Application of Sweetwater as Potential Carbon Source for Rhamnolipid Production by Marine *Pseudomonas aeruginosa* UMTKB-5

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Abstract: Rhamnolipid (RL), the major component of biosurfactant is commonly produced via bacterial fermentation from selected carbon sources. Biosynthesis of RL is initiated by nitrogen limitation and presence of excess carbon source. RL is completely biodegradable and non-toxic. Applications of RL include the production of fine chemicals, enhancement of biodegradation, food industries and pharmaceutical products. In this study, efforts were taken to biosynthesize RL using Pseudomonas aeruginosa UMTKB-5 isolated from marine sediment. The bacterium was fed with cane sugar refinery by-product, sweetwater as sole carbon source and 5 different types of nitrogen sources. Three different of carbon to nitrogen (C/N) ratios were tested in this study. The sweetwater was first characterized for its components. Sweetwater is mainly comprised of water (79.9 wt%) and glycerol (10.3 wt%). The total sugar content is 17.4 wt% and mainly comprises of sucrose, glucose and fructose. Biosynthesis of RL was carried out in 50 mL shaken-flask cultures, incubated at 30˚C for 72 h at 200 rpm. Sulfuric acid was used to hydrolyze rhamnose groups of RL in the culture supernatant into methyl furfural. The hydrolyzed sample containing rhamnose was reacted with orcinol (1-3-dihydroxy-5-methylbenzene). The concentration of RL produced was measured spectrometrically at 421 nm. The surface tension was measured using Du Nouy Ring method. The result obtained showed that production of RL using sweetwater was in the range of 42 – 50 mg/L. The cell biomass was recorded in the range of 329 – 729 mg/L. The lower surface tension (47.26 mN/m) activity occurred when ammonium chloride with C/N ratio of 35 was applied. The findings of this study demonstrate the potential application of agro-industrial by-product, sweetwater as a renewable carbon feedstock for RL production via bacterial fermentation.

Key words: Rhamnolipid, *Pseudomonas aeruginosa*, sweetwater, cane sugar refinery by-product.

1. Introduction

Biosurfactants are reported as the amphipathic compound produced extracellularly by a variety of microorganisms including yeast, bacteria and filamentous fungi [1]. These naturally occurring surface active compounds are getting much attention as an alternative for synthetic surfactant. With regards to its antimicrobial properties, biodegradability, low toxicity as well as ecological acceptability, biosurfactantsoffer several potential industrial applications. Biosurfactants are applied in the food industry,

cosmetic industries [2], agricultural fields, especially as biological control of pathogens and in accelerating biodegradation of petroleum [3], [4]. Glycolipids and lipopeptides are the most commonly isolated biosurfactants [5]. Rhamnolipid (RL), which belongs to the glycolipids class, is a naturally occurring biosurfactant which is composed of rhamnose sugar molecules and β-hydroxyl alkanoic acids (lipid). Its amphiphilic property enables the molecules to concentrate at interfaces, thus reducing the surface tension of aqueous media [4]. Jarvis and Johnson (1949) first isolated RL from *P. aeruginosa* [6].

RL production from *Pseudomonas* sp. has high surface activity, thus, becoming an attractive target for biotechnology research [7]. The RL produced by *P. aeruginosa* in liquid cultures are mainly rhamnosyl-β-hydroxydecanol-β-hydroxydecanoate(mono-RL) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-RLs) [8]. RL biosynthesis generally involved three major parts, which are biosynthesis of a lipid moiety, biosynthesis of sugar moiety and enzymatic reactions [9]. RL biosynthesis begins with the transfer of two rhamnose moieties from TDP-L-rhamnose. For mono-RL synthesis, the enzyme rhamnosyl-transferase 1(Rt 1) catalyzes the rhamnose transfer to β-hydroxydecanol-β-hydroxydecanoate. Meanwhile, di-RLs are synthesized by the enzyme rhamnosyl-transferase 2 (Rt 2) from TDP-L-rhamnose and mono-RL *P. aeruginosa* produces RL during stationary growth phase in limiting concentration of nitrogen sources and excess of carbon sources [10]. Previous studies have reported the production of RL by *P. aeruginosa* using various carbon sources. For example, using vegetable based oils such as, soybean oil [11] and corn oil [12], glycerol [13] as well as industrial by-products or wastes [14].

