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SOLVENT-LESS APPROACH FOR THE RECOVERY OF PALM-BASED SOPHOROLIPIDS BIOSURFACTANT VIA SALTING-OUT METHOD

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ABSTRACT

A suitable recovery process aimed at purifying the sophorolipids (SL) biosurfactant with eliminating of solvents was studied. Two routes of recovery strategies to extract the SL from the culture medium were investigated: (1) organic-solvent extraction, (2) non-solvent extraction via salting-out methods. The recovered SL produced by yeast culture Starmerella bombicola *(ATCC 22214), supplemented with glucose and refined, bleached and deodorised (RBD) palm olein as the primary and secondary carbon sources, yielded in 53 g litre^{-1} and 99 g litre^{-1}, respectively. The SL were produced as a mixture of lactonic and acidic forms, as qualitatively predicted by the appearance of seven spots on the thin layer chromatography plates. By high performance liquid chromatography (HPLC) analysis, salting-out via precipitation method successfully reduced acidic compounds and resulted in lactonic-rich SL in the final product in which the purity was approximately 75%. This was revealed by the identification of five major structures and diacetylated lactonic SL (688 g mol^{-1}) as the dominant compound. In conclusion, salting-out is recommended as a potential downstream processing for recovery of palm-based SL.*

Keywords: sophorolipids, biosurfactant, salting-out, RBD palm olein, *Starmerella bombicola*.

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INTRODUCTION

Most yeast surfactants have been typically identified as glycolipids. Glycolipid biosurfactants are carbohydrates attached to a long-chain aliphatic acids or hydroxyaliphatic acids. The most interesting glycolipids are the sophorolipids (SL) produced in the form of extracellular oily secretion that are heavier than water (Van Bogaert and Soetaert, 2011). Production of SL has been reported to improve when vegetable oils are supplied along with glucose as carbon sources (Wadekar *et al*., 2012b). However, very few articles have been published on the use of palm oil as a carbon source even though palm oil is known as the most promising renewable resource for various industrial applications.

For initial recovery of biosurfactant from the fermentation broth, conventional methods such as organic solvent extraction are often applied, whereby solvents such as ethyl acetate are used to remove the hydrophilic compounds (Hubert *et al*., 2012) and hexane to remove residual lipophilic (Van Bogaert and Soetaert, 2011). Although these methods offer a simple separation of biosurfactant from impurities, they generate large amounts of organic wastes, which are impractical for large scale operation and may be harmful towards human and

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the environment. Hence, further improvement and development of efficient methods for the production and extraction of SL biosurfactant is necessary.

This research is focused at improving downstream processes to recover the SL with high purity and to reduce the use of solvents. Briefly, this study was carried out to produce a SL biosurfactant from refined, bleached and deodorised (RBD) palm olein as a secondary carbon source using *Starmerella bombicola* culture. A solvent-less approach was investigated for the recovery of the crude SL. Subsequently, the qualitative and quantitative information of the crude SL and salt treatment SL were identified using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and mass spectrometric (MS) techniques.

MATERIALS AND METHODS

Materials

For the production of SL, two types of media were employed: (1) yeast malt agar (Difco Lab, USA) as the growing media for yeast, and (2) basal medium containing KH_2PO_4 (Mallinckrodt, Mexico), NaCl (Mallinckrodt, Mexico), $MgSO_4.7H_2O$ (Ajax Finechem, Australia), $CaCl_2$ (Fisher Scientific, United Kingdom) and supplemented with yeast extract (Difco Laboratory, USA) and D-glucose (J. Kollin Chemicals, United Kingdom). Refined, bleached and deodorised (RBD) palm olein, supplied by the Malaysian Palm Oil Board (Malaysia), was used as the carbon source for the yeast culture.

For extraction and characterisation, the reagents used were analytical grade; while for analysis and chromatography, the reagents were HPLC grade. The reagents for extraction were: (i) solvent - ethyl acetate (Merck, Germany) and n-hexane (Merck, Germany), (ii) salts - ammonium sulphate (BioBasic, Canada) and aluminium sulphate (Merck, Germany), and (iii) buffer - KH_2PO_4 (BioBasic, Canada) and K_2HPO_4 (BioBasic, Canada). The reagents for characterisation were chloroform (Merck, Germany), methanol (Merck, Germany), acetic acid (Merck, Germany), acetonitrile (Sigma Aldrich, USA) and formic acid (Merck, Germany). The standard sample used was a commercial SL, *Sopholiance* (France).

