodamine B, exhibited that all samples showed high positive lipolytic activity. While, phenol red, was positive only for two of the sampling points. Biodegradation studies showed, the bacteria could degrade the oil after 3 days incubation.**

Index Terms—**Bacteria potential, biodegradation, galing river, identification and wastewater pollutant.**

I. Introduction

The Earth may be covered by over 70% water, but only 2% of that water is freshwater that we can consume directly. Furthermore, from this 2% of freshwater not all can be used. Slightly over 30% of the Earth's freshwater come from groundwater sources, rivers, and lakes. The rest is from ice caps and glaciers. This means the freshwater which we can easily obtain and consume only comes from 0.6% of Earth's total water. The demands for water usage in Malaysia are increasing every year and at alarming high. Malaysia's consume more than 300 liters of water per person per day compared to 150 liters per person per day by Singaporeans and water consumption per capital per day increases about 7.6 liters per year [1]. The increases in water consumption in Malaysia does not parallel with the water reserves whereas Malaysia's water reserves per capital per day is declining at a rate of 5.8 liters per year. At this rate, Malaysia would be left with nearly no water reserves by 2025.

The success in the economic growth and industrialization in Malaysia has led to environmental problems with ever increasing land, air, and water pollution [2], [3]. According to the Environmental Quality Report 2010 by the Malaysian Department of Environment (DOE), water quality in Galing River are in class IV for Big Galing and class III for small Galing. This report shows that Galing River is one of the most polluted river in Pahang state. With the increases of development along the river bank, it is hard to preserve the water quality in a safe class. Galing River that located in the heart of Kuantan city has been mounted with pollutant released by the industry, commerlised and also housing area.

The use of microorganisms to remove or reduced inorganic sulfur compounds in nature has been considered as a potential alternative [4]. Certain types of bacteria are resistance to toxicity degradation in waste water. *Bacillus sp, Clostridium sp, Staphylococcus, Mycobacterium, Escherichiacoli, Providences, Erwinia, Proteus vulgaris, Pseudomonas aeruginosa and Acinetobacter radioresistens,* are bacteria identified can endure degradation metal mercury [5]. Kanu *et al.* was reported about isolation, identification and analysis of bacteria *Pseudomonas aeruginosa sp in* waste water and the same bacteria have shown good potential for use in waste water treatment, biochemical oxygen demand (BOD) and lipid degraded after 12 days [6].

To date, no comprehensive investigation on the identification and characterization of bacteria in the river source itself in Malaysia especially in the Galing River has been carried out. The objective of the present study was to identification different bacteria isolates in river water sample and characterize it according to their abilities as a biodegradation agent for water pollutantas.

II. Study Area and Issues

A. Study Area

Fig. 1. Map showing four location sampling station (G1) Kilang jaya gading, (G2) Setali area, (G3) Galing street 29 & 33 and (G4) Vistana hotel.

The study area which is Galing River catchment is situated in Kuantan city with the catchment area of 22.7 km^2 and Area Galing River long to 7.7 km. The catchment area falls under

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the jurisdiction of the Majlis Perbandaran Kuantan (MPK), showed in Fig. 1.

B. Topography

The topography around Galing River is mostly a flat-plain area. Galing River flow downstream merging with River Kuantan before going into the South China Sea. It has several tributary rivers, namely Small Galing River and Son River Galing. Table I and Fig. 1 show the sub-catchment area that have been decided and identified in this project.

TABLE I: SUB-CATCHMENT OF GALING RIVER

No	Subcatchment
1	Semambu villages, industrial areas Semambu
2	Galing Hill/ Semambu
3	Semambu/Air Putih
4	Air Putih
5	Kuala Kuantan, Galing Hill
6	Air Putih
7	Sekilau Hill, Malay Reserve Great Space, Kg. Great leisure
8	Seri Kuantan, Kg. Across Shower
9	Protector hill, Alor Akar

III. MATERIALS AND METHODS

A. Collection of Water Samples

Water samples were collected from Galing River (Kuantan). There are four sampling stations that are G_1 (Kilang Jaya Gading, Semambu Industrial street 3/2 - N. 3°.84718'' E. 103° 33316''), G_2 (Setali area, Srisetali alley 63 - N. 3°.84409'' and E. 103° 33221''), G_3 (Galing Street 29 & 33 - N. 3°.83296'' and E. 103° 33047'') and G_4 (Vistana hotel - N. 3°.8174'' and E. 103°. 33705''). The samples were collected using sterile glass bottles with methods of *Depth Integrated Sample*. The water samples were preserved at 4 °C temperature before the commencement of the experiment.

B. Isolation and Identification of Bacterial Isolates

The preparation of media from commercial products is simple and straight forward. For agar plate's preparation, 24 g of nutrient agar (NA) in 1L distilled water, then dispensed and sterilized for 20 minutes at 121 ℃. Then the agar was poured into a petri plate, to make an agar plate nutrient. Agar pours containing 15 ml of media are used to prepare agar plates.