Although biosurfactant exhibit such important advantages, they are not extensively employed due to the relatively high production cost [15]. Carbon sources are vital for RL production and are one of the high cost absorbing factors. A possible strategy to reduce production cost would be using renewable carbon source such as agro-industrial waste and by-products as a substrate. This study highlights the application of sweetwater, a novel substrate for RL production by a marine isolate, *P. aeruginosa* UMTKB-5.

2. Methodology

2.1. Bacterial Strain and Inoculum Preparation

P. aeruginosa UMTKB-5 (GenBank accession number: KT194193.1) was previously isolated from marine sediment [16]. *P. aeruginosa* UMTKB-5 was grown overnight at 30 °C on nutrient rich (NR) agar. Two loop-full of culture were inoculated in NR medium consisting of the following components: per liter; 10 g peptone, 10 g meat extract and 2 g yeast extract [17]. Approximately 14 g/L of bacteriological agar was added for NR agar. Pre-culture was incubated on a reciprocal shaker at 200 rpm, 30 °C.

2.2. Characterization of Sweetwater

The sweetwater was collected from Gula Padang TerapSdn. Bhd. (Kedah, Malaysia). The sweetwater was sent to TPM Biotech Sdn. Bhd. and ITS Testing Services (M) Sdn. Bhd., for sugar content and other analysis.

2.3. RL Biosynthesis

Cultivation was carried out in 50 mL mineral salts medium (MSM), per liter: 2.80 g of KH_2PO_4, 3.32 g of Na_2HPO_4 and 0.25 g of hydrated $MgSO_4.7H_2O$ using 250 mL Erlenmeyer flasks [17]. The nitrogen source concentration was fixed at 0.25 g/L and the following types were used; NH_4Cl, $(NH_2)SO_4$, NH_4NO_3, NH_2CONH_2 and $NaNO_3$ [18]. Carbon to nitrogen ratios (C/N) of 20 and 35 were applied. Approximately 3.5 mL (200 mg/L) of bacteria pre-culture was transferred into 46 ml of MSM supplemented with 1 mL/L of trace elements (TE). TE components are as follows: per liter; 0.22 g $COCl_2.H_2O$, 9.70 g $FeCl_3$, 7.80 g $CaCl_3$, 0.12 g $NiCl_2.6H_2O$, 0.11 g $CrCl_3.6H_20$ and 0.16 g $CuSO_4.5H_20$ [19]. The cultures were incubated at 30 ºC, 200 rpm for 72 h. Cells were harvested by centrifuging at 4 ºC, 9000 rpm for 5 min using *HIMAC CR 22N* (Hitachi,

Tokyo) high-speed refrigerated centrifuge machine. The cell pellets were kept in -80°C deep freezer, *MDF-U5537* (Sanyo, Japan). On the other hand, the supernatants were subjected to orcinol assays.

2.4. Quantification of RL

The concentration of RL was determined by orcinol assay using the method proposed by [20], [21] with some modifications. Approximately 400 µL of culture supernatant was extracted twice with 750 µL of diethyl ether. After being a vortex for 3 min, the fractions were evaporated to dryness and 400 µL of pH 8 phosphate buffer was added. To 100 µL of each sample, 900 µl of orcinol reagent (0.19% w/v of orcinol in 53 % v/v of H_2SO_4) was added. After heating for 30 min at 80 ºC, the samples were cooled at room temperature. The optical density of samples was determined using a spectrophotometer, *Varioskan™ Flash Multimode Reader* (Thermo Scientific, USA) at 421 nm. The rhamnolipid concentration was calculated from the standard curve prepared with L-rhamnose and corrected with the multiplication factor, 2.25 [22].

2.5. Lyophilization and Cell Dry Weight Measurement

The lyophilization of cell pellets was done using *Freezone 4.5 Freezer Dry System* (Labconco, USA). Cells were frozen at -80°C was subjected to lyophilization for 72 h before determining the cell dry weight (CDW). The CDW was measured using a *CP 2245* (Sartorius, Germany) electronic balance.

2.6. Surface Tension Measurement

The supernatants were then subjected to surface activity measurements using *Sigma-701* (Attension, Finland) tensiometer. The Du-Nouy-Ring method was used to measure the surface tension [9]. The procedure was carried out at room temperature.

2.7. Statistical Analysis

The data analysis was conducted using the SPSS software (version 20.0). One-way ANOVA was applied to determine any significance difference between nitrogen sources and the RL production. The results are considered significant if $p < 0.05$.