Microbial Culture and its Maintenance

The yeast *S. bombicola* ATCC 22214, was obtained from the American Type Culture Collection (Rockville, Maryland). The strain was grown on yeast malt agar (Difco Laboratory, US) at 30°C, maintained at 4°C and sub-cultured every four weeks.

Production of Sophorolipids

S. bombicola inoculum was prepared by mixing a loopful of colony grown on Difco Agar culture into 0.1 litre sterile basal medium with the composition as follows: KH_2PO_4, 0.5% (w/v); $MgSO_4.7H_2O$, 0.1% (w/v); NaCl, 0.01% (w/v) and $CaCl_2$, 0.01% (w/v), supplemented with glucose, 10% (w/v) and yeast extract, 5% (w/v) and incubated at 30ºC for 48 hr. The 0.1 litre inoculum was then subsequently transferred into 1 litre sterile production medium containing glucose, 10% (w/v); RBD palm olein, 10% (v/v); yeast extract, 5% (w/v); and freshly prepared basal medium, 75% (v/v, pH 5.5). The culture was then incubated for 168 hr at 30°C and 210 rpm in an orbital incubator shaker (Hotech 707, Taiwan). This experiment was analysed accordingly in means value of triple batches.

Recovery of Sophorolipids through Organic-solvent Extraction

This extraction process was done according to the method reported by Gao *et al*. (2013) and Hubert *et al*. (2012). Organic-solvent extraction was employed as the conventional recovery method to isolate SL from the growing medium. Four cascade unit operations were conducted. The first step, liquid-liquid extraction was conducted by adding ethyl acetate solvent into the pooled growing media from the same batch medium at the ratio of 1:1 (v/v) and shaken vigorously. To ensure that the product was completely recovered, the extraction process was repeated three times with an equal volume of ethyl acetate to the lower aqueous layer of medium (1:1, v/v). The second step involved evaporation where the pooled upper solvent layer was evaporated at 77°C using N-100 rotary evaporator with aspirator pump (Eyela, Tokyo). The resulting crude product and residual oil were introduced to the third step which was the liquid-liquid extraction by washing three times with n-hexane at the ratio of 1:1 (v/v). As a final step, the mixed product was evaporated at 68ºC. The extracted SL were dried at 105ºC overnight to complete the removal of hexane, and dried again in desiccators over anhydrous Na_2SO_4 for 48 hr before weighing.

Recovery of Sophorolipids through Non-solvent Extraction via Salting-out Method

Alternative to the conventional method, a recovery method that eliminates the use of organic solvent was developed. This route is referred to as the bioseparation route (Bailey and Ollis, 1986) with three recovery steps starting with centrifugation to precipitation via salting-out method, and finally buffer exchanging through dialysis.

The non-solvent extraction route initially involved solid-liquid extraction via centrifugation to eliminate the un-metabolised yeast cells, residual oil and residual growth medium. This was done by modification of the method reported by Nunez *et al*. (2001). A 1 litre sample of a single batch medium was directly introduced to centrifugation using an EBA 21 centrifuge (HettichLab, Germany) at 13 000 rpm for 30 min at room temperature. The crude SL were collected and stored at 4ºC overnight to allow the SL to separate as a honey-like phase at the bottom of the flask. The yeast biomass that appeared on the top of the flask was washed several times with a copious amount of distilled water followed by centrifugation to ensure the entire residual yeast cells were removed. The mean values of extracted crude SL (g litre^{-1}) of triple batches were calculated.

Following the centrifugation step, the crude SL were recovered by precipitation with gentle mixing with aluminium sulphate [$Al_2(SO_4)_3$] salt for 1 hr until all the salt dissolved. This step was conducted with several modifications as originally described by Schenk *et al.* (1995). The solid precipitate containing the residual fatty acid and salts was discarded, and the supernatant containing the desired SL was left overnight in a 10ºC phosphate buffer (50 mM, pH 6.5) until saturated. The supernatant was collected and subjected to the third step, *i.e*. dialysis.

