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DUAL-PRODUCTION OF POLYHYDROXYALKANOATE AND RHAMNOLIPID BY *Pseudomonas aeruginosa* UMTKB-5 USING INDUSTRIAL BY-PRODUCTS

(Dwi-Produksi Polihidroksialkanoat dan Ramnolipid oleh *Pseudomonas aeruginosa* UMTKB-5 dengan Penggunaan Hasil Sampingan Industri)

Noor Fazielawanie Mohd Rashid[1], Tan Suet May Amelia[1], Al-Ashraf Abdullah Amirul[2,3,4],
Kesaven Bhubalan[1,4,5]*

[1]*Faculty of Science and Marine Environment,*
Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[2]*Centre for Chemical Biology,*
Universiti Sains Malaysia, 11900 Bayan Lepas, Penang, Malaysia
[3]*School of Biological Sciences,*
Universiti Sains Malaysia, 11800 Gelugor, Penang, Malaysia
[4]*Malaysian Institute of Pharmaceuticals and Nutraceuticals,*
National Institutes of Biotechnology Malaysia, 11700 Gelugor, Penang, Malaysia
[5]*Institute of Marine Biotechnology,*
Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

**Corresponding author: kesaven@umt.edu.my*

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Abstract

The biodegradability and biocompatibility of polyhydroxyalkanoate (PHA) bioplastic and rhamnolipid (RL) biosurfactant have encouraged their application in medicine, packaging, and bioremediation. However, the resources used to produce these two substances contribute to high manufacturing costs. Therefore, we applied the dual-production approach and fed the same renewable and economical carbon and nitrogen sources into singular cultivation media to produce both PHA and RL simultaneously. By-products from oleochemical (glycerol and glycerine pitch) and sugarcane (molasses and sweet water) industries were used to produce mcl-PHA and RL from *Pseudomonas aeruginosa* UMTKB-5. Furthermore, we also attempted the introduction of plasmid pBBR-PC1020 into transformant *P. aeruginosa* UMTKB-5 to produce 20-50% scl-mcl PHA with better properties. The PHA and RL yields were compared between wild-type and transformant strains with the use of seven carbon sources and six nitrogen sources. With glycerol as the carbon source and urea [$CO(NH_2)_2$] as the nitrogen source, the wild-type strain had produced the highest amount of PHA and RL at 0.24 ± 0.01 and 2.31 ± 0.01 g/L, respectively. The overall molecular weights (M_w) of the polymers produced ranged from 4×10^5 to 5×10^5 Da. Characterisation analysis on the RL congeners produced were identified as mono- and di-RL.

Keywords: *Pseudomonas aeruginosa*, polyhydroxyalkanoate, rhamnolipid, glycerine pitch, glycerol, dual-production

Abstrak

Biopolimer polihidroksialkanoat (PHA) dan biosurfaktan ramnolipid (RL) yang mempunyai ciri-ciri biodegradasi dan keserasian bio telah mendorong penggunaannya dalam perubatan, pembungkusan, dan bioremediasi. Walau bagaimanapun, sumber yang digunakan untuk menghasilkan kedua-dua bahan ini menyumbang kepada kos pembuatan yang tinggi. Justeru, kami mengaplikasikan pendekatan dwi-produksi dan memberi sumber karbon dan nitrogen sama yang boleh diperbaharui dan murah ke dalam media kultur tunggal untuk menghasilkan PHA dan RL secara serentak. Hasil sampingan dari industri oleokimia (gliserol dan gliserin) dan tebu (molase dan air manis) digunakan sebagai sumber karbon untuk menghasilkan mcl-PHA dan RL dari *Pseudomonas aeruginosa* UMTKB-5. Selanjutnya, kami juga memperkenalkan plasmid pBBR-PC1020 kepada transforman *P. aeruginosa* UMTKB-5 untuk menghasilkan 20-50% scl-mcl PHA dengan sifat yang lebih baik. Hasil PHA dan RL dibandingkan antara strain jenis liar dan transforman dengan penggunaan tujuh sumber karbon dan enam sumber nitrogen. Dengan gliserol sebagai sumber karbon dan urea [$CO(NH_2)_2$] sebagai sumber nitrogen, strain jenis liar telah menghasilkan jumlah PHA dan RL tertinggi masing-masing pada 0.24 ± 0.01 dan 2.31 ± 0.01 g/L. Berat molekul (Mw) polimer yang dihasilkan secara keseluruhan adalah dari 4×10^5 hingga 5×10^5 Da. Analisis pencirian atas kongener RL yang dihasilkan telah dikenal pasti sebagai mono- dan di-RL.

Kata kunci: *Pseudomonas aeruginosa*, polihidroksialkanoat, ramnolipid, sisa gliserin, gliserol, dwi-produksi

Introduction

Plastic pollution and the depletion of finite sources, such as natural gas and crude oil, from the manufacturing of fossil-based plastic have urged the research for alternative materials. One of the potential substitutes of plastic, polyhydroxyalkanoate (PHA), is known as a naturally occurring, biodegradable, and biocompatible polymer [1]. Polyhydroxyalkanoate is biosynthesised by microbes, such as fungi, algae, and bacteria, as an energy storage compound in cell cytoplasm [2]. This biopolymer is classified by their branching monomers. Short-chain-length PHA (scl-PHA) consists of 3-5 carbon atoms in the monomer units, such as poly(3-hydroxybutyrate) [P(3HB)] and poly(4-hydroxybutyrate) [P(4HB)]. Medium-chain-length PHA (mcl-PHA) comprises more than 6 carbon atoms, such as poly(3-hydroxyhexanoate) [P(3HHx)] and poly(3-hydroxydecanoate) [(3HD)] [3]. Polyhydroxyalkanoate can also be categorised into a homopolymer PHA that comprises one type of hydroxyalkanoate [e.g. P(3HB)] or a heteropolymer PHA that comprises more than one type of hydroxyalkanoate [e.g. poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV)]. The diverse types of PHA allow the polymer to be tailored for multiple applications such as agriculture, packaging, and biomedicine [4]. For example, mcl-PHA is elastomeric; scl-PHA is crystalline, brittle, and stiff; while scl-mcl-PHA, has the combined properties of both types of PHA, is less crystalline, higher toughness, and higher degradation rate [5-7].