River water samples was taken to the laboratory and stored overnight at 4 ℃. 1 ml of each water sample was diluted from 10^{-1}, 10^{-3} and 10^{-8} dilution factor. Then, 100 µl from each dilution tube was inoculated into a prepared petri dish containing sterile nutrient agar. The plates were inverted and placed in the incubator at 37 ℃ for 24 hours.

Independent colonies that appear to be composed of only one cell type were chosen. An inoculating loop was filmed and cooled, and used to gently transfer the colony by touching, and re-streaking onto a new nutrient agar plate. The plates were then incubated at 37 ℃ for 24 hours and after accounting of colonies. The development colonies were counted in plates and average number of colonies per three plates was determined. The number of total bacterial count (CFU) per ml sample was determined. The bacterial isolates were kept on nutrient agar at 4 °C and recultured every 4 weeks.

To identify population growth in bacteria, the liquid medium was prepared with 8 grams of nutrient broth media (NB), and suspended in 1 L distilled water. The suspension is mixed toughly in an Erlenmeyer flask. Then it is dispensed and sterilized for 20 minutes at 121 ℃. Inoculums were prepared in nutrient broth by using an inoculating loop from independent colonies from pure culture. Microbial growth was monitored at regular intervals by measuring the optical density (OD) at 600 nm using several dilutions in various time and day incubation.

C. Cultivation and Morphological Characteristics of Bacteria Isolates

After the successful isolation from the samples, a number of bacteria were sub-cultured and their morphology was observed. Then the purified bacteria were subjected to Gram-staining procedure in order to classify them (Bergey's Manual Determinative Bacteriology). The morphology observation and Gram-staining results were analyzed together. The general characteristics of bacterial colonies were then described in terms of size, shape, margin, elevation, consistency, color, transparency and give an accurate description of the colonies.

D. Screening Potential of Lypolytic Bacteria as Biodegradation Oil

Tween 20 agar plates were prepared as follows; the culture medium contained peptone 1 % (w/v), NaCl (0.5 % w/v), $CaCl_2$ (0.01 % w/v), agar (2 % w/v) and tween-20 (1 % v/v) and the mixture was adjusted at pH 6.0 using 1 M NaCl. About 20 ml of the medium was poured into each petri dish and the organism inoculated. The appearance of visible clear zone was used as an indicated of lypolitic activity.

Phenol red agar plate was also used to assess the test organisms. Phenol red agar plates were prepared using phenol (0.01 % w/v) along with 1 % (v/v) olive oil, 0.1 % (w/v) $CaCl_2$, 2 % (w/v) agar and the pH adjusted to 7.3 - 7-4. About 20 ml of the medium was poured into each petri dish and the organism inoculated. Changes in color of phenol red were used as an indicator of the enzyme activity.

Agar plate containing Rhodamine B 0.001 % (w/v), nutrient broth 0.8 %, NaCl 0.4 % (w/v), agar 1 % (w/v) and olive oil 2 % was prepared in distilled water, adjusted pH of 6.5. The assay incubated at 55 °C for 18 h and lypolitic activity was identified as an orange halo around colonies under UV light at 350 nm.

E. Waste Vegetable Oil (WVO) Degradation

The preparation media stone mineral salt solution (SMSS) in 100 gr there are: $NaCO_3$ (5 gr/L), NH_4NO_3 (2.5 gr/L), $Na_2HPO_4.7H_2O$ (1 gr/L), KH_2PO_4 (0.5 gr/L), $MgSO_4.7H_2O$ (0.5 gr/L), $MnCL_2$ (0.2 gr/L), extract yeast (BVT) (0.1 gr/L) and waste vegetable oil (WVO) (2% in 100 mL media SMSS). The media was shaker in 2000 ppm until four days at temperature 40 ℃.

Every media SMSS will added the bacteria was prepared in incubator shaker for 12 h, and the varieted the solution on 500, 750, and 1000 µL than centrifuges.

Variation of bacteria in any media that has been in incubation SMSS then enter in conical flash and shaker for 4

days, and in the lid using a light-tight aluminum foil in order. then will be tested every day optical density with a wavelength of 600 nm and the obtained values SMSs degradation bacteria in media with variations of bacteria and waste vegetable oil.

