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Research Article

Rhamnolipid production by *Pseudomonas aeruginosa* UMTKB-5 using bio-oil derived from microwave co-pyrolysis of hospital plastic wastes and its characterization

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Abstract
Rhamnolipid (RL) is a bacteria-derived biosurfactant known for its eco-friendly properties, but it is expensive to produce due to high costs required for pure substrate-based carbon sources. Recently, microwave co-pyrolysis (MCP) for converting wastes to value-added products has been the spotlight. The MCP of hospital plastic wastes (HPW) and used cooking oil (UCO) can produce alkane-rich bio-oil with potential to be a carbon source for microbial transformation. Therefore, this study investigated the best parameters for RL production by *Pseudomonas aeruginosa* UMTKB-5 using bio-oil and the characterisation of the RL. MCP of HPW and UCO showed higher heating rate (10-17 °C/min) generating alkane-rich bio-oils characterised using Gas Chromatography-Mass Spectrometry (GCMS). 1g/L of U1H2, urea, 7% (v/v) inoculum size and 200 rpm agitation speed were the best parameters (producing up to 5.000±0.793 mg/L RL). The RL was characterised using Fourier Transform Infrared Spectroscopy (FTIR) and Ultra Performance Liquid Chromatography (UPLC). It comprises of RL functional groups and congeners, Rha-C_{10}-C_{10} and RhaRha-C_{10}-C_{10}. The RL produced by the strain has more hydrophilicity making it a suitable candidate for bioremediation and washing detergents. Hence, this study proves that MCP of HPW and UCO derived bio-oil is a suitable carbon source for RL biosynthesis.

Keywords: Rhamnolipid, *Pseudomonas aeruginosa*, microwave co-pyrolysis, bio-oil, hospital plastic waste

Introduction

Rhamnolipid (RL) is a microbial biosurfactant and is one of the most extensively studied glycolipid biosurfactant. RL is an amphiphilic molecule where the hydrophilic head is made of one or two molecules of rhamnose sugar while the hydrophobic part is comprised of hydroxy fatty acid chains [1]. There are two types of RL namely mono-RL and di-RL where the former has one rhamnose sugar while the latter has two [2]. The difference in rhamnose sugars gives rise to various characteristics such as antimicrobial ability [3], emulsification, critical micelle concentration (CMC) and cleaning efficiency [4] [5]. RL is commonly biosynthesised by *Pseudomonas aeruginosa,* a robust strain that is known to utilise oily substrates rich in alkane and fatty acids for RL production [6] [8]. RL is used in pharmaceutical and medical applications because it exhibits antitumor,

antiviral and antimicrobial activity [9]. If RL is so great, why is chemical surfactant still the preferred choice? The reason lies behind the expensive production costs of RL as well as in the difficulty of producing it in large quantities [10]. Production of RL requires state-of-the-art technology, complicated upstream and downstream processing, and purification process. According to Kee et al. [11], the feedstock for bacteria fermentation was estimated to account for 50% of the total production cost, hence researchers have been looking for cheaper and sustainable ways to lower the cost of RL production.

Recently, microwave co-pyrolysis (MCP) has been gaining traction as the go-to method to recycle wastes. MCP is a process of decomposing two or more materials in an inert environment using microwaves [12]. It generates three products which are bio-oil, syngas and char. These products are known as value-added products which can be utilised several ways [13]. Bio-oil, one of the products of MCP is considered renewable energy. This attracted much attention as the world is shifting towards greener alternatives to reduce the carbon footprints. As of now, bio-oils are widely used as fuel for combustion, binding agent to make pellets and briquettes [14]. Bio-oils are notorious for the instability during storage and transport, aging, oxidation, constant reactions in the bio-oil, and acidity which makes it a less suitable candidate for applications in furnaces, boilers, engines and more [14]. Recently, scientists have discovered that MCP has a potential to pyrolyse the plastics to produce value-added products. Mahari et al. [15] recently reported that MCP of hospital plastic wastes (HPW) and used cooking oil (UCO) can produce bio-oil that was suitable as bacterial feedstocks. HPW is one of the most severe types of plastic wastes due to their biohazardous nature. Normally these HPW were incinerated or landfilled which is both cheap and environmentally deteriorating. UCO are usually collected by private ventures and sold to companies that manufactures biodiesel and soaps using UCO.

Hence, this study aims to investigate the feasibility of bio-oil derived from microwave co-pyrolysis as a carbon source for RL biosynthesis by *P. aeruginosa* UMTKB-5 and determine the optimum parameters for RL production.

Materials and Methods

Preparation of feedstock materials

This method was adapted from Mahari et al. [15] with slight modifications. The feedstocks used in this study were used cooking oil (UCO) and HPW. The UCO was collected from household usage after chicken and potato-based materials twice and sieved to remove solid particles. HPW was provided by Penang KPJ Healthcare, Malaysia. The donated HPW comprise of only intravenous fluids drip bottles made of low-density polyethylene (LPDE). The HPW in this study was not contaminated with blood and other bodily fluids of patients. As a precautionary measure, the HPW cleaned with disinfectant and Decon90. The HPW was then rinsed with water and dried in an oven for 2 hours at 30-40 °C to eliminate the water content. The HPW was then cut into small pieces (3-4 mm^2) in order to fit in the insertion of the reactor.

Production of bio-oil via microwave co-pyrolysis

The microwave co-pyrolysis system was set up according to Mahari et al. [16] with slight modifications. The production of bio-oil was performed under N_2 environment with a flow rate of 0.5-1.0 l/min. Due to the low microwave absorbing capability of the feedstocks, activated carbon was used to facilitate the absorption of microwaves. Approximately 100 g of activated carbon was added into the reactor. A varying amount of HPW and UCO with a combined weight of 100 g were added into the reactor to achieve a total weight of 200 g. This was to produce UCO/HPW bio-oils with ratios of 1:1, 1:2 and 2:1. These bio-oils will be known as U1H1, U1H2 and U2H1, respectively. As a control, HPW and UCO were individually pyrolysed. The microwave power was fixed at 700 W. The pyrolysis ended when no volatiles were emitting from the microwave. The yield of the bio-oil was calculated using Eq. 1.

$$Yield\ of\ bio-oil\ (wt.\%) = \frac{Weight\ of\ bio-oil\ (g)}{Weight\ of\ feedstock\ (g)} * 100\% \quad (1)$$

Characterisation of bio-oil

Elemental analysis of the bio-oil was executed using a Elementar Unicube elemental analyser to determine the carbon, hydrogen, nitrogen and sulphur composition in the bio-oil. The chemical composition of the bio-oils was determined using gas chromatography-mass spectrometry (GCMS). 1 ppm of bio-oil was dissolved in GCMS grade Supelco SupraSolv® n-Hexane. The analysis of the bio-oil was performed using a Shimadzu GCMS-2010QP Ultra equipped with GC-2010 Gas Chromatograph with AOC-20i Auto Injector and AOC-20s Auto Sampler and SH-Rtx-5 (Crossbond 5% diphenyl / 95% dimethyl polysiloxane) (30 m x 0.25 mm x 0.25 µm) fused silica column. The mobile phase used was helium gas. The parameters for GCMS operation adapted from Mahari et al. [15]. The concentration of each compound was calculated as a percentage of the total area of all the peaks in the analysis.