Certain bacterial PHA-producers also biosynthesise rhamnolipid (RL), which is the most studied biosurfactant [8-10] with emulsifying and antimicrobial properties. It is commonly composed of rhamnose (Rha) sugar molecules and β-hydroxyl alkanoic acids moieties. The properties of RL depends on homologue composition and distribution, bacterial producer, culture conditions, and medium types. *Pseudomonas* spp. Have been receiving research attention for biosurfactant applications since the RL produced from this genus showed high reduction of surface tension [11]. A marine isolate, *Pseudomonas aeruginosa* UMTKB-5 was previously reported to produce high RL concentration particularly from renewable agro-industrial by-products as substrates, which were molasses and sweetwater [12].

Some strains in the *Pseudomonas* genus have been reported to produce RL and PHA simultaneously [13-16]. Both PHA and RL are produced during imbalanced growth conditions, particularly under excess carbon sources and limitation of multivalent ions, such as nitrogen and phosphorus. Their yield is also influenced by the type of carbon source, type of nitrogen source, pH, and age of culture. The biodegradability, biocompatibility, and low toxicity of both PHA and RL encourage their usage in various industries. For example, RL has been researched in bioremediation, pharmaceuticals, cosmetics, and food industry [17-19], while PHA is widely used in pharmaceuticals, medical, packaging, and aquaculture industries [20-23].

However, low productivity, high downstream processing and raw-material costs caused a bottleneck for the commercialisation of PHA and RL. Carbon source accounts for about 30 to 50% of total production cost [24], hence alternative renewable resources, such as agro-industry by-products, have been researched to replace conventional substrates [25,26]. The renewable resources tested to produce PHA and RL from bacteria were wastes or by-products from biofuel and biochemical (glycerol and glycerine pitch), sugar cane (molasses), frying oil (waste lipids), cheese (whey), water treatment (municipal wastewater), starch (cassava wastewater), and other industries that use lignocellulosic raw materials [8, 13, 27-28]. Therefore, we attempted to construct transformant *P. aeruginosa* UMTKB-5 (harbouring PHA-synthesising gene (*phaC*) from *Cupriavidus* sp. USMAA1020) to biosynthesise scl-mcl PHA, and then compared the PHA and RL yields between wild-type and transformant *P. aeruginosa* UMTKB-5 with the use of seven carbon sources (common sugars and agro-industrial by-products) and six nitrogen sources through simultaneous production in a single production medium (Figure 1).

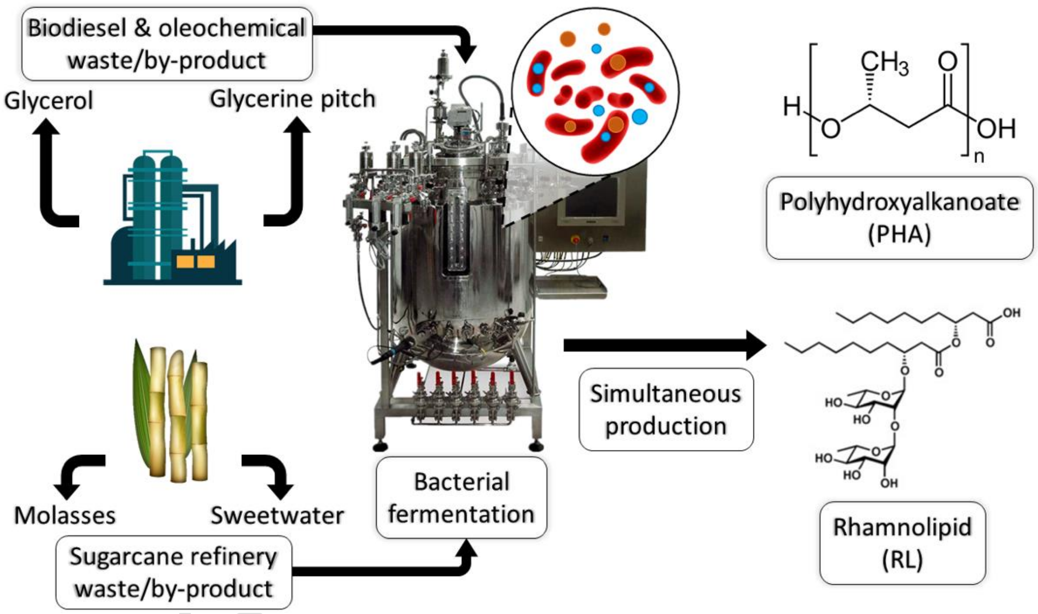

Figure 1. Illustration showing the simultaneous production of PHA and RL from industrial by-products using bacterial fermentation

Materials and Methods

Bacterial strains, plasmids, carbon sources

The wild-type *P. aeruginosa* UMTKB-5 strain (GenBank accession number: KT194193.1), isolated from marine sediment at Pulau Bidong (Terengganu, Malaysia), was obtained from the culture collection of Institute of Marine Biotechnology, Universiti Malaysia Terengganu [12]. Several types of carbon sources were used to produce PHA and RL. Bacteriological-grade sucrose (HiMedia, India) and 100% refined-grade glycerol (Bendosen, Norway) were purchased from commercial supplier. Meanwhile, glycerine pitch, generally comprising glycerol, ash, fatty acids, and organic salts, was obtained from Oleochemicals Industry, Penang, Malaysia [29]. On the other hand, molasses and sweetwater were obtained from cane sugar refinery company Gula Padang Terap Sdn. Bhd. (14006-V), Kuala Nerang, Kedah, Malaysia. Molasses typically consist of dry solids, ash, sugars (fructose and sucrose), and nutrients (phosphorus and nitrogen). Sweetwater is an agricultural by-product that still contains residual sucrose after filtration by a sugar cane refining process.

Biosynthesis of PHA and RL was carried out at a concentration of 20 g/L carbon substrate. The amount of sugars in the molasses and sweetwater used in this study were calculated to be 50.9 and 18.4 wt.%, respectively [12, 25].