3. Results and Discussion

3.1. Characterization of Sweetwater

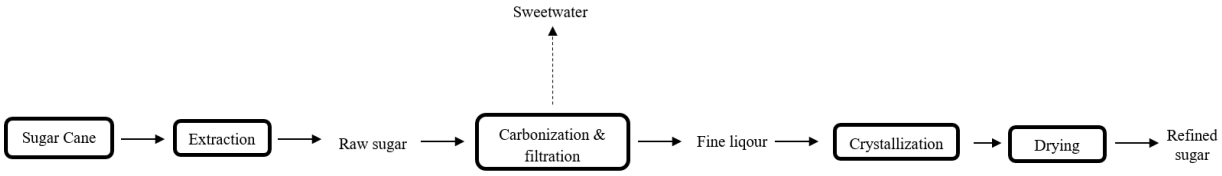

Fig. 1. Cane sugar refinery process.

Sweetwater is one of the main by-products of cane sugar refining process. Sweetwater is also described as water that still contains sucrose from anywhere in the refinery. Based on the sugar refining process (Fig. 1), sweetwater is produced after undergoing carbonization and filtration as waste. Table 1 shows the composition of sugars, glycerol, and water in sweetwater. Sucrose was detected as the highest remaining sugar component with 17.4 wt%. Then former is followed by glucose (0.6 wt%) and fructose (0.4 wt%). Besides this, glycerol was also recorded in sweetwater with a concentration of 10.3 wt%. Hence, sweetwater is a promising cost-effective carbon feedstock comprising the consortia of carbon sources from the waste. To date, there are few published reports on the usage of sweetwater as a carbon substrate for RL production.The physical parameter of sweetwater is stated in Table 2. The pH of sweetwater is acidic but

the strain was still able to produce RL. The pH is important parameter because it involves the chemical reactions of the living cells [23]. The previous study showed that the maximum RL production occurred when pH was in the range of 6 to 6.8 [24]. In addition, sweetwater was able to dissolve completely in liquid (98.90%). This makes sweetwater a water-soluble substrate.

Table 1. The Components of Sweetwater

Compositions in sweetwater	Contents	Method
Glucose (wt%)[a]	0.6	AOAC 982.14 (HPLC)
Fructose (wt%)[a]	0.4	AOAC 982.14 (HPLC)
Sucrose (wt%)[a]	17.4	AOAC 982.14 (HPLC)
Glycerol content (wt%)[b]	10.3	Titration
Water content (wt%)[b]	79.9	Karl Fischer

[a] Analyzed by TPM Biotech SdnBhd, Malaysia
[b] Analyzed by ITS Testing Services (M) SdnBhd, Malaysia

Table 2. The Properties of Sweetwater

Test	Value	Method
pH value[a]	4.86	AOAC 32.1.20
Solubility test (%)[a]	98.90	QC/0054
Alkalinity as NaOH (% w/w)[b]	0.015	APHA 2130 B
Specific gravity, 37°C[b]	1.078	ASTM D 1052
Dynamic viscosity, 37°C (mPa.s)[b]	1.36	Brookfield viscometer
Total residue at 160°C (% w/w)[b]	14.3	Oven drying

[a] Analyzed by TPM Biotech SdnBhd, Malaysia
[b] Analyzed by ITS Testing Services (M) SdnBhd, Malaysia

3.2. Evaluation of Different Nitrogen Sources and C/N Ratio

Table 3. Production of RL by P. aeruginosa UMTKB-5 Using Different C/N Ratio and Nitrogen Sources[a]

C/N ratio	Nitrogen sources	CDW[b], mg/L	RL concentration[c], mg/L
20	NH_4Cl	469.0 ± 10	47.8 ± 3
	$(NH_4)_2SO_4$	446.0 ± 30	46.7 ± 0
	NH_4NO_3	499.0 ± 70	44.6 ± 3
	NH_2CONH_2	329.3 ± 14	44.3 ± 2
	$NaNO_3$	476.0 ± 90	45.8 ± 2
35	NH_4Cl	458.0 ± 80	45.2 ± 3
	$(NH_4)_2SO_4$	564.0 ± 60	45.2 ± 2
	NH_4NO_3	723.0 ± 35	42.8 ± 1
	NH_2CONH_2	666.0 ± 20	50.1 ± 6
	$NaNO_3$	535.0 ± 33	44.1 ± 4
50	NH_4Cl	649.0 ± 10	45.4 ± 3
	$(NH_4)_2SO_4$	546.0 ± 33	45.6 ± 2
	NH_4NO_3	658.0 ± 88	43.5 ± 1
	NH_2CONH_2	729.0 ± 52	46.3 ± 2
	$NaNO_3$	543.0 ± 21	43.4 ± 1