Bacterial strain isolation and preservation

Pseudomonas aeruginosa UMTKB-5 was used in this study. The strain was previously isolated from the marine sediments collected from Pulau Bidong, Terengganu, Malaysia [17]. The partial 16s rRNA gene sequence of the *P. aeruginosa* UMTKB-5 has been deposited in the GenBank database at the National Centre for Biotechnology Information (NCBI) (Accession number: KT194193.1) [18]. The strain was acclimatized in nutrient rich (NR) broth (per liter distilled water: 10 g peptone, 10 g HM extract, 2g yeast extract and if agar, 14 g bacteriological agar powder) with 1 g/L of bio-oil for 10 cycles (each cycle for 24 hours). For long term storage, the strain was preserved in 40% (v/v) glycerol at -80 °C. For maintenance purposes, the strain was streaked on bio-oil smothered NR agar incubated at 30 °C for 24 hours and stored in 4 °C.

One-factor-at-a-time optimization

This step was adapted from Azran et al. [17] and Azemi et al. [18] with slight modifications. *P. aeruginosa* UMTKB-5 was activated in 50 mL NR broth and cultivated for 7 hours at 30 °C at 200 rpm. After 7 hours, 1 mL of the culture was inoculated into fresh 100 mL NR broth for preculture cultivated at 30 °C at 200 rpm for 16 hours. To determine the best combination of carbon and nitrogen sources, the cultivation of RL was done in 50 mL mineral salts medium (MSM), per liter distilled water: 2.80 g KH_2PO_4, 3.30 g Na_2HPO_4, 0.25 g nitrogen source (urea, ammonium chloride, and ammonium suphate), 2.00 g bio-oil, 1mL trace elements and 1.00 mL of 0.001 M $MgSO_4.7H_2O$ using 250 mL conical flasks. Approximately 7% of preculture was inoculated into the MSM (n = 6). The culture was incubated for 72 hours at 30 ºC, 200 rpm in *Ecotron CH-4103* (INFORS HT, Switzerland) incubator shaker. For bio-oil concentration determination, 1 g/L, 2 g/L, 3 g/L and 4 g/L were used. For inoculum sizes, 5%, 7% and 10% (v/v) were used. For agitation speed, 150 rpm, 200 rpm and 250 rpm were used. The parameters that record the highest RL concentration was selected to be used in the subsequent tests.

Residual bio-oil determination

The residual bio-oil was analysed to determine the composition of the bio-oil that was utilised by *P. aeruginosa* UMTKB-5. The extraction of residual bio-oil was adapted from Shi et al. [19] with modification. Approximately 25 mL of n-hexane was added into the culture and mixed vigorously for 5 min. The top n-hexane layer was carefully isolated into a beaker and was allowed to dry overnight leaving behind the residual bio-oil. The residual bio-oil was then prepared and characterised using GCMS [15]. The composition of the residual bio-oil was then compared with the stock bio-oil.

Biomass determination

This step was done at 72nd hour to determine the biomass of the bacteria. At 72nd hour, approximately 1 mL of culture was pipetted into a pre-weighed 1.5 mL microcentrifuge tube and spun down at 9000 rpm, 4°C for 5 minutes using Eppendorf 5810R Refrigerated Centrifuge. The cell pellet was washed with 1 mL hexane by resuspension using IKA Vortex 3. The cell pellet was centrifuged again at the same parameters. This step was done to remove excess oil on the cell pellet [20]. The cell pellet was washed twice by resuspension with distilled water. The cell pellet was centrifuged at the same parameters, supernatant was discarded and stored in Eppendorf U410 deep freezer at -80ºC overnight before freeze-drying in Labconco Freezone 4.5 Freeze Dryer (Labconco, USA) at -35ºC. The dried cell was weighed using Sartorius M-Pact AX224 Analytical Balance [18].

Rhamnolipid quantification

Quantification of RL concentration in supernatant was done using orcinol assay. The orcinol reagent, per 100 mL, consist of 53% concentrated sulphuric acid and 0.19% w/v orcinol powder in distilled water [18]. This method was adapted from Ballot [21] with slight modifications. Approximately 400 µL of supernatant was added with 750 µL diethyl ether and vortexed. The RL containing upper fraction was isolated. This process was repeated twice. The organic fraction was be left in a fume hood to dry completely. A 400 µL of pH 8 phosphate buffer was added to re-dissolve the RL precipitate. Approximately 900 µL of 0.19 % orcinol reagent and 100 µL of samples were mixed and placed water bath for 30 minutes at 80ºC. The samples were then left to cool to room temperature in a dark environment. The samples were analysed using SpectraMax ID5 Multi-Mode Microplate Reader at 421 nm. The rhamnose concentration was calculated by inserting the absorbance into the equation determined using the standard curve prepared with L-rhamnose. The concentration was then multiplied with a correction factor of 2.25 to compensate for the extra mass.

Extraction of RL

This part of the experiment was to produce crude RL for characterisation purposes. The 5 L conical flasks were used in this experiment. The culture parameters were according to the results obtained from the above experiments. The culture was centrifuged to remove the cells and vacuum filtered with PTFE hydrophilic filter paper to remove the bio-oil. The cell-free supernatant was then subjected to solvent extraction [22]. The supernatant was mixed with methanol:acetone:chloroform, 1:1:1 by volume and agitated at 200 rpm, 30°C for 5 hours. The mixture was then transferred into a separating funnel and

allowed to separate into two layers. The RL-rich bottom layer was contained in a glass container and left to dryness in a fume hood at room temperature. The RL layer was then collected in a 15 mL centrifuge tube with a plastic scrapper. A 10 mL of ethyl acetate was mixed with the RL and centrifuged at 10000 rpm for 10 minutes to remove any debris. The supernatant was transferred to a Bijou bottle using pipette avoiding the pellet. The supernatant was left to dry in the fume hood. The RL was then subjected to characterisation.