Construction of transformant *P. aeruginosa* UMTKB-5

Prior biosynthesis, the *P. aeruginosa* UMTKB-5 transformant strain was constructed using the Pbbr-PC1020 plasmid, a Pbbr1MCS-2 derivative harbouring approximately 1.7 kb fragment of PHA synthase gene from *Cupriavidus* sp. USMAA1020 [30,31]. Transformant *P. aeruginosa* UMTKB-5 was constructed using benchtop- and microcentrifuge-based approaches based on the method proposed by Chuanchuen et al. [32]. The concentration of the plasmid was determined at an absorbance of 260 nm using Biophotometer Plus (Eppendorf, Germany). Transformation was carried out by adding 100 to 400 ng (2-5 µL) of plasmid DNA into 100 µL of prechilled competent cells. Sterile distilled water was used as negative control. The mixture was incubated in ice for 15 minutes, then heat-shock at 37 ºC for 3 minutes, followed by an addition of 500 µL of LB broth. The tubes were incubated on a shaking incubator at 37 ºC for 1 hour. 100 µL of the mixture was plated onto the LB agar containing 50 µg/mL of kanamycin (Merck, Darmstadt, Germany) to maintain plasmid stability. The plates were incubated at 37 °C overnight and observed.

Biosynthesis of PHA and RL

To prepare activated pre-culture, 2 loopfuls of wild-type *P. aeruginosa* UMTKB-5 bacterial cells were inoculated into 50 mL NR broth (HiMedia, India) and cultivated at 30 ºC, 200 rpm using HIMAC CR 22N (Hitachi, Japan) centrifuge machine for 11 hours. The transformant *P. aeruginosa* UMTKB-5 was cultivated for 8 hours with 50 µg/mL kanamycin added for plasmid maintenance. Using one-stage cultivation, biosynthesis of PHA and RL was carried out aerobically at 200 rpm, 30 ºC for 72 hours in a 5-L Biostat® C plus (Sartorius, Germany) bioreactor (2.5-L working volume) with 40% dissolved oxygen and pH 7 [13]. The pH was maintained by adding either 5 M of KOH (R&M, UK) or HCl solutions. Growth profiling was done for 96 hours with sampling at 12-hours intervals. Biosynthesis was carried out and compared between the use of seven types of carbon sources (glucose, fructose, sucrose, glycerol, glycerine pitch, molasses, and sweetwater) and six different types of nitrogen sources [$NH_4H_2PO_4$, $CO(NH_2)_2$, $NaNO_3$, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$] to test for the source that produced the highest yield. Carbon sources were added into the mineral salt's medium prior fermentation. The concentration of nitrogen source applied was 2.5 g/L, whereas the carbon source applied was based on a C/N ratio of 50. The mineral salts medium consisted of KH_2PO_4 (R&M, UK), Na_2HPO_4 (R&M, UK), and NH_4Cl (Bendosen, Hmbg) [33]. The cells were harvested by centrifugation (9000 rpm, 4 ºC) after 72 hours of fermentation. The collected supernatant was subjected to RL analysis, and the cell pellet was lyophilised using FreeZone 4.5 (Labconco Corporation, USA) freeze dry system for 72 h prior to PHA detection using gas chromatography analysis. The lyophilised cells were weighed using Sartorius CP2245 electronic balance (Sartorius, Germany) to determine cell biomass.

PHA characterisation: Gas chromatography analysis

The methanolysis was performed according to the modified method of Braunegg et al. [34]. Approximately 8-10 mg of lipolyzed cells were weighed and filled into screw-capped test tubes. A volume of 2 mL of methanolysis solution and 2 mL of $CHCl_3$were added into the tube, and then sealed with Teflon tape. The samples were heated at 100 ºC on a heating block for two and a half hours. The tube was slightly inverted at every 30 minutes interval to homogenise the mixture.

After heating, the solution was cooled at room temperature for 15 minutes. A volume of 1 mL distilled water was added into the tubes and vortexed for 30 sec to encourage the formation of two distinct phases. The upper layer consists of water, while the bottom layer consists of $CHCl_3$ and hydroxyalkanoate methyl ester. By using 1endoza pipette, the bottom layer was transferred into tube containing anhydrous sodium sulphate (Na_2SO_4) (R&M, UK) to absorb the remaining water. Prior to injection, 0.5 mL of the solution was then transferred into GC vials containing 0.5 mL of CME solution.

The GC analysis was run using Trace GC ultra (Thermo Scientific, Italy) equipped with a flame ionisation detector (FID) and a fused silica capillary column (30 m length x 0.32 mm internal diameter, 0.25 µm film (Supelco, SPB-TM1, USA) with conditions: injector temperature 200 °C, detector temperature 280 °C, initial column temperature 50 ºC, final column temperature 280 ºC, air flow 2 mL/min, total flow 10 ºC/min, and analysis time 30 min. The concentration of PHA in the sample was calculated using the formula equation below [35]:

$$\text{Concentration of C6} = \frac{(\text{Area of C6 x concentration IS})}{(\text{Area IS x } R\text{fC6})}$$

where area C6 is peak area for the C6 monomer, area IS is peak area for the internal standard, methyl benzoate, concentration IS is the amount of methyl benzoate per mL dichloromethane (DCM) and *R*fC6 is the relative response factor for the C6 methyl-ester

Gas permeation chromatography analysis

The molecular weight data was obtained by using GPC (Shimadzu, Japan), refractive index detector (RID-0A) equipped with Plgel 5 µm MIXED-C column, 600 x 7.5 mm (polymer Laboratories, Ltd, UK) [36].

Approximately 1 mg of polymer sample was dissolved in 1 mL of $CHCl_3$ (HPLC grade). The solution was then filtered using 0.45 µm PTFE syringe filter (Sartorius, Germany). The sample was analysed to get the molecular weight (M_w), number average molecular weight (M_n) as well as polydispersity index (M_w/M_n). The $CHCl_3$ at a flow rate of 1.0 mL/min was used. Polystyrene standards with a low polydispersity were used as standard.

Differential scanning calorimetry analysis

DSC was run by using *Perkin-Elmer Pyris I* (Perkin Elmer, USA) equipped with nitrogen cooling accessory. Approximately 5-8 mg of samples was heated from -50 to 200 ºC. The rate of heating applied was 10 ºC/min. The glass transition temperature (T_g), melting temperature I and heat of fusion (ΔH_m) were determined from the graph [36].