The protocol for this method was described by Jin *et al*. (2012) and Bollag *et al*. (1996). The homogeneous mixture of recovered SL was delivered into the dialysis tube and double knots were tied at the other ends of the tubing. The dialysis tube was placed in a beaker containing excess volume of phosphate buffer (50 mM, pH 6.5, 10ºC). The buffer was gently stirred to encourage efficient transfer of solute throughout the dialysis period. For the recovery of SL, dialysis was conducted for four days in $(NH_4)_2SO_4$ and seven days in $Al_2(SO_4)_3$ to achieve the targeted pH of 6.5. Finally, the precipitated SL were refrigerated overnight at 10ºC to solidify into paste form and then weighed.

Analysis and Characterisation

Determination of R_f value of sophorolipids. TLC is a simple method for profiling and detection of glycolipids in a sample. This was carried out according to the method described by Wadekar *et al.* (2012a) using TLC Silica gel 60, 2.5 x 7.5 cm (Merck, Germany), but with some modifications on visualisation solution. The developing solvent used was a mixture of 65: 15: 2 (v/v/v) chloroform/methanol/water. The localisation of the separated zones was performed by spraying visualisation solution containing 95% ethanol and 5% sulphuric acid (v/v). The plate was then heated in an oven at 120ºC for 20 min. Later, the retention factor (R_f) values of SL spots were calculated.

Identification of sophorolipids. HPLC method was carried out using C18 reverse phase column, RP 18 Lichrospher 100 (150 mm x 4.6 mm, 5 μm particle sizes) from Merck (Germany) and the HPLC unit was equipped with an evaporative light scattering detector (ELSD). The HPLC parameter set-up was as follows: column temperature, 37ºC; evaporation temperature, 50ºC; and sensitivity gain selected was 500, under atmospheric pressure. Mobile phase was composed of different concentration of eluent A (100% acetonitrile; (v/v)) and eluent B (100% filtered distilled water; (v/v)). These eluents were mixed to the desired percentage, set by eluent A in the HPLC menu, which were: in isocratic mode for the first 15 min of 30%, a linear gradient elution for the next 45 min, starting with 30% until 70%, and in isocratic mode for the next 15 min as to have 100%. The solvent flow rate was kept constant at 0.8 ml min^{-1} and the injection volume was 30 μl.

For identification of individual structure, the sample was analysed to the ionised molecules according to their mass to charge ratio (m/z), detected by a mass analyser equipped with electron spray ionisation (ESI-MS). The C18 reverse phase column was used in Dionex Ultimate 3000 Analytical HPLC connected to a Bruker mictoTOF-Q mass spectrometer equipped with an electrospray ionisation (ESI) source. The mobile phase consisted of filtered water/formic acid [99.5:0.5%; (v/v)] (eluent C) and acetonitrile [100%; (v/v)] (eluent D). The elution condition was conducted to the desired percentage, set by eluent D as followed: in isocratic mode for 5 min until 50%, in gradient mode for 20 min until 60%, in gradient mode for 20 min until 100%, in isocratic mode for 10 min with 100% and a further equilibration for 10 min. The LC-MS parameter set-up was as followed: scan began at 600 m/z and ended at 750 m/z, the ion polarity was in negative mode, ESI capillary voltage at 3500 V, nebuliser set at 3.5 bar, dry heater set at 195ºC and dry gas set at 8 ml min^{-1}. The flow rate was constant at 0.8 ml min^{-1} and the injection volume was 40 μl, and the sample was maintained at 10ºC. Data acquisition and mass spectrometric evaluation were performed with Bruker Daltonics Data Analysis Software 3.4. The predicted structure was drawn using ACD/Chemsketch software.

RESULTS AND DISCUSSION

Production of Sophorolipids using RBD Palm Olein

This study investigated RBD palm olein as the potential renewable and low cost lipophilic substrate in the production of SL. RBD palm olein is abundant in Malaysia and free from impurities, which promotes a safe application especially

towards consumer health. This study was conducted in shake flasks using RBD palm olein and glucose as the lipophilic and hydrophilic substrates. *S. bombicola* seed culture grew well in a sterilised basal medium supplemented with 10% (w/v) glucose, 5% (w/v) yeast extract and 10% (v/v) RBD palm olein at 30°C and 210 rpm in a shaker incubator. The SL production was measured at intervals of 24 hr.