Characterisation of RL

Chemical profiling of RLs was performed by high resolution mass spectrometry (HRMS) using Waters UPLC system (Waters Technologies Co., Ltd., Milford, MA, USA) coupled with a Quadrupole-Time-of-Flight (QToF) mass spectrometer (SYNAPT^{TM} XS). One microliter of solution was injected into the UPLC system. The separation of the components was performed using an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 50 mm) with the column oven temperature held at 40 °C. LCMS-grade H_2O and acetonitrile (MeCN) containing 0.1% (vol/vol) formic acid (HCOOH) were prepared as Solvent A and B, respectively and were used as mobile phase. To facilitate the fragmentation of RL electrospray ionization (ESI) was used in the negative ionization mode. The functional groups of the RLs were analysed using Shimadzu IRAffinity-1S Fourier Transform Infrared Spectrophotometer. Some RL was placed on the ATR accessory platform completely covering the zinc selenide (ZnSe) diamond. The RL was scanned 45 scans to determine the functional groups at a resolution of 4 cm^{-1} and a spectral range of 600 to 4600 cm^{-1}. The spectra were processed using LabSolutions IR.

Statistical analysis

All the data in the experiment were analysed and presented as means ± standard deviation. The data were evaluated statistically using interaction tests, post-hoc test, and correlation using R Statistics.

Results and Discussion

Bio-oil production and its characterisation:

Temperature profile

The temperature profile of the microwave co-pyrolysis of the bio-oils were shown in **Figure 1**. The temperature profile shows the synergistic effects between the feedstocks during pyrolysis which would affect the heating rate and yield of the bio-oil. This occurrence was also observed in Mahari et al. [15]. During the microwave co-pyrolysis and pyrolysis, the pyrolytic volatiles were formed around 5-9 mins. The maximum temperature recorded occurred at different times. For co-pyrolysis, their maximum temperature of 420 – 442 °C was observed between 25-30 min while UCO and HPW, respectively had their maximum temperature of 381 °C and 456 °C observed at 50th min and 55th min of the experiment (**Figure 1**). It was also observed that the heating rate of co-pyrolysis was higher (10-17 °C/min) compared to pyrolysis (7-8 °C/min). This suggests co-pyrolysis has a positive synergistic effect on the feedstock to reach a higher heating rate compared to pyrolysis. This finding was also similarly reported by Mahari et al. [15]. At approximately 40 °C, UCO have a lower specific heat capacity, approximately 2184 J/kg/°C [23] while HPW made of LDPE has a specific heat capacity of approximately 2600 J/kg/°C [24]. Due to this UCO was able lower down the overall melting point of the feedstock facilitating decomposition of HPW at a lower temperature resulting in higher heating rate.

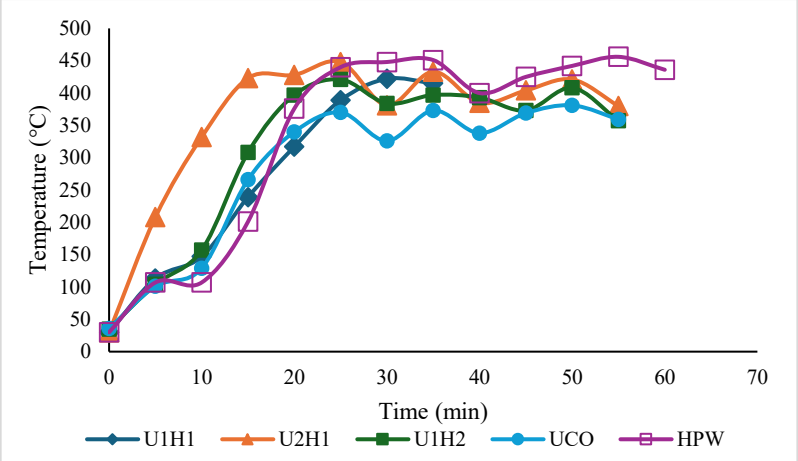

Figure 1. The temperature profile of microwave pyrolysis of the bio-oils. UCO:HPW ratio – 1:1 = U1H1; 2:1 = U2H1; 1:2 = U1H2. Individually, UCO and HPW are control

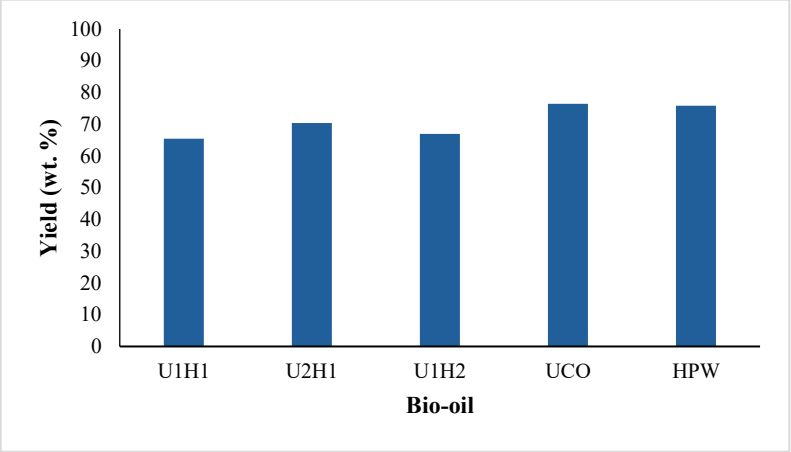

Figure 2. Yield of bio-oils after microwave co-pyrolysis. UCO:HPW ratio – 1:1 = U1H1; 2:1 = U2H1; 1:2 = U1H2. UCO and HPW bio-oils serves as control

As shown in **Figure 1**, the HPW had the longest pyrolysis duration. This was because HPW is a polymer that can withstand elevated temperatures and have exceptional thermal stability [25]. Hence, extended heating time and higher temperatures were needed to break the long HPW polymer chains [15]. According to Mahari et al. [26], during co-pyrolysis the degradation of plastics begins with the removal of hydrogen atom from the chain creating highly reactive and unstable free radicals of hydrogen ($H^{\cdot}$) and hydrocarbon ($R^{\cdot}$). These radicals could react with oxygen molecules from UCO to form peroxy-radical ($RO_2^{\cdot}$). The $RO_2^{\cdot}$ would then randomly remove a hydrogen atom from a polymer chain to form hydrogen peroxide (ROOH) which would then split up to generate two radicals, $RO^{\cdot}$ and $OH^{\cdot}$. These radicals will continue propagating breaking polymer chains into smaller molecules (eg. light hydrocarbons). In pyrolytic polymer thermal degradation, there are two types of carbon bond scission known as random chain scission (reduces polymer chains into smaller molecules) and chain end scission (occurs at gas/liquid interface and produces volatiles).