Thermogravimetric analysis

TGA was conducted using thermal analysis system TGA/DSC 1 Mettler Toledo according to Guo et al. [37] with modification. Samples of approximately 15 mg were used for each measurement. Samples were heated at a flow rate of 20 mL/min from room temperature to 600 ºC using argon gas.

RL characterisation

Direct amount of RL in the sample were assessed by orcinol assay according to the method described by Koch et al. [38]. The orcinol reagent was prepared by adding the concentrated sulphuric acid, H_2SO_4 (98% w/w) and 0.19% of orcinol (3,5-dihydroxytoluene) to distilled water. The final concentration of acid was 53% w/w. A total of 100 mL of orcinol reagent was prepared by weighing 0.19 g of orcinol powder, 99% purity (R&M, UK), and mixed with 46 mL of distilled water. Finally, 54 mL of 98% w/w of H_2SO_4 was added into the solutions drop wise. For sample preparation, 400 µL of supernatant was added with 750 µL of diethyl ether (Merck, USA). Approximately 900 µL of prepared orcinol reagent was added to 100 µL of sample prior to determination of RL concentration. The optical density of 421 nm wavelengths was determined using spectrophotometer *UV1601PC* (Shimadzu, Japan).

The RL was further extracted using the method as proposed by Yin et al. [39]. The supernatant was mixed with ethyl acetate (Merck, Germany) at a ratio of 1:1 and vigorously shook for 3 minutes. The upper fraction was collected, and the solution was concentrated using rotary evaporator (Buchi, Switzerland) at 40 °C. Crude RL was obtained. A total of 30 mL of 0.05 M sodium bicarbonate was used to dissolve the crude RL. After adjusting to pH 2, the solution was kept overnight before being lyophilised for 72 hours.

The liquid chromatography mass spectrometry (LCMS) analysis was run using LCMS-QtoF model 6560 *UPHLC* (Agilent, USA) with a G4220A binary pump, a G4212A diode array detector, a G4226A HiP auto sampler and a G1316C column compartment. Column POROSHELL 210 EC-C18 (4.6 mm x 100 mm, internal diameter 2.7 µm) [ChromTech, Inc., Minnesota, USA] was used for LCMS with conditions: gas temperature

225 °C, gas flow 12 L/min, nebulizer 20 psig, sheath gas temperature 400 °C, sheath gas flow 12 L/min, nozzle voltage 500 V, fragmentor 365 V, mobile phase flow 0.40 mL/min, column temperature 40 °C. For solvent A or aqueous solvent, 2 Mm ammonium acetate in Milli-Q water, pH 8.0–8.5 was prepared. For solvent B or organic solvent, 2 Mm ammonium acetate in acetonitrile, pH 8.0–8.5 was prepared. Different gradient of mobile phase ranged from 30 to 70% was applied. Approximately 32 mg pure RL was dissolved in 1.5 mL of acetonitrile (I). A total of 10 µL of sample was auto injected using the autosampler. The syringe was automatically rinsed with ACN after each injection.

Results and Discussion

Effect of carbon sources on the yield of PHA and RL

The PHA and RL was biosynthesised at an incubation shaking speed of 200 rpm and a temperature of 30 ºC for 72 hours with a fixed concentration of carbon source at 20 g/L. Based on the GC analysis data, the wild-type strain was able to produce PHA from 0.05 to 0.31 g/L for all carbon sources tested, with the exception of sucrose and sweetwater (Figure 2a). Data showed that the highest PHA concentration was obtained when the strain was fed with glycerol as sole carbon source, accounting to 0.31 ± 0.01 g/L. This is approximately 10 wt.% in terms of contents of cell biomass. The PHA concentration obtained in the present study was much better compared to *P. aeruginosa* L2-1 (0.143 g/L) using the same concentration of carbon source [15]. Therefore, it can be concluded that the accumulation of PHA seems to be strain dependent. When the strain was supplemented with sugars, the strain was able to produce PHA with the exception of sucrose. The utilisation of sucrose could have been sufficient for the bacterial growth but not PHA production, which may be due to the energy conversion and lack of a utilisation pathway. In other words, the use of sucrose as a carbon source causing low PHA yield could be explained by bacterial energy conservation where much was used for sucrose conversion, growth, and RL production, hence limited intermediates were channelled for PHA production. In a previous study, *Ralstonia eutropha* was unable to utilise sucrose as a carbon source for PHA production until a sucrose utilisation pathway was introduced through the addition of a gene that encodes b-fructofuranosidase [40]. Also, Ali and Jamil [41] reported that *P. aeruginosa* IFS and 30N produced 0.013 and 0.039 g/L of PHA when the said strains were fed with sucrose as sole carbon sources. Thus, it can be concluded that the utilisation of sucrose as carbon source for PHA production was strain and genome dependent.

On the other hand, when molasses and sweetwater was used, the value was lower compared to other reported strains. For example, *P. aeruginosa* NCIM and *B. megaterium* UMTKB-1 were capable of producing 4.14 ± 0.12 and 0.29 ± 0.01 g/L of PHA, when the strains were supplemented with molasses and sweetwater, respectively [6, 25]. This is the first report of utilisation of sweetwater for PHA production in *Pseudomonas* strain. The lower PHA concentration obtained using molasses and sweetwater as carbon sources could be due to the additional nitrogen and lower glycerol contents. Molasses contains approximately 41% and 32% of sucrose and glycerol, respectively [25]. However, molasses can contain up to approximately 9.5% of nitrogenous compounds [42]. Meanwhile, sweetwater contains approximately 17% and 10% of sucrose and glycerol, respectively [12]. Here, the glycerol present in molasses and sweetwater only supported the growth of cell.