RL, rhamnolipid. Data shown are means of triplicates. [a]Incubated for 72 h at 30°C at 200 rpm in MSM, [b]Dried cell pellets was weighed after 72 h of lyophilization, [c]RL concentration was quantified using orcinol assays

Here, *P. aeruginosa* UMTKB-5 was tested for RL production using sweetwater as a sole of carbon source. *P. aeruginosa* is previously known as the primary producer of RL [9]. Several factors are known to influence

the production of RL including nutritional and environmental parameter [23]. Excess carbon source and limitation of nitrogen sources is the important key in the biosynthesis of RL [9]. Here, effort was taken to evaluate RL production using sweetwater with a combination of different nitrogen sources. The concentration of nitrogen sources was fixed to 0.25 g/L of concentration. Results of the different C/N ratio are stated in Table 3. The RL production was in the range of 42 – 50 mg/L. However, the one-way ANOVA testproves that there was no significance different in the RL concentrations from three different C/N ratios, $p = 0.183$, ($p > 0.05$).

It is important to study C/N ratio as it is known to play an important role in optimizing RL production [24]. Nevertheless, the results prove the ability of *P. aeruginosa* UMTKB-5 to successfully convert sweetwater into RL. As in the previous study of glycolipid biosurfactant production, Kim and coworkers recognized that the organic nitrogen such as urea, NH_2CONH_2 give support growth of the cells [25]. Urea was found to promote cell growth as higher cell biomass (Table 3). Increase in CDW was recorded (329 – 729 mg/L) based on different C/N ratios. Fig. 2 illustrates the metabolic pathway of RL [26], [27]. The different carbon components found in sweetwater are isomerized into D-glucose-1-phosphate, the precursor for sugar moiety in RL [9]. On the other hand, lipid moiety is attained from fatty acid *de novo* synthesis.