Figure 1 shows a growth profile of *S. bombicola* exhibiting a short lag phase for the first 24 hr, and followed by a logarithmic growth phase at 36 hr until 72 hr of cultivation. The maximum growth rate, μ_{max} was calculated at 0.462 hr^{-1} and the doubling time, t_d was 1.498 hr. During this phase, the average cell composition and size would not be affected by the increase of time as this was a period of balanced growth. However, the pH of the culture medium dropped drastically from pH 5.5 to pH 3.0 which could have resulted from the ammonium consumption and the fatty acid generated in the media (Hu and Ju, 2001b). This similar finding was also reported previously by Van Bogaert *et al*. (2007), as a reduction of pH showed a good condition of the fermentation medium as this would protect the medium against contamination. Stationary growth phase was achieved at approximately 84 hr. During this stationary (resting) phase, production of SL was still observed which indicated that the process of SL production was non-growth associated, even though the SL were also obtained during log (growth) phase (Daverey and Pakshirajan, 2009).

The average yield of the total crude SL after 168 hr was 68.9 g $litre^{-1}$, with the cell growth yield in the fermentation, $Y_{X/S}$ at 1.9 g cells g^{-1} substrate; and the product formation over substrates, $Y_{P/S}$ at 7.89 g products g^{-1} substrate. Thus, fresh RBD palm olein supplied for laboratory used has shown the ability to produce higher amount of SL as compared to the previous studies using different quality of RBD palm olein substrate, *i.e.* used frying oil, and generated 10.73 g $litre^{-1}$ of SL after 200 hr fermentation (Wadekar *et al*., 2012a). Utilisation of palm olein as a secondary substrate in a bioreactor has also been reported by Fleurackers (2006), where SL yield was 50 g $litre^{-1}$ after 14 days fermentation. A higher SL yield of 300 g $litre^{-1}$ was reported from rapeseed oil. However, the fermentation was done in a two-stage process, thus making this production more expensive as compared to the palm olein (Rau *et al*., 2001).

Solvent Extraction as Conventional Recovery Strategy

The current downstream processing steps for the recovery of SL contributed to the high total cost of production. The most commonly used method is the conventional solvent extraction using ethyl acetate, which produces clean SL (Hubert *et al*., 2012). In this study, 4.4 litres of organic solvent was used in the recovery of SL per 1.6 litre fermentation medium with a total yield of 53.3 g $litre^{-1}$ SL (*Figure 2a*). This preliminary investigation showed that ethyl acetate can be used to recover crude SL from *S. bombicola* culture grown on RBD palm olein substrate. The yield was higher than other previous works reported on the recovery of SL from palm oil substrate with yield of 5.6 g $litre^{-1}$ (Wadekar *et al*., 2012b) and 15 g $litre^{-1}$ (Fleurackers, 2006). Lower SL recovery was reported when using other types of vegetable oils such as sunflower, jatropha, karanja and neem oil at 6.6 g $litre^{-1}$, 6 g $litre^{-1}$, 7.6 g $litre^{-1}$ and 2.63 g $litre^{-1}$, respectively (Wadekar *et al*., 2012a, b). The use of excessive volumes of solvent in the recovery of SL by the conventional method was also in agreement with Wadekar *et al*. (2012b). This excessive use may contribute to a high risk of hazardous waste pollution, causing the whole