The RL production was also found to vary depending on the carbon sources used. In general, wild-type *P. aeruginosa* UMTKB-5 produced RL ranging from 0.12 ± 0.02 to 1.94 ± 0.03 g/L. The highest RL concentration was obtained when the strain was supplemented with fructose as sole carbon source, accounting for 1.94 ± 0.03 g/L. The performance of *P. aeruginosa* UMTKB-5 with fructose at 20 g/L was much better compared to *P. aeruginosa* PA14 (1.34 ± 0.03 g/L) [43] using the same carbon concentration, thus concluded that RL production was strain dependent. Glycerol was noted to produce the second highest amount of RL, accounting for 1.11 ± 0.03 g/L. However, the value was lesser compared to other *P. aeruginosa* strains as reported by Santos et al. [44] and Rooney *et al*. [45], accounting for 2.0 and 2.3 g/L, respectively.

For transformant strain, it was clearly observed that insertion of *phaC* gene into wild-type *P. aeruginosa* UMTKB-5 resulted in the increasing significantly ($p < 0.05$) of polymer accumulation, with exception for molasses and sweetwater (Figure 2b). When glucose was fed as carbon source, PHA concentration increased fourfold to 0.25 ± 0.02 g/L. The PHA concentration was increased six-fold when sucrose was utilised accounting for 0.35 ± 0.01 g/L. While, using glycerol and glycerine pitch, PHA accumulation increased to 0.78 ± 0.03 and 0.45 ± 0.02 g/L, respectively. In contrast, molasses and sweetwater only accumulated trace amount (<0.05 g/L) of polymer produced. Using glycerol, the transformant *P. aeruginosa* UMTKB-5 was able to produce approximately 24.0 ± 3.0 wt.% of PHA (0.78 ± 0.03 g/L), slightly higher compared to engineered *Pseudomonas putida* KT2440 that produced 21.0 wt.% PHA [46].

On the other hand, RL production was lower significantly ($p < 0.05$) in transformant strain, compared to wild-type strain for all the carbon sources tested. When glucose and fructose were utilised, the RL concentration decreased to 0.170 ± 0.001 g/L (six-fold) and 0.33 ± 0.05 g/L (six-fold). Using glycerol and glycerine pitch, the RL concentrations were reduced to 0.37 ± 0.07 (threefold) and 0.23 ± 0.03 (fourfold) g/L, respectively. Using molasses and sweetwater, RL concentration decreased to 0.03 ± 0.01 and 0.04 ± 0.01 g/L. The introduction of *phaC* gene increased PHA production but decreased RL production. The decrease in RL production clearly indicated competition for intermediates between PHA and RL biosynthesis pathways, since the lipid moieties derived from fatty acid biosynthesis are involved in both pathways [47].

Composition of PHA

The PHA monomers accumulated by wild-type *P. aeruginosa* UMTKB-5 strain was different according to the substrate used, with the chain length (between C_6 and C_{10}) as shown in Table 1(a). 3-hydroxyhexanoate (HHX) [C_6] was the only major type of PHA found when sugars (glucose, fructose) and sugar cane by-products (molasses, sweetwater) were utilised as the sole substrate. The strain was able to accumulate high compositions of 3-hydroxydecanoate (HD) [C_{10}], ranging from 50 to 90 mol% when glycerol and glycerine pitch were used. Besides that, 3-hydroxyoctanoate (HO) [C_8] was also produced using these carbon substrates. The finding of this study agrees with Hori and co-workers [48], such that HHx, HO and HD monomers were accumulated by *P. aeruginosa* utilising decanoate, glucose and ethanol as substrates. On the other hand, Marsudi and co-workers [16] also obtained PHA monomer compositions of HHx, HO and HD when *P. aeruginosa* IFO03924 utilised 13.8 g/L of glycerol as carbon substrate. In addition, *P. aeruginosa* IPT169 was able to produce PHA monomer composition of HHx, HO, HD and HDd when the strain was fed with corn oil as carbon substrate [49].

The PHA monomer composition from transformant *P. aeruginosa* UMTKB-5 was different compared to the wild-type strain. The transformant strain showed successful accumulation of P(3HB) ranging from 20 to 50% for all the carbon sources examined, with exception of molasses and sweetwater [Table 1 (b)]. Glycerine pitch gave the highest P(3HB) monomer composition, accounting for 49%. This was possible because this genetically modified strain harbours the PHA synthase gene (*phaC*) of *Cupriavidus* sp. USM1020, a strain well-known for P(3HB) production.

The reported compositions of PHA monomer were very similar to those provided with glucose and fructose as sole substrates. However, no P(3HB) was detected when molasses and sweetwater were fed. This is mainly due to the low concentration of glycerol contained in both molasses and sweet water to trigger P(3HB) production. In the present study, the glycerol present in molasses and sweetwater supported cell growth. However, it was insufficient to generate intermediate into production of P(3HB).

In the present study, the monomer composition of PHA from the transformant strain was similar to that from the study by Cepades et al. [50], whereby PHA composed mainly of 3HB, 3HHx and 3HD were produced when non-naturally occurring *P. aeruginosa* harbouring PHA synthase gene from *Aeromonas* sp. was provided with glucose as the only carbon source. The type of microorganism, environmental condition, and carbon substrate affect PHA composition and production of scl or mcl fatty acids [51]. Rehm et al. [52] noted that the *phaC*, carbon sources and metabolic routes play important roles in determining the compositions of PHA.