IV. Results and Discusion

A. Identification and Quantifying of Bacterial Consortium

Quantity study of bacteria isolated from Galing River by using the plate count method. The series dilutions plated in order to achieve the desired range of colonies per plate (30 – 300). The total bacterial count (CFU/ml river water) in site G_1 fluctuated from 0.21×10^{-2} to 5.3×10^{-8}, G_2 fluctuated from 1.1×10^{-2} to 22.3×10^{-8}, G_3 fluctuated from 2.37×10^{-2} to 19.8×10^{-8} and G_4 fluctuated from 0.17×10^{-2} to 0.1×10^{-8}. These results indicated the number colonies of the samples was various within each dilution, the lowest count was at site G_4 and the number of colonies became higher at G_2, and G_3 sites, respectively (Table II). The total colonies count value was different between both four sites of sampling area was indicated the content of bacteria in each area sampling is influenced by the pollutant [7].

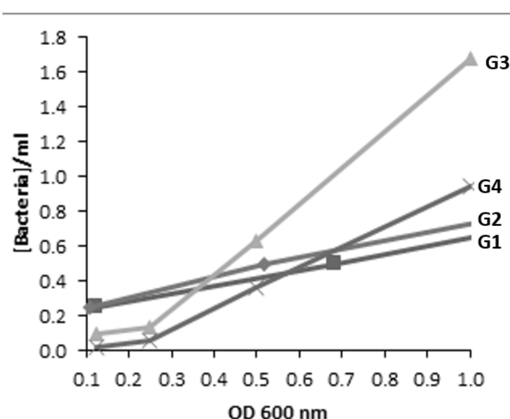

Fig. 2. Optical density absorbency 600 nm of bacteria at four sampling area (G_1, G_2, G_3 and G_4).

TABLE II: Bacterial Population with Dilution Factor in Agar Plate Medium at Four Sites Sampling

Dilution factor	CFU/ml			
	G_1	G_2	G_3	G_4
10^{-1}	0.2×10^{-2}	1.1×10^{-2}	2.37×10^{-2}	0.17×10^{-2}
10^{-3}	6×10^{-4}	202×10^{-4}	16.2×10^{-4}	1×10^{-4}
10^{-8}	5.3×10^{-8}	22.4×10^{-8}	19.8×10^{-8}	0.17×10^{-8}

TABLE III: Identifications Strain in Four Samples River Water

No	Strain	Location Sampling				Characteristic	
		G_1	G_2	G_3	G_4	Gram-Stain	Shape
1	S1	+	+	+	+	Positive	Clustered Cocci
2	S2	+	0	+	+	Positive	Bacilli/ Rod
3	S3	+	+	+	+	Negative	Bacilli/ Rod
4	S4	+	+	0	0	Negative	Bacilli/ Rod
5	S5	+	+	0	+	Positive	Bacilli/ Rod
6	S6	+	+	+	+	Negative	Bacilli/ Rod
7	S7	+	+	+	0	Positive	Bacilli/ Rod

Note: (+) Detection, (0) Not Detection

Population growth in bacteria is increase in the quantity of cells and is depend upon the availability of nutrients in the environment. Turbidity measurement or optical density of the cell suspensions was related to cell number. Fig. 2 shows the variation of optical density in each sample was measured at wavelength 600 nm. The results show the optical density of samples has correlation with the number colonies per unit (CFU). The turbidity each various samples were lowest in G_4 and the turbidity became higher at sites G_1, G_2, and G_3, respectively.

B. Morphological Characteristics of Bacterial Isolates

The Gram reaction screening of the isolated sample was carried out. The isolates were identified with seven bacteria namely *S*1, *S*2, *S*3, *S*4, *S*5, *S*6 and *S*7 (Tabel III). Based on the Table III, all strains can be found in sampling point G_1 and for sampling point G_2 only *S*2 is not detected from the list. In point G_3 two strains of bacteria are not detected that is *S4* and *S*5, while in point G_4 there two strains are not identified that is *S*4 and *S*7. Sampling point G_1 that located in the area of industrial which also situated the poultry factory which flushes out their untreated waste water directly to the river. This may contribute to the high strain factor in that sampling point. The picture of Gram straining was shown in the Fig. 3 a-f, the different color and morphology.

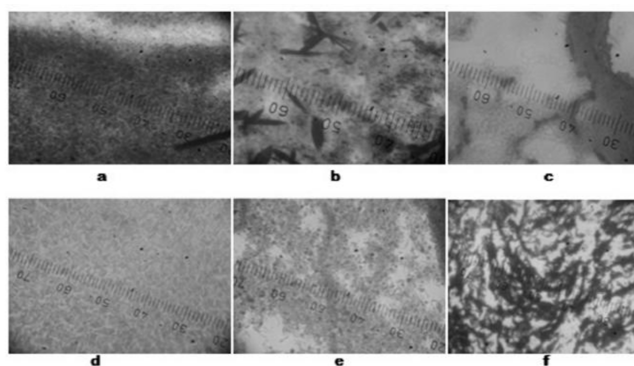

Fig. 3. The different color and morphology of bacteria from sample water a *S*1, b *S*2., *S*5, c *S*3, d *S*4, e *S*6 and f *S*7.