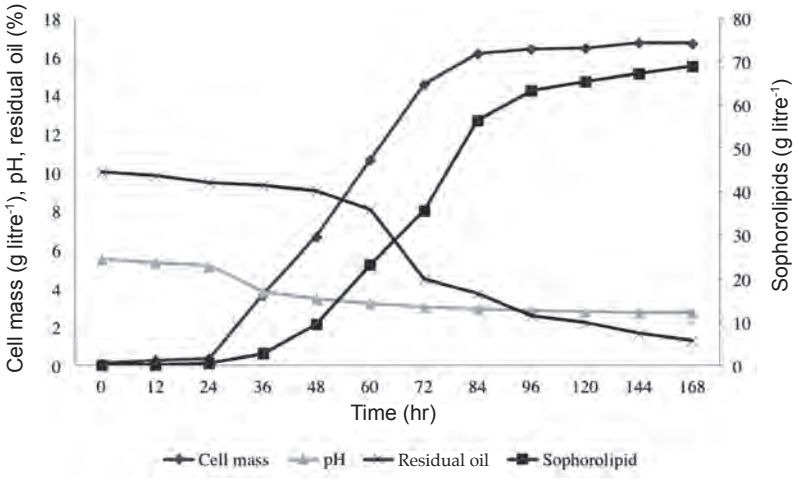

Figure 1. A growth profile of producing sophorolipid (SL) with the yeast growth curve, pH reduction and oil consumption in a shake flask study containing of 10% refined, bleached and deodorised palm olein (v/v), 10% glucose (w/v), basal medium and 5% yeast extract (w/v).

process to be less 'green'. One of the approaches to make the process greener is by substituting the solvent with a milder one. Ethanol has been used as an alternative to ethyl acetate by Fleurackers *et al*. (2010), but this has resulted in low SL yield at only 15 g litre^{-1}. The ratio of sample to solvent was also much higher (1:3; (v/v)).

Recovery of Sophorolipids through Non-solvent Extraction Route via Salting-out Method

Alternative to the conventional extraction, a non-solvent extraction method was chosen for recovery of SL. This route is important to overcome the negative effects arising from the conventional extraction method. The routes chosen must have a similar function to the conventional extraction, *i.e*. the ability to remove the impurities from the growing medium and the lipophilic compounds.

In this study, centrifugation was investigated as a first step to separate SL from other components of the fermentation broth. The broth was centrifuged at 13 000 rpm at room temperature for 30 min. *Figure 2b(i)* shows a product of centrifugation comprising of four distinct phases, which were SL layer, biomass (yeast and other cell debris) layer, medium layer and oil layer positioned from bottom to top, respectively. Gao *et al.* (2013) also reported that their SL phase was found to form at the bottom layer, heavier than the biomass. This could be due to the introduction of a high centrifugation temperature, thus triggering SL to solidify in the broth.

The SL layer separated by centrifugation formed honey-like behaviour/viscosity when stored at 4ºC (Pekin *et al*., 2005). Through this investigation on centrifugation method, a total SL production of 68.9 g litre^{-1} was obtained with product yield coefficient over substrate, $Y_{p/s}$ of 6.41 g g^{-1} and overall productivity was 0.41 g litre^{-1} per day. This shows that the recovery process by direct centrifugation is successful and superior to the earlier conventional extraction method in which about 15.67 g litre^{-1} of product loss was recorded.

Following the centrifugation, salting-out method was chosen for this study to precipitate the desired SL from the impurities and hydrophilic compounds. The appropriate value of salt concentration to be added during the salting-out experiment was screened initially. This was done to figure out the most efficient percentage saturation of salt to recover SL during salting-out process. Two types of salts were investigated for their suitability in enhancing the SL recovery; these were $Al_2(SO_4)_3$ and $(NH_4)_2SO_4$. The employment of $Al_2(SO_4)_3$ salt was justifiable based on previous studies (Schenk *et al*., 1995) whereas $(NH_4)_2SO_4$ was chosen independently because the salt was easy to procure, cheaper in price, and most important of all, this chemical is considered safe to use. It also had been used in the past for the isolation of polymeric biosurfactant from a recombinant *Acinetobacter calcoaceticus* RAG-1 strain by other researchers (Shabtai and Gutnick, 1986). Precipitation using $Al_2(SO_4)_3$ salt gave total SL production (g litre^{-1}) of 70.15, 95.96, 93.46 and 84.30 for 1%, 2%, 3%, and 5%, (w/v) of salts, respectively. This was considered high as compared to Schenk *et al*. (1995) where only 2.25 g litre^{-1} biosurfactant produced from *Pseudomonas aeruginosa* was recovered after using 2% (w/v) $Al_2(SO_4)_3$ salt. Whereas, $(NH_4)_2SO_4$ salt resulted in total SL production (g litre^{-1}) of 70.57, 86.80, 98.46, 91.59 and 76.81 for 0%-20%, 20%-40 %, 40%-60%, 60%-80%, and 80%-100% saturation, respectively.