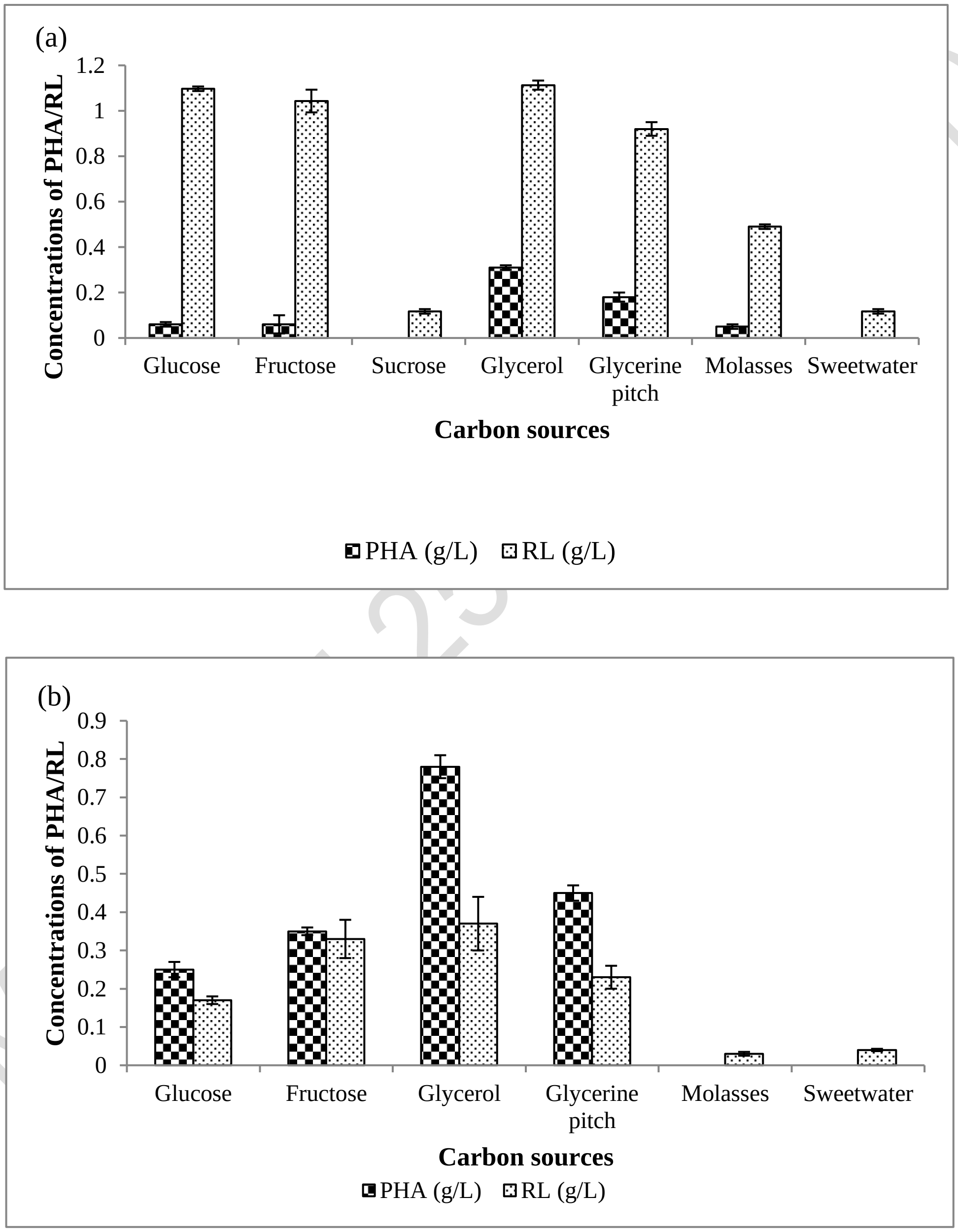

Figure 2. Simultaneous production of PHA and RL by wild-type (a) and transformant (b) *Pseudomonas aeruginosa* UMTKB-5 strains using different carbon sources

Table 1. Composition of PHA produced by (a) wild-type and (b) transformant *P. aeruginosa* UMTKB-5 utilising different carbon sources

Carbon Source (g/L)	**^{a}Relative Amount of Monomer in PHA (%)**		
	HHx	**HO**	**HD**
Glucose	100	0	0
Fructose	100	0	0
Glycerol	7.8	4.4	87.8
Glycerin pitch	17.0	24.0	59.0

Carbon sources (g/L)	**^{b}Relative amount of monomer in PHA (%)**			
	HB	**HHx**	**HO**	**HD**
Glucose	27.6	72.4	0	0
Fructose	34.4	65.6	0	0
Glycerol	34.0	46.4	11.8	7.8
Glycerin pitch	49.9	30.3	9.2	10.5

HB: 3-hydroxybutyrate, HHx: 3-hydroxyhexanoate, HO: 3-hydroxyoctanoate, HD: 3-hydroxydecanoate

Effect of nitrogen sources on the yield of PHA and RL

Glycerol at a C/N ratio of 50 was selected as the sole carbon source for subsequent comparison of the production of PHA and RL between different nitrogen sources since it is an economical and renewable resource and has also produced one of the highest PHA yields (Figure 2). The highest production of PHA and RL was achieved when wild-type *P. aeruginosa* UMTKB-5 was supplemented with $CO(NH_2)_2$ as nitrogen source and glycerol as sole carbon source, accounting for 0.242 ± 0.011 and 2.308 ± 0.010 g/L, respectively (Table 2). The nitrogen source that provided the highest PHA concentration of 2.242 g/L was $CO(NH_2)_2$. Our findings agreed with a study by Tripathi *et al*. [6], which also obtained the highest PHA concentration (2.89 g/L) using the same nitrogen source, $CO(NH_2)_2$.

For RL production, $CO(NH_2)_2$ gave the highest RL concentration (2.308 ± 0.010 g/L), where all carbon sources tested had significant difference in the amount of PHA produced ($p < 0.05$). However, Moussa et al. [9] obtained the highest RL concentration at 0.34 g/L when $NaNO_3$ was used compared to $CO(NH_2)_2$, which was 0.3 g/L. The carbon source that yielded the most PHA was different between the two mentioned studies, which could be due to the difference in the concentration and type of carbon source, the concentration of nitrogen source supplemented into the culture medium, and incubation time. $CO(NH_2)_2$ exists as an uncharged polar molecule, which is easily assimilated into *P. aeruginosa* [53].

Characterisation of PHA from wild-type *P. aeruginosa* UMTKB-5

The thermal properties of the polymers produced by *P. aeruginosa* UMTKB-5 utilising glycerol and glycerine pitch as sole carbon sources were shown in Table 3. The T_m of PHA polymers produced by wild-type *P. aeruginosa* UMTKB-5 using glycerol and glycerine pitch was approximately 48 to 50 ºC. The value was almost similar with polymers obtained from *P. aeruginosa* [54]. On the other hand, Guo et al. [37] obtained T_m range of 54 to 55 ºC when *P. mendocina* strains were grown on 20 g/L of glucose as carbon source and 0.7 g/L of $(NH4)_2SO_4$ as nitrogen source. Conversely, in this study, the T_g could not be detected from the endothermic peaks in each DSC trace, which indicates that the samples are amorphous.