C. Screening Potential of Lipolytic Bacteria as Biodegradation Oil

The qualitative methods have been widely used for the screening potential of the lipolytic activity. In this study, samples were screened using Tween 20 agar plate, Rhodamine B and phenol red with in 3 days incubation, the microorganism showed lipolytic activity. Screening using Tween 20 agar plates showed precipitated around the organism. From the result in Table IV, there are two areas was positive has lipolytic activity (B and C). Agar plates containing olive oil and Rhodamine B appear opaque and are pink colored. After incubation sample was monitored by irradiating plate with UV light at 350 nm. Microbial colonies began to show an orange fluorescence and continuing incubation time orange fluorescent halos were formed around the colonies. From the result in Table III, was shown that all of sample area was positive has lipolytic activity.

Phenol red has an end point at pH 7.3 - 7.4, where it is red and a slight decrease in pH (7 - 7.1) turns it to yellow. The yellow zone around culture confirmed lipolytic activity. From the result in Table IV, showed two sample areas (G_1

and G_2) exhibited positive lipolytic activity. Fig. 4 shows the lipolytic activity of the sample indicated the bacteria population in the sample as biodegradation agent.

TABLE IV: SCREENING OF BACTERIA USING TWEEN 20 AGAR PLATE RHODAMINE B AND PHENOL RED IN DEGRADATION LIPID

Media agar	G_1	G_2	G_3	G_4
Tween 20	-	+	+	-
Rhodamine B	++	++	+	+
Phenol Red	++	++	-	-

Note: (++) Major, (+) Moderate, (-) Minor

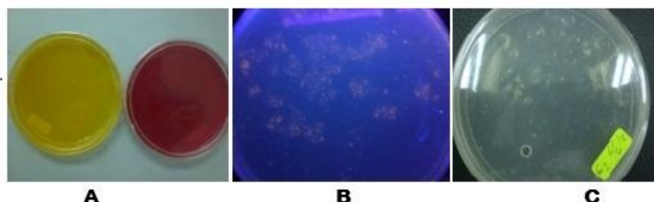

Fig. 4. A Phenol Red, B Rhodamin B and CTween 20.

D. Waste Vegetable Oil (WVO) Degradation Studies

The experiment was performed to study the biodegradation of waste oil by bacteria from the water Galing river. Inoculate in nutrient broth was prepared to increase cell biomass and then mixed and used as a consortium. The cultures that were incubated for 4 days showed a high degree of degradation more than those (cultures) at 1, 2, and 3 days. The relationship between degradation rate of WVO, bacterial growth and incubation time is an important consideration when evaluating the efficacy of oil degrading process. As shown in Fig. 5, the cell density was relatively low on the first day then increased markedly from the second day to the third day. In the following three days cell density remains relatively constant and decreased in the fourth day. The degradation rate was only a few percent on the first day, and increased linearly in the next days. The growth curve indicated that the bacteria were in the adaptation phase on the first day when bacteria could not grow and reproduce immediately. The bacteria would take a period of time to grow in the new culture medium. The second day and the third day was the logarithmic growth phase, when bacterial metabolism was dynamic and synthesis of new cellular material was fast. Bacteria entered into the stationary phase from the fourth day.

V. CONCLUSION

From the identification and characterization of bacteria in Galing River based on observations using gram staining, and use the microscope and is supported by the study of literature is based on the drawings it was found that the bacteria identified from four different sampling points included Strain *S*1, *S*2, *S*5 and *S*7 was positive strain and *S*3, *S*4 and *S*6 was negative strain, for the specific of the strain is still in progress. However, screening using Tween 20, Rhodamine B, phenol red, and biodegradable studies indicated the bacteria population has potential as biodegrdation agent. Utilization of bacteria potential in Galing River as biodegradation agent in wastewater treatment is an excellent idea, which not only reduce the cost but also the environmental friendly process.

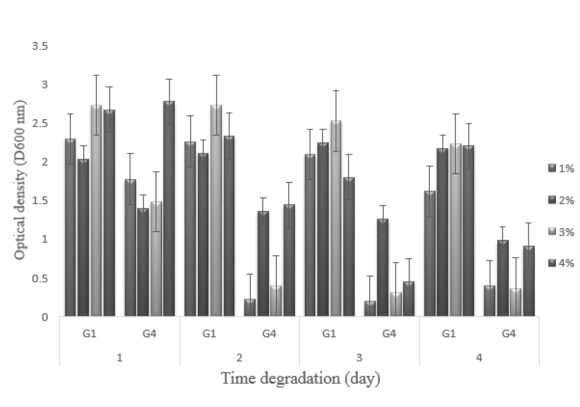

Fig. 5. The degradation Waste Vegetable Oil (WVO) based differences bacteria presentations and time per days.

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