The study also showed that the highest SL production was contributed by 2% (w/v) of $Al_2(SO_4)_3$ salt and 40%-60% (w/v) of $(NH_4)_2SO_4$ salt. But, $Al_2(SO_4)_3$ used a bigger volume of buffer (1.4 litre) and took seven days for completion compared to $(NH_4)_2SO_4$ salts at 0.8 litre and four days. Salting-out using 2% (w/v) of $Al_2(SO_4)_3$ and 40%-60% (w/v) of $(NH_4)_2SO_4$ gave productivity of 13.71 g litre^{-1} per day and 24.62 g litre^{-1} per day, respectively, and both

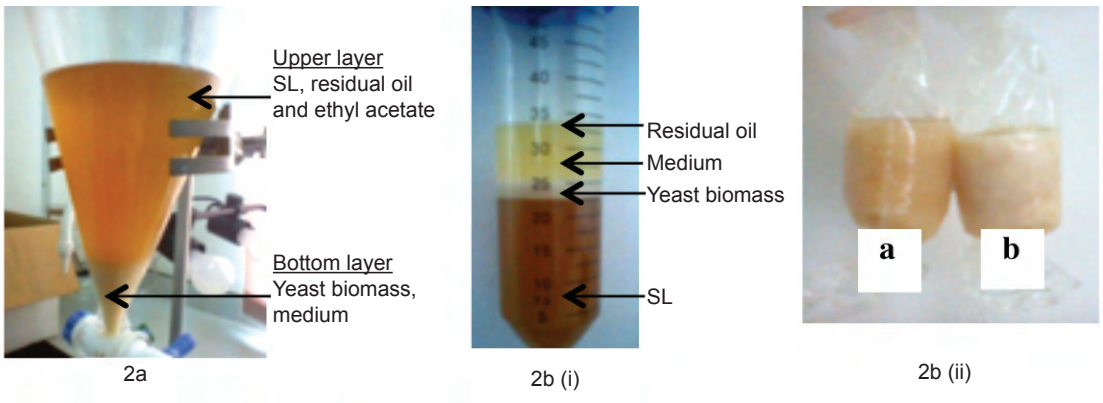

Figure 2. The comparison of sophorolipid (SL) recovered by two routes of extraction method [2a: solvent-extraction method; 2b: non-solvent extraction method (i) centrifugation method; (ii) salting-out method by (a) $Al_2(SO_4)_3$ salts, (b) $(NH_4)_2SO_4$ salts].

final products appeared in a paste-like behaviour with pale brown colour [*Figure 2b(ii)*]. Thus, the use of $(NH_4)_2SO_4$ is convenient and superior to $Al_2(SO_4)_3$ as a salt in promoting salting out of the residual fatty acids and other impurities from the SL mixture. The pH of SL products was observed to be similar to previously reported on commercial SL, *Sopholiance* with a pH range of 6–6.5 (Develter and Fleurackers, 2008). Recovery of SL using precipitation method was also studied by Fleurackers (2006). He used two types of chloride salts, CaCl and LiCl, and reported that 80% of unconverted fatty acid (oleic acid) was removed as insoluble calcium soap which best met the future cleaning technologies as it had a higher proportion of acidic structure.

Determination on the Presence of Sophorolipids Compounds by TLC

In this study, preliminary detection of SL produced from *S. bombicola* culture was performed using TLC. This technique may provide information on possible structural types of glycolipids present whether lactonic or acid. Results from different recovery methods investigated in this study were compared accordingly (*Figure 3*). The comparison was done by calculating the R_f value of each spot observed on the plates (*Table 1*).

Spots, visualised as dark purple, proved the existence of glycolipid compounds (Fuchs *et al*., 2011), while the yellow spots appearing in the top region of the plates showed the existence of other lipids (Asmer *et al*., 1988). Since these were also observed in all the TLC plates of the current study, it indicated that the recovered SL still contained impurities. There were seven spots on the control plate (*Sopholiance* in Lane A of *Figure 3*) identified as glycolipids compounds (spot *a* to spot *g*). The three dominant compounds of *Sopholiance* were spots *c*, *d* and *e*, in which their R_f values were calculated at 0.24, 0.39 and 0.52, respectively. When compared with other TLC plates, as represented by crude SL in Lane B, solvent-extracted of SL in Lane C, salt-treatment of SL with $Al_2(SO_4)_3$ in Lane D and salt-treatment of SL with $(NH_4)_2SO_4$ in Lane E, it was found that spot *f* was the dominant spot, giving its R_f value of 0.64. In the initial observation, spots *a, b* and *c* with R_f values of 0 – 0.24 were identified as an acidic form of SL, whereas the remaining spots with R_f 0.35 – 0.75 corresponded to lactone form of SL. Similar TLC spots were obtained in studies by Ribeiro and colleagues (2012), where TLC spots with R_f of 0.71 and 0.77 were identified as diacetylated lactonic SL; a spot with R_f of 0.52 was monoacetylated lactonic SL, and a spot with R_f of 0.37 was diacetylated acidic SL.