Bio-oil yield

During pyrolysis, it was observed that vigorous pyrolysis volatiles were formed from 10 minutes onwards around 200-300 °C which could be linked to rapid decomposition of the feedstocks. These volatiles were condensed and collected as bio-oils. The yield of the bio-oils obtained varies based on the ratio of the feedstock (**Figure 2**). The highest yield obtained is from the UCO (76.43 wt. %) while the lowest was U1H1 at 65.46 wt. %. The co-pyrolysis yielded lesser oil compared to pyrolysis which was reported differently by Mahari et al. [15, 16]. In current study, the duration of pyrolysis was 55 to 60 mins while Mahari et al. [15, 16] reported a duration of 30 mins. Due to the long residence time in current study, in co-pyrolysis bio-oils could have undergone more secondary cracking reactions due to the interactions between the two feedstocks forming gas compared to pyrolysis resulting in lesser bio-oil yield.

Characterisation of bio-oil

The elemental compositions of the bio-oils are shown in **Table 1**. The bio-oils comprised of carbon, hydrogen, nitrogen and oxygen (O). Current finding shows excessively high O content, up to 34.00%, in all the bio-oils. As reported in Mahari et al. [15, 16, 26] co-pyrolysed bio-oils have reportedly lower O content compared to pyrolysed UCO. The possible explanation to this occurrence was oxidation of bio-oil during storage. A study by Roponggi et al. [27] reported that lipids can solubilise O_2 very well. The O_2 was introduced during the repeated opening and closing of the bio-oil container which was inevitable due to the need of inoculation for biosynthesis in an aerobic environment for the screening tests. Furthermore, assuming the bio-oils were O_2 lacking (low partial pressure O_2 (pO_2)) after co-pyrolysis the transfer of atmospheric O_2 into the oil was greater due to the difference in pO_2.

Figure 3 shows the chemical composition of the bio-oils. The main components of the bio-oils are alkane, alkene, alcohol, fatty acid, ester, aromatic, ether and others. U1H1 stood out as the composition of "Others" was quite substantial. This 75.93% of "Others" consisted of varying siloxane species. Alkane composition was only at 8.86% which was the lowest among the bio-oils. The reasons behind this were unknown. Apart from U1H1, the major components in the bio-oils were alkane (50 – 59%), followed by "Others". It should be noted that the siloxane species made up most of "Others". The

siloxane species could be derived from the ceramic coating on the frying pans which gives the "non-stick" property. These ceramic coatings in the non-stick cookware were actually silicon-organic compound [28]. This thin layer coating can chip off when in contact with physical abuse releasing the silicon-organic compounds to undergo hydrolysis to produce siloxane species. Another minor probability is from the bleeding of the GCMS capillary column. The capillary column is made of 95% dimethyl polysiloxane and rated "low-bleed" by the manufacturer which could result in a small percentage of siloxane species detected in the samples.

The bio-oils also contain oxygenated compounds such as carboxylic acid, ester, ether and alcohol. This also explains the high content of O in the elemental composition. According to Lam et al. [29] oxygenated compounds were undesirable as it promotes polymerisation reaction during storage leading to lower bio-oil stability, decreased energy content and increase in oil viscosity. *P. aeruginosa* are well documented on their utilisation of fatty-acid rich carbon sources such as palm fatty acid distillate [30], guava seed oil [31], canola oil [32].

One-factor-at-a-time optimization

The carbon and nitrogen sources are vital in biosynthesis of RL which would have a direct effect on the quality and yield of RL. Thus, it is crucial to determine the suitability of carbon and nitrogen source combinations. According to **Figure 4**, highest RL concentration at 5.170±1.2124 mg/L was observed when UCO was used as carbon source followed by U1H2 at 5.000±0.7937 mg/L and the lowest was U1H1 at 0.550±0.1732 mg/L. On the other hand, the bacterial biomass was highest (1.92-2.20 g/L) when U2H1 was used as the carbon source and lowest (0.50-0.70 g/L) when U1H1 was used. U1H1 has exceptionally high content 'Others' (75.93%) which could be unsuitable for bacterial growth. Statistical analyses shows that the carbon source has a significant effect on biomass ($p < 0.05$) and RL concentration ($p < 0.05$). Dunn's test shows that the presence and concentration of UCO affects the biomass and RL concentration.

Table 1. Elemental composition of bio-oils

UCO:HPW	C	H	N	O
U1H1	60.71	8.40	0.30	30.60
U2H1	63.90	8.71	0.32	27.07
U1H2	57.65	8.05	0.30	34.00
UCO	65.93	9.07	0.03	24.97
HPW	73.50	10.27	0.29	15.94

UCO:HPW ratio – 1:1 = U1H1; 2:1 = U2H1; 1:2 = U1H2. UCO and HPW bio-oils serves as control