It is important to determine the physical properties of biopolymer produced to determine its suitability in a particular application. To measure the molecular mass of mcl-PHA produced by *P. aeruginosa* UMTKB-5, both number-average molecular weights (*Mn*) and polydispersity (*Mw/Mn*) were measured using GPC. The *Mn* of the polymer is important in determining its strength as well as ability to be shaped [1].

Using glycerol as carbon source, the weight-average molecular weight (*Mw*) and the number-average molecular weight (*Mn*) were $4.0\mathrm{x}10^5$ and $1.4\mathrm{x}10^5$ Da, respectively. While when glycerine pitch was utilised, *Mw* and *Mn* of $4.7\mathrm{x}10^5$ and $4.19\mathrm{x}10^5$ were obtained, respectively. The physical properties produced from glycerol was comparable with the polymer produced by *P. aeruginosa* ATCC, which obtained *Mw* of polymer ranged from $7.7\mathrm{x}10^4$ to $1.88\mathrm{x}10^5$ Da, when the said strain was grown on odd chain fatty acids as carbon sources [54]. Polydispersity (PI) (*Mw/Mn*) of PHA using glycerol and glycerine pitch obtained in the present study was 2.85 and 4.19, respectively. Hori et al. [48] obtained PI of 2.0 when *P. aeruginosa* IF03924 was grown on glucose as carbon source. On the other hand, PI of 1.8-2.0 was obtained when *P. putida* G016 was grown on Sodium terephthalate (TA) and waste glycerol (WG) in fed-batch fermentation [55]. These values are typical for mcl-PHA polymer. However, in this study, a slightly higher PI was obtained when glycerine pitch was fed as carbon source. However, it was at the same level as that mcl-PHA synthesised by *P. aeruginosa* strains [48].

On the other hand, thermal degradation studies were carried out for mcl-PHA polymers produced by *P. aeruginosa* UMTKB-5 utilising glycerol and glycerine pitch as carbon sources using thermogravimetric analysis (TGA). TGA was used to measure the degradation temperature of polymers [56]. Results obtained in the present study showed that the degradation rate of the polymers were 310 ºC (Table 3). It was higher in comparison with degradation temperatures of mcl-polymers of *P. putida* ranging from 280 to 290 ºC [55]. The different in degradation temperatures probably due to the different in culture condition used in each study such as carbon and nitrogen source, temperature, pH and agitation [57]. Kenny et al. [55] used MSM medium supplemented with Sodium terephthalate (TA) and waste glycerol (WG) from biodiesel manufacture as carbon sources. *P. aeruginosa* 47T2 obtained degradation temperature of 295 ºC when the strain was grown on waste cooking oil supplemented with $CO(NH_2)_2$ as nitrogen source [58]. Different monomers ratios of the polymers and also different in culture conditions used affected the thermal properties of the polymer produced [59]. The properties of produced polymers can be tailored to be applied in various industries ranging including packaging materials or highly elastictic ones, because the polymers are produced from renewable carbon sources [60].

Characterisation of RL congener from wild-type *P. aeruginosa* UMTKB-5

Rhamnolipids comprise one or two β-hydroxy fatty acids of varying chain lengths (C_8–C_{22}) linked to one or two rhamnose rings [39]. Common RL congeners (i.e., substance structurally related to RL by origin and function) include di-RL, α-l-rhamnopyranosyl-α-l-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RhaRha C10C10) and RhaRhaC10, as well as mono-RL homologs, RhaC10 and RhaC10C10. Other homologs were found to be present within the *m*/*z* range of 475–677 depending on the number of rhamnose ring, fatty acid chain, and carbon [39].

Mass spectrometry analysis revealed the presence of mono- and di-RL moieties in the RL mixture. Three major components of *m/z* 649, 621 and 675 were detected when using glycerol supplemented with $CO(NH_2)_2$. These corresponded to the congeners $RhaRhaC_{10}C_{10}$, $RhaRhaC_{10}C_8$ and $RhaRhaC_{10}C_{12:1}$ at 38.14%, 25.69%, and 17.14% respectively. The LCMS also detected $RhaRhaC_8$ (*m/z* 451), $RhaRhaC_{10}C_{10:1}$ (*m/z* 647), $RhaC_{10}$ (*m/z* 333) and $RhaRhaC_{12}$ (*m/z* 507) in their biosurfactant mixture (Table 4). On the other hand, when glycerol supplemented with NH_4NO_3 was used, five major components of *m/z* 649, 621, 479, 675, and 677 were detected. These corresponded to $RhaRhaC_{10}C_{10}$, $RhaRhaC_{10}C_8$, $RhaRhaC_{10}$, $RhaRhaC_{10}C_{12:1}$, and $RhaRhaC_{10}C_{12}$ at 25.64%, 21.76%, 12.92%, 10.83%, and 10.49% respectively. A few minor components were also detected, which were $RhaRhaC_8$ (*m/z* 451), $RhaRhaC_{10}C_{10:1}$ (*m/z* 647), $RhaC_{10}$ (*m/z* 333) and $RhaRhaC_{12}$ (*m/z* 507) (Table 4).

The result in Table 4 shows that $RhaRhaC_{10}C_{10}$ was the predominant component in the biosurfactant mixture of glycerol supplemented with $CO(NH_2)_2$) and NH_4NO_3, accounting for relative abundance 38.14% and 25.64% respectively, using negative electrospray ionisation. In addition, the di-RL formed 96.81% and 95.53% in glycerol that respectively utilised $CO(NH_2)_2$) and NH_4NO_3. The result obtained in this study agrees with Siddharta et al. [61], whereby $RhaRhaC_{10}C_{10}$ was found as an abundance mixture (42.6%) when *P. aeruginosa* was provided with glycerol. Another study by Lotfabad et al. [62] also produced $RhaRhaC_{10}C_{10}$ (35.93%) as an abundance mixture when *P. aeruginosa* MR01 was supplemented with glucose as carbon source. On the other hand, Aparna et al. [63] also obtained the same range of *m/z* when *Pseudomonas* sp. 2B was grown on different carbon sources and analysed by negative mode. A mixture of RL with molecular ions at *m/z* 333,479, 504, 505, 532, 650 and 678 were produced. The results obtained were very similar with the RL congener produced in this study.