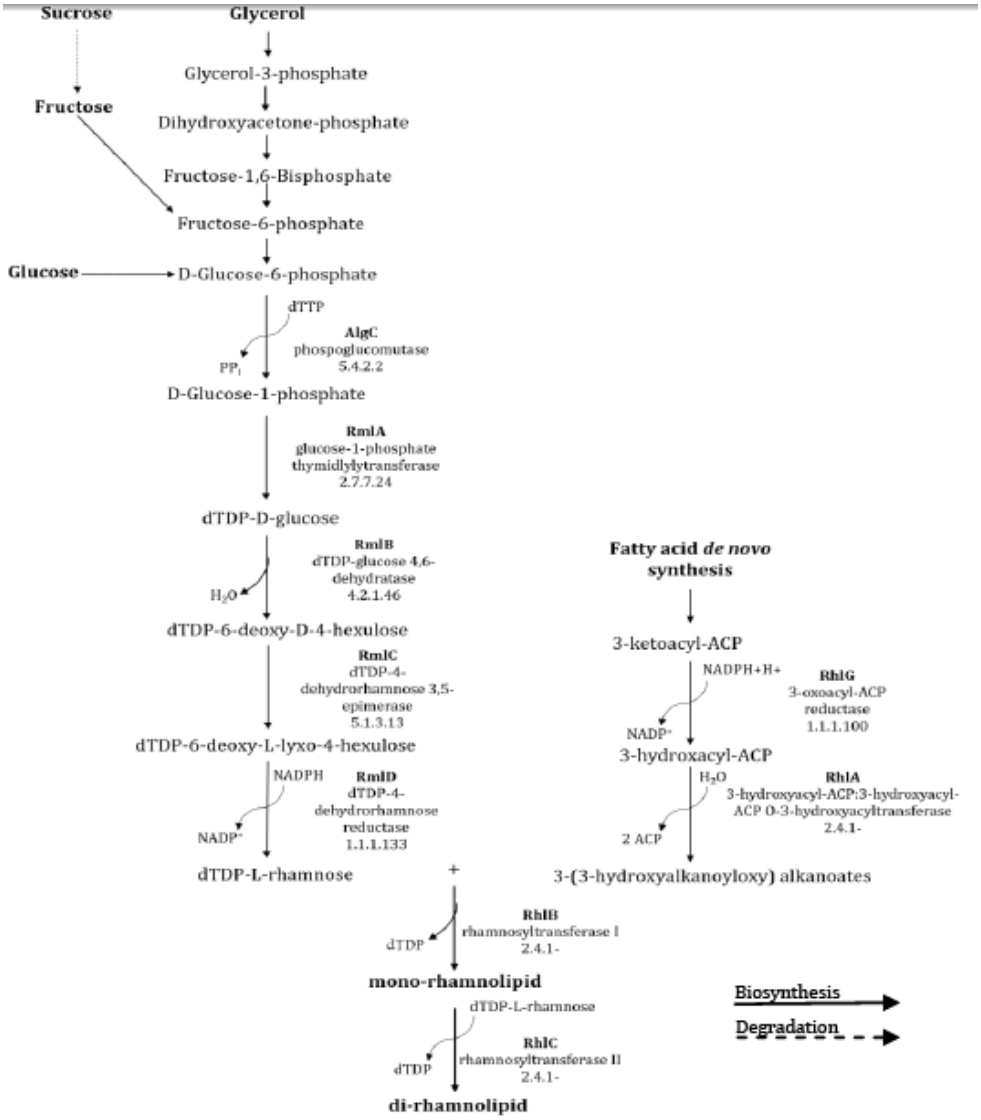

Fig. 2. RL biosynthesis pathway in *P. aeruginosa*.

3.3. Surface Tension of RL Produced from Sweetwater

The distilled water was selected as negative control in conducting surface tension measurement of RL produced by *P. aeruginosa* UMTKB-5. The surface tension of water is 72 mN/m [28]. All of the RLs produced from different nitrogen sources with different C/N ratio showed a sharp decrease in surface tension when compared with the control (Fig. 3). The range of surface tensions measured was between 47 – 59 mN/m. The lowest surface tension of 47.26 mN/m was recorded when NH_4Cl used as nitrogen source with C/N ratio of 35. The surface activity is the crucial properties of surfactant [29]. This phenomenon occurs when the surfactant is able to decrease the intermolecular force between molecules of liquid [29]. Biosurfactant like RL with high surface activity can be utilized in various application including enhancement of biodegradation, food industry, cosmetic and pharmaceutical applications that are related with surface chemistry [30]-[32]. Efforts are being taken to well document the production and characteristics of the RL produced, in order to suffice commercial interest. Studies on the optimization of RL using statistical approach has been carried out [33]. The characterization of the RL congeners using mass spectrometry is also considered important as different mono-RL and di-RL might affect emulsification property and antimicrobial activity [34], [35].

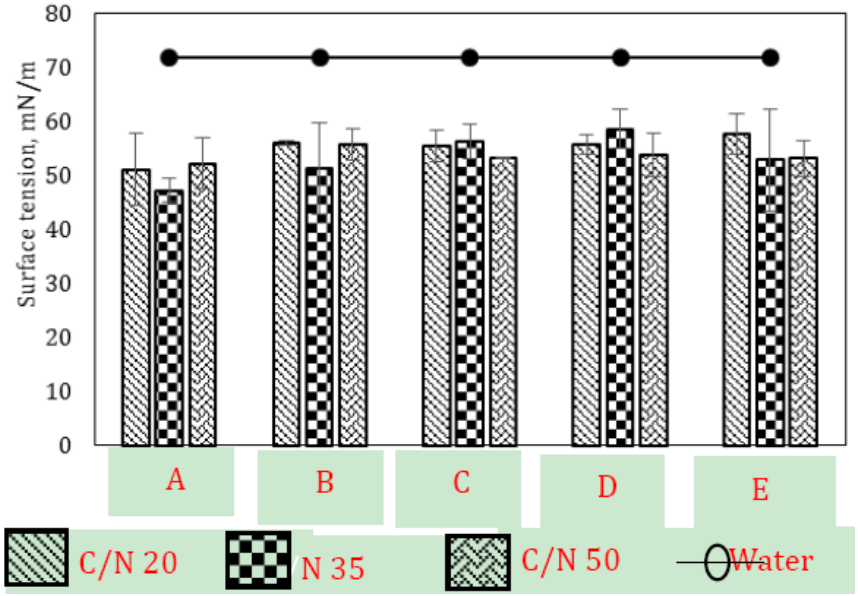

Fig. 3. Surface tension activity of RL based on the different C/N ratio. A: NH_4Cl; B: $(NH_4)_2SO_4$; C: NH_4NO_3; D: NH_2CONH_2; E: $NaNO_3$.

4. Conclusion

Based on the findings, it can be concluded that sweetwater could be a potential substrate for RL production. Application of this agro-industrial by-product as carbon feedstock could reduce the overall cost of RL production. The bioconversion of waste to wealth is a promising approach in development of sustainable biotechnological processes.

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