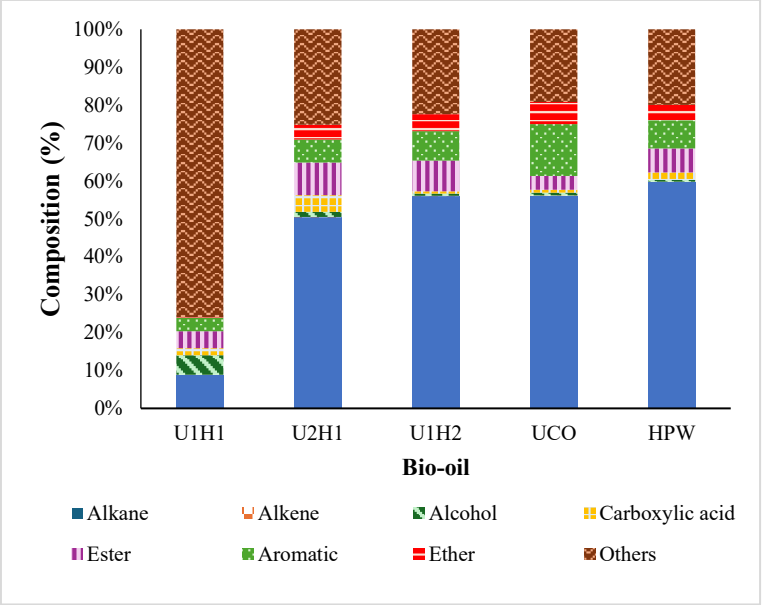

Figure 3. Chemical composition of bio-oils. UCO:HPW ratio – 1:1 = U1H1; 2:1 = U2H1; 1:2 = U1H2. UCO and HPW bio-oils serves as control

Besides, **Figure 4** depicts RL production was observed only in UCO bio-oil but not HPW bio-oil. However, RL production (up to 5.000 mg/L) was observed when U1H1, U2H1 and U1H2 were used as carbon source. This shows possible synergy between UCO and HPW during co-pyrolysis to generate bio-oil feasible for RL production by *P. aeruginosa*. Similar observation was reported by Mahari et al. [15] where in their study *Bacillus megaterium* produced polyhydroxyalkanoate only in the presence of UCO and HPW co-pyrolysed bio-oil.

Figure 4 also demonstrates higher RL concentration when urea was used as the nitrogen source (1.020-5.170 mg/L) over AC and AS. Statistics has shown that nitrogen source has no significant effect on RL concentration (p = 0.052, more than 0.05). It should be noted that urea is an organic nitrogen while the ammonium chloride and ammonium sulphate are inorganic. Chai & Adnan [33] has shown that organic nitrogen can enable higher biomaterial yield than their inorganic counterpart. Additionally, the variation in RL concentrations when *P. aeruginosa* UMTKB-5 was cultured with different nitrogen sources might be attributed to the bacterium's preference for a specific nitrogen source for growth and enzyme production. In another comparative study by Azemi et al. [18] *P. aeruginosa* UMTKB-5 was reported to also yield highest RL concentration (up to 46.3±2 mg/L) when urea was used as a nitrogen source.

Pseudomonas spp. are reported to be very efficient in bioremediating several environmental organic pollutants such as petroleum hydrocarbons, pesticides, dyes and waste cooking oils [34]. According to **Figure 3**, all the bio-oils were rich in alkane except U1H1. Since the bio-oils closely mimics the composition of petroleum hydrocarbons the degradation of bio-oil by *P. aeruginosa* should follow the n-alkane degradation pathway ultimately producing one of the precursors, β-hydroxydecanoyl-β-hydroxydecanoic acid (HAA) through fatty acid β-oxidation pathway. However, when HPW bio-oil was used as a carbon source was not able to produce RL due to the presence of cyclopentanecarboxyamide derivatives. This compound inhibits the fatty acid synthase enzyme (FAS) [35]. FAS is a key component in *de novo* fatty acid synthesis. It is a multi-enzyme protein so by inhibiting this protein the synthesis of fatty acid will be halted. Hence, this finding suggests that the *de novo* fatty acid synthesis pathway is crucial for HAA production rather than the fatty acid β-oxidation pathway. This finding is in agreement with Gutiérrez-Gómez et al. [36].

The bio-oils generated in this study contain numerous substances that have not been assessed for their impact on microorganisms, which could potentially hinder their growth and production [37]. Since U1H2 bio-oil and urea had the highest RL concentration (**Figure 4**) it was selected for the subsequent experiments. Aside from the carbon and nitrogen sources, there are many factors that contribute to the biosynthesis of RL. In current study, bio-oil concentration, inoculum size, and agitation speed were investigated. The U1H2 bio-oil was tested at four concentrations (1, 2, 3, and 4 g/L, respectively). The use of 1 g/L of U1H2 bio-oil showed the highest RL concentration at 3.350±0.8660 mg/L followed by 4 g/L, 3 g/L, and 2 g/L showed had recorded the lowest RL concentration at 1.475±0.1500 mg/L. The observation was not expected as it was initially assumed that the RL concentration would decrease with the increase of bio-oil concentration. Due to the many unknown substances in the bio-oil [37] higher concentrations would mean more potentially toxic constituents, inhibitory agents or insoluble bio-oil causing difficulties *P. aeruginosa* UMTKB-5 to access the nutrients in the culture medium or rendering the bacteria to function normally. A study by Eraqi et al. [38] and Alyousif et al. [6] reported that increasing the concentration of hydrophobic carbon sources has an inhibitory effect on microbial growth and activity.

For inoculum size determination, v/v at 5%, 7% and 10% were investigated. *P. aeruginosa* UMTKB-5 produced highest RL concentration (4.450±0.2121 mg/L) when 7% v/v inoculum size was used. Tukey's test shows the inoculum size does has no significant effect on the RL concentration (p > 0.05). Current findings show that the larger inoculum size (10% v/v) did not observe RL production. The possible reason could be due to severe competition causing the rapid depletion of limited nutrients by the increased number of bacterial cells disrupting the growth and RL production. This finding was similar to El-Housseiny et al. [39, 40] where they observed a similar trend as well. Not only that, a larger inoculum size could have also caused rapid accumulation of metabolic by-products possibly leading to pH imbalance and less efficient matter transfer resulting in cell stress and ultimately death which further amplify the culture toxicity [41]. This phenomenon impacts the RL production by the bacteria. The 5% v/v inoculum size observed a lower RL concentration could be due to lower cell numbers requiring longer time to grow to optimum number then produce RL [39].