This study demonstrated that the biosurfactant produced by *P. aeruginosa* UMTKB-5 was indeed RL, in which $RhaRhaC_{10}C_{10}$ and $RhaRhaC_{10}C_8$ were the predominant mixtures compared to mono-RL. Most of the authors reported di-RL as the predominant component of RL biosurfactants from *P. aeruginosa* [15,62]. Déziel et al. [64] found that di-RL has higher CMC concentration, thus having higher surface activity over mono-RL. Having these great properties, di-RL is widely used in the bioremediation of poly-aromatic hydrocarbon (PAH) to treat contaminated sites [62]. The applications of RL depend on the proportion and type of monomers produced by bacterial species [65]. The RL is being produced as a mixture of different molecules [65]. The difference in the proportion of RL mixture produced in this study in comparison with those reported in the literature could be explained by the difference of carbon source, nitrogen source, medium and extraction technique used [8, 63]. In addition, *P. aeruginosa* strains or species can also affect the proportion of RL-congener produced [64, 65].

Table 2. Effect of various nitrogen sources on the production of PHA and RL by wild-type *P. aeruginosa* UMTKB-5[a] using glycerol as sole carbon source at a C/N ratio of 50

Nitrogen Sources	**CDW (g/L)**	**Rhamnolipid (g/L)[b]**	**PHA Content (wt.%)[c]**	**PHA Concentration (g/L)[d]**	**PHA Composition (mol%)**		
					HHx	**HO**	**HD**
$NH_4H_2PO_4$	1.13 ± 0.12	1.534 ± 0.260	7.10 ± 0.78	0.080 ± 0.001	4.5	25.5	70
$CO(NH_2)_2$	2.48 ± 0.13	2.308 ± 0.010	9.78 ± 0.21	0.242 ± 0.011	4.5	26.9	68.5
$NaNO_3$	1.54 ± 0.04	1.309 ± 0.100	6.10 ± 0.52	0.010 ± 0.001	3.8	25.8	70.4
NH_4Cl	1.84 ± 0.07	0.856 ± 0.110	6.40 ± 0.48	0.115 ± 0.010	3.9	26.2	69.8
$(NH_4)_2SO_4$	1.12 ± 0.06	0.967 ± 0.060	7.47 ± 0.47	0.082 ± 0.012	3.3	27.7	69.1
NH_4NO_3	1.96 ± 0.05	1.541 ± 0.090	4.36 ± 0.81	0.085 ± 0.011	7.9	24	68.1

$NH_4H_2PO_4$, Ammonium dihydrogen phosphate; $CO(NH_2)_2$, urea; $NaNO_3$, Ammonium nitrate; NH_4Cl, Ammonium chloride; $(NH_4)_2SO_4$, Ammonium sulphate; NH_4NO_3, Ammonium nitrate; HHx, 3-hydroxyhexanoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate

[a] Incubated for 72 h at 30 ºC at 200 rpm in MSM

[b] RL concentration as determined by orcinol assay

[c] PHA content in freeze-dried cells were determined by gas chromatography

[d] PHA concentration = PHA content x CD

Table 3. Thermal properties and molecular weights of PHA polymers produced by wild-type *P. aeruginosa* UMTKB-5 using glycerol and glycerine pitch

Carbon Sources	**Thermal Properties**[a]				**Molecular Weights**[e]		
	T_g (°C)[b]	T_m (°C)[c]	ΔH_m (J/g)[d]	T_d (°C)[e]	M_n (x 10^5 Da)	M_w (x 10^5 Da)	M_w/M_n
Glycerol	ND	48.7	4.3	310	1.4	4.0	2.85
Glycerin pitch	ND	49.8	2.6	310	1.1	4.7	4.19

[a] Thermal properties determined by DSC

[b] Tg- glass transition temperature

[c] T_m- melting temperature

[d] ΔH_m, enthalphy of fusion

[e] Determined by TGA

[f] M_w, weight average molecular weight; M_n, number average molecular weight

ND = not detected

Table 4. Structural and relative abundance of RL homologue produced by *P. aeruginosa* UMTKB-5 after 72 h of cultivation determined by LCMS analysis

Congener	**Pseudomolecular ion (*m/z*)**	**Glycerol + $CO(NH_2)_2$**[a]		**Glycerol + NH_4NO_3**[b]	
		Retention Time	**Relative Abundance (%)**	**Retention Time**	**Relative Abundance (%)**
$RhaRhaC_{10}C_8$	621	2.573	25.69	2.563	21.76
$RhaRhaC_8$	451	2.585	9.00	2.568	9.23
$RhaRhaC_{10}C_{10:1}$	647	2.87	2.88	2.833	9.82
$RhaRhaC_{10}C_{10}$	649	3.208	38.14	3.152	25.64
$RhaRhaC_{10}$	479	-	-	3.18	12.92
$RhaC_{10}$	333	3.725	3.19	3.612	4.47
$RhaRhaC_{10}C_{12:1}$	675	3.771	17.14	3.679	10.83
$RhaRhaC_{12}$	507	4.411	3.96	4.347	2.52
$RhaRhaC_{10}C_{12}$	677	-	-	4.387	10.49

[a] The strain was cultured using glycerol supplemented with $CO(NH_2)_2$) as nitrogen source

[b] The strain was cultured using glycerol supplemented with NH_4NO_3 as nitrogen source

Conclusion

Overall, *P. aeruginosa* UMTKB-5 strains have exhibited successful bioconversion of oleo/agro industrial by-products into value added PHA and RL in a single cultivation medium. Glycerol was found to be the most preferred by-product for *P. aeruginosa* UMTKB-5 and it resulted in one of the highest concentrations of PHA and RL simultaneously. However, the mutant strain could not be further tested for PHA and RL production due to the instability of the plasmid. A possible way to overcome this is by homologous recombination of the gene into the bacterial chromosome. The application of dual-production and inexpensive renewable carbon sources will considerably reduce the final production cost for these biomaterials. To make the industrial production of PHA and RL viable, significant efforts in strain optimisation and bioengineering are needed to produce high yield of biomaterials using economical carbon sources.

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