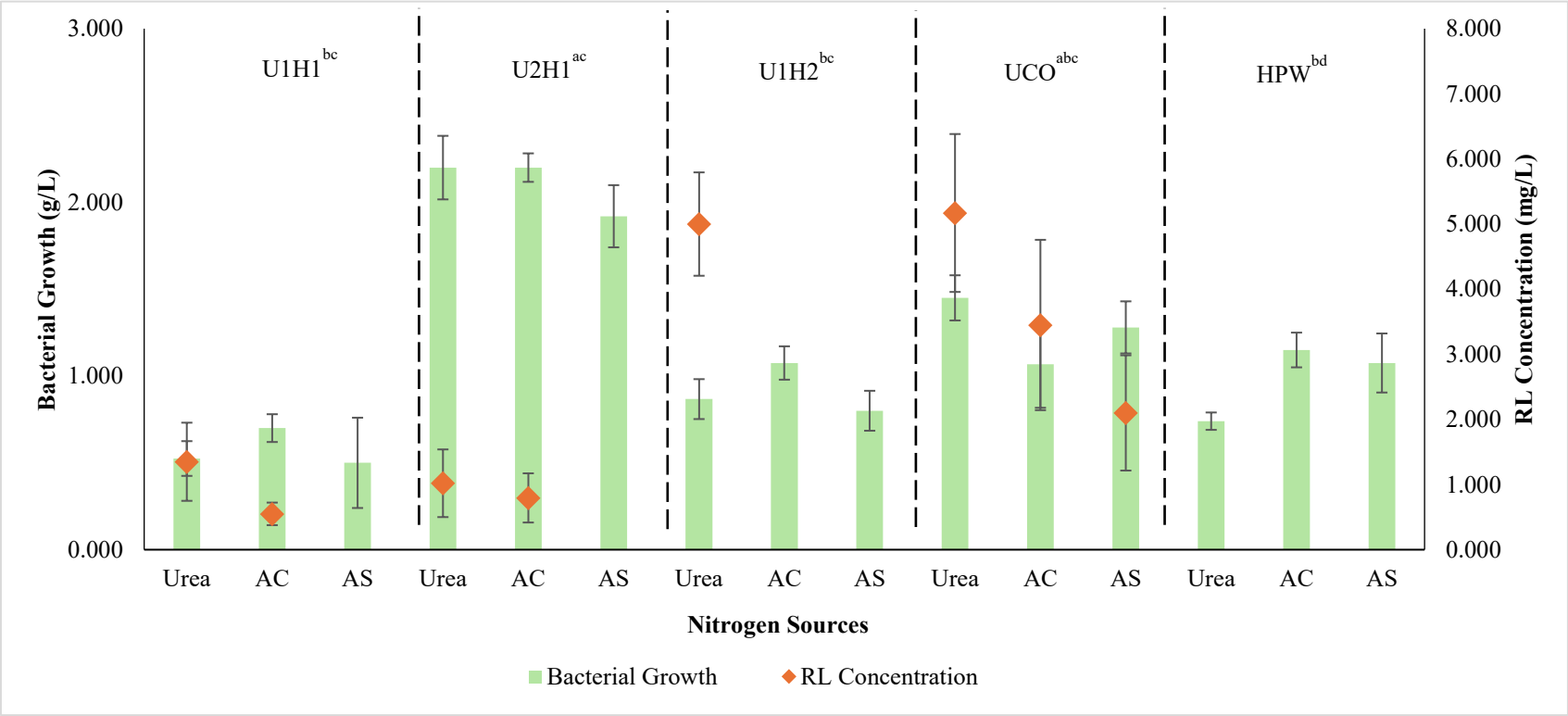

Figure 4. Biomass (measured by cell dry weight) and RL concentration of *P. aeruginosa* UMTKB-5 before and after at different combinations of carbon and nitrogen sources. [a-b] significantly different biomass (Dunn's test, $p \leq 0.005$), [c-d] significantly different RL concentration with carbon sources (Dunn's test, $p \leq 0.005$). Same superscripts are not significantly different. The carbon sources are bio-oil of varying UCO:HPW ratio labelled as U1H1 (1:1), U2H1 (2:1) and U1H2 (1:2). The nitrogen sources are urea, ammonium chloride (AC) and ammonium sulphate (AS). UCO and HPW bio-oils serves as control.

Table 2. The best parameters for biosynthesis of RL by *P. aeruginosa* UMTKB-5

	Parameters
Carbon source	U1H2
Nitrogen source	Urea
Bio-oil concentration	1 g/L
Inoculum size	7%
Agitation speed	200 rpm

For agitation speed, 150, 200, and 250 rpm were investigated. Highest RL concentration was recorded at 200 rpm at 4.600±0.6245 mg/L followed by 150 rpm and 250 rpm. Statistical analysis shows that agitation speed influences the RL concentration produced by *P. aeruginosa* UMTKB-5 (p <0.05). Through observation, higher agitation speed resulted in slight foaming and broke the bio-oil clumps into much smaller bits. The bio-oil bits were also found in the foam which could have also impacted the RL production. Kumar and Das [42] mentioned that agitation speed plays a role in mass transferability of components of the medium and oxygen. Wei et al. [43] reported an increase in cell biomass, RL production and increased dissolved oxygen (DO) when the agitation speed was increased from 50 to 200 rpm then decline at 250 rpm due to heavy foaming causing reduced oxygen transfer into the culture. In current study, the occurrence of foaming could have impacted the oxygen transfer into the culture causing reduced RL concentration. Dissolved oxygen is an important factor in RL production and bacterial growth since the marine *P. aeruginosa* UMTKB-5 in current study prefers aerobic conditions. Moreover, higher agitation speeds could also induce stress onto the bacterial culture resulting in lower RL yields as well as cell viability [44]. At lower speed (150 rpm) the oxygen transfer would be lower which could be one of the reasons for decreased RL production. At lower speeds, the dispersion of bio-oil would also be less efficient. Hence, the best parameters for RL production by *P. aeruginosa* UMTKB-5 using U1H2 bio-oil were as shown in **Table 2**.

Residual Bio-oil Determination

For residual bio-oil determination, a new experiment was conducted with the best parameters shown in **Table 2**. **Figure 5** shows the composition of U1H2 bio-oil before and after the biosynthesis. Quite clearly there was a decrease in alkane content (4.60%) after biosynthesis by *P. aeruginosa* UMTKB-5. Besides, esters content also showed noticeable reduction (1.92% from 8.17%) by *P. aeruginosa* UMTKB-5. The content of 'Others' slightly increased after biosynthesis recording a 28.74% from 22.38% by *P. aeruginosa* UMTKB-5.

Muriel-Millán et al. [45] reported that *P. aeruginosa* GOM1 was able to degrade 96% of the n-alkanes in their study after a 30-day incubation period. The alkanes could be used for RL synthesis via the n-alkane degradation pathway. According to Galitskaya et al. [46], the biodegradation of alkanes decreases in the following order: n-alkanes > branched alkanes > cyclic alkanes. In the U1H2 bio-oil, it was observed that there was a higher content of branched alkane (36.68%) than linear alkane (19.31%). Surprisingly, the residual oil showed a decrease in branched alkane content (12.40% and 26.10%, respectively) while increase in n-alkane (10.10% and 17.10%, respectively). This suggests that the strain may be the able to degrade branched alkane into n-alkane. Besides, a slight increase in fatty acid content was observed. It was assumed that the increase was due to the hydrolysis of triglycerides to utilise the glycerol for gluconeogenesis leaving behind the free fatty acids. The GCMS results also show that the concentration of a predominant fatty acid in the U1H2 bio-oil, palmitic acid, decreased by 49% hinting possible utilisation by *P. aeruginosa* UMTKB-5.

Characterisation of RL:

FTIR

The functional groups of RL produced using U1H2 were analysed using ATR-FTIR. **Figure 6** shows the spectra for RL by *P. aeruginosa* UMTKB-5. Stretching C-O bands were detected in the IR spectrum at 1118 and 1037 cm^{-1}, asymmetric O-C-O band at 1037 cm^{-1}, deformation C-OH band at 1037 cm^{-1}, deformation C-H band at 1456 cm^{-1}, strong symmetric stretching C=O band of carboxylate group at 1720 and 1649 cm^{-1}. The spectrum showed stretching (-C-H) vibration of $-CH_2$ and $-CH_3$ groups at 2926 and 2852 cm^{-1}. A broad peak at 3377 cm^{-1} for symmetric O-H stretching was observed. The findings in this study have demonstrated that the RL produced using bio-oil as a carbon source has similar functional groups compared to RL produced using other carbon sources such as glycerol [47], soybean meal [49], and crude rice bran oil [48]. Sabturani et al. [47] mentioned that the important absorption spectra were located at 3308.46, 2922.9, 2857.09 and 1730.96 cm^{-1}. These absorption bands indicates that the biosurfactant produced by *P. aeruginosa* UMTKB-5 belonged to a glycolipid group. Moreover, the FTIR spectra of the biosurfactant were in good conformation with the FTIR spectra of rhamnolipids reported by Safari et al. [48], Dabaghi et al. [49] and Sabturani et al. [47].

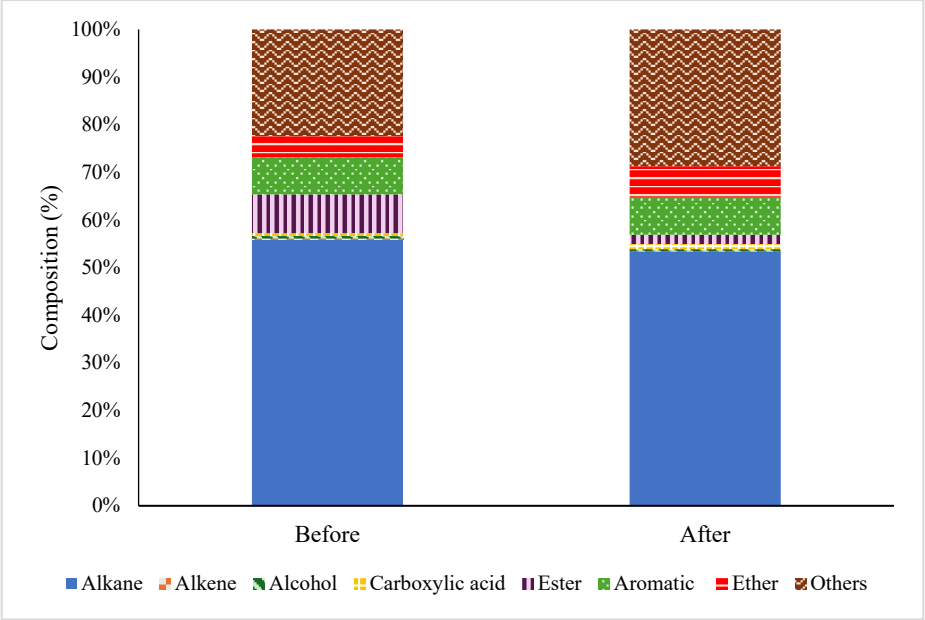

Figure 5. Composition of U1H2 before and after utilisation by *P. aeruginosa* UMTKB-5. The residual bio-oil was bio-oil recovered from the culture after 72 hours of biosynthesis

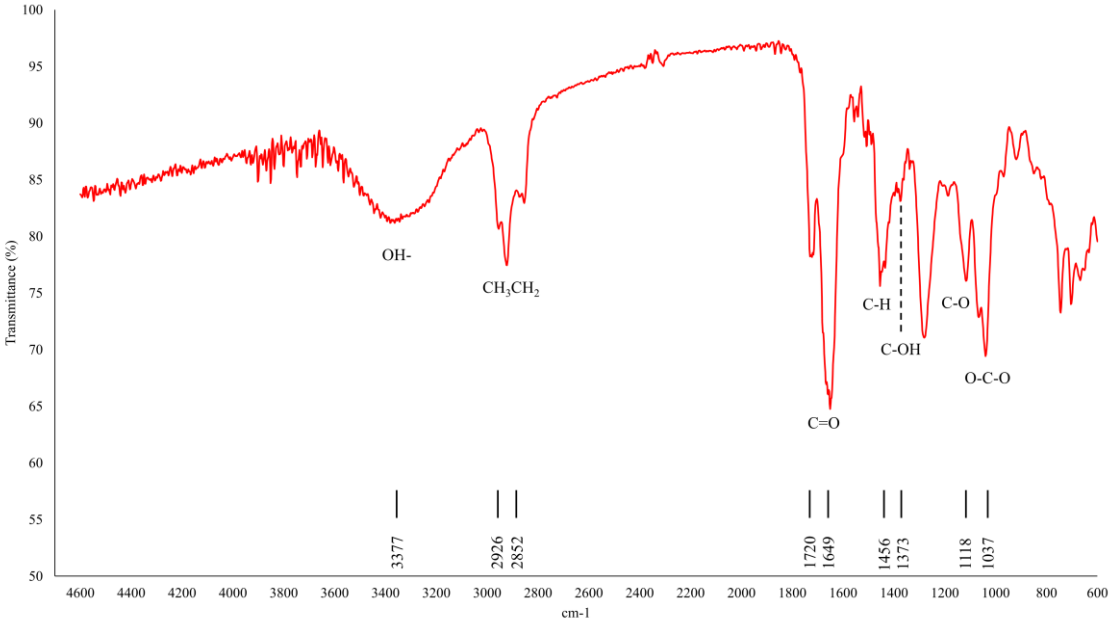

Figure 6. FTIR spectrum showing the functional groups in RL produced by P. aeruginosa UMTKB-5

UPLC-QToF-ESI-MS/MS

To further confirm that the biosurfactant was RL UPLC-QToF-ESI-MS/MS was used. Table 3 shows the RL congeners of the rhamnolipid produced by *P. aeruginosa* UMTKB-5. According to literatures, the common *P. aeruginosa* produced RL congeners were the mono-RL, Rha-C_{10}-C_{10} and di-RL, RhaRha-C_{10}-C_{10} [50][51]. Both these congeners were found at 503 *m/z* and 649 *m/z*, respectively which agreed with current study. Moreover, it appears that these two congeners had the highest intensity at 4.99e5 and 4.00e6 (Table 3), respectively in the biosurfactant. The ratio of di-RL to mono-RL produced by *P. aeruginosa* UMTKB-5 was 87.57:12.43. The major molecule was RhaRha-C_{10}-C_{10} accounting for 74.1% of total RL. Table 3 shows the fragment ions of the congeners. The fragment ions obtained in this study were in agreement with the findings reported by

Table 3. List of different RL congeners, intensity and fragments produced by *P. aeruginosa* UMTKB-5 in the biosurfactant

Compound	This study			Heyd et al. [52]	
	$[M-H]^-$ (*m*/*z*)	Fragment ions (*m*/*z*)	Intensity	$[M-H]^-$ (*m*/*z*)	Fragment ions (*m*/*z*)
Rha-C_{10}	333.1913	169.1342	7.93e4	333	169
Rha-C_8-C_{10}	475.2904	333.2021, 305.1697, 169.1267, 141.0945	1.13e4	475	333,305,169,141
Rha-Rha-C_{10}	479.2487	169.1352	4.05e5	479	169
Rha-C_{10}-C_{10}	503.3228	339.2813, 333.2191, 169.1350	4.99e5	503	339, 333, 169
Rha-C_{10}-C_{12}	531.3532	361.2209, 333.1895, 197.1517, 169.1203	7.71e4	531	367, 333, 197, 169
Rha-Rha-C_8-C_{10}	621.3488	451.2186, 311.2216, 169.1228, 141.0914	1.51e5	621	451, 311, 169, 141
Rha-Rha-C_{10}-C_8		479.2505, 311.2216, 169.1228, 141.0914			479, 311, 169, 141
Rha-Rha-C_{10}-C_{10}	649.3799	479.2497, 169.1227, 339.2528	4.00e6	649	479, 339, 169
Rha-Rha-C_{10}-C_{12}	677.4117	479.2498, 367.2845, 197.1538, 169.1225	1.75e5	677	479, 367, 197, 169
Rha-Rha-C_{12}-C_{10}		507.2816, 367.2845, 197.1538, 169.1225			507, 367, 197, 169

Heyd et al. [52]. Therefore, the biosurfactant produced in this study was RL.

The properties of RL are usually determined by the nature of their congeners. The varying number of hydrophobic/hydrophilic residues and their mixtures have various physicochemical characteristics that could result in a range of uses [4]. According to Zhao et al. [3] they reported that both mono- and di-RL possess antimicrobial activity but the mono-RL is more superior. It was mentioned that the fatty acid chain in mono-RL occupy larger molecular volume resulting in enhanced lipophilic properties leading to stronger cytolysis properties. However, when it comes to washing capability di-RL prevails. In a study by Li et al. [4] they reported that di-RL exhibited better oil-washing properties than mono-RL. It was noted that in the event of two identical fatty acid chain length RL the dual rhamnose sugars in di-RL will have greatly enhanced the hydrophilicity over mono-RL.

The influence of the rhamnolipid congeners towards interfacial tension were investigated by Gong et al. [53]. It was reported that the higher percentage of di-RL resulted in decrease in interfacial tension. In terms of CMC, mono-RL have a lower CMC than di-RL [54]. This is due to the increased hydrophobicity caused by the presence of one rhamnose sugar over the di-RL with two rhamnose sugars. Due to this, mono-RL molecule tends to aggregate into micelles at lower concentrations [54]. Low CMC levels are a great trait in RL, but it may lack the hydrophilicity to be used in certain applications.

In summary, the RL produced by *P. aeruginosa* UMTKB-5 has significantly higher ratio of di-RL. The RL produced would be more hydrophilic suggesting it could be a great candidate in washing applications such as bioremediation and washing detergents. Hence, current findings have successfully characterised RL produced by *P. aeruginosa* UMTKB-5.

Conclusion

For the first time, bio-oil has been proven to be a feasible feedstock for RL production. Besides, HPW was now being transformed into a new product that is biotechnologically important and eco-friendly. Microwave co-pyrolysis of UCO and HPW exhibited positive synergistic effect which effectively increased the heating rate (10-17°C/min). Using elemental analysis and GCMS, the bio-oils in this study were successfully characterised which allowed deeper understanding on bacteria utilisation. The alkane-rich bio-oils were promising carbon sources as *P. aeruginosa* UMTKB-5 was successful in the bioconversion of bio-oil into value-added RL up to 5.000 mg/L. The best parameters were 1 g/L of U2H1, urea, 7% (v/v) inoculum size and 200 rpm agitation speed. Moreover, by using state-of-the-art equipment, ATR-FTIR and UPLC-QToF-ESI-MS/MS, the RL produced by *P. aeruginosa* UMTKB-5 was successfully characterised. It consisted of numerous congeners where Rha-C_{10}-C_{10} and RhaRha-C_{10}-C_{10} were the prominent ones. The RL in this study was suited for bioremediation and used in making washing detergents due to its increased hydrophilic characteristics. The bio-oil in this study has numerous unknown compounds which could have affected the RL production by the bacteria. Several possible ways to overcome this would be tuning the bio-oil using a catalyst to reduce the composition of unknown compounds. The use of bioengineered strains that could be more robust towards the harsh environment of the bio-oil while increasing RL production. Lastly, to further understand the bio-oil's potential as a carbon source, upscaling to a fermenter would be a great approach to understanding the interactions of bio-oil and the strains in a fermenter environment. Conclusively, this

study has successfully proved that RL is a sustainable resource derived from waste.

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