Identification of Individual Sophorolipids Compound by HPLC and LC-ESI-MS

The chromatographic separation for this study was performed using C18 reverse phase column with an elution step performed at linear gradient and acetonitrile/water as mobile phase. The total retention time (RT) for a complete separation process took approximately 40 min, with the acidic

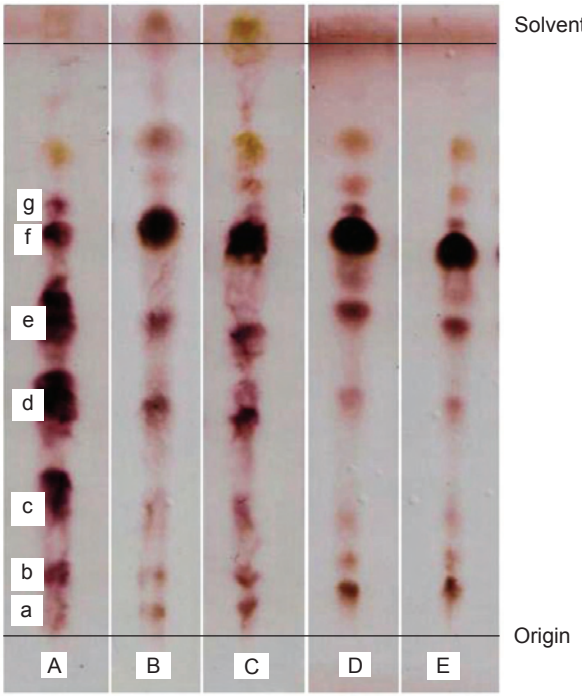

Figure 3. Identification of sophorolipid (SL) compounds: (A) standard Sopholiance, *(B) crude SL, (C) solvent extracted of SL, (D) salt treatment of SL with $Al_2(SO_4)_3$ and (E) salt treatment of SL with $(NH_4)_2SO_4$.*

TABLE 1. COMPARISON OF THE R_f VALUES OF COMMERCIAL SOPHOLIANCE (SL), SOLVENT EXTRACTED OF SL, CRUDE SL, SALT TREATMENT OF SL WITH $AL_2(SO_4)_3$, AND SALT TREATMENT OF SL WITH $(NH_4)_2SO_4$

SL product	R_f values				
Spot	*Sopholiance*	Solvent extracted	Crude	Salt treatment [$Al_2(SO_4)_3$]	Salt treatment [$(NH_4)_2SO_4$]
g	0.67	0.7	0.73	0.75	0.74
f	0.64	0.62	0.64	0.67	0.66
e	0.52	0.46	0.50	0.54	0.53
d	0.39	0.34	0.37	0.41	0.41
c	0.24	0.20	0.20	0.23	0.23
b	0.10	0.10	0.10	0.16	0.16
a	0.04	0.06	0.04	0.11	0.11

structure eluted first (peak appearance from RT 0 to 25 min) and followed with the lactonic structures (peak appearance from RT 26 to 45 min). These chromatographic separation profiles were also in agreement with other previous studies (Ribeiro *et al*., 2012).

The average of the major peak from all types of recovered SL was similar at RT 33 min. However, this peak showed decreasing intensity as the number of recovery process increased. The relative peak area of 662, 125 μV min^{-1} was obtained for solvent extracted sample with percentage SL compound of 76%. Conversely, when SL sample employed the centrifugation method, the relative peak area decreased to 555, 297 μV min^{-1}, with the percentage of the total SL compound at 67.53%. For samples that underwent salting-out process, the relative peak area continued to decrease to half of the crude SL peak area, *i.e*. 380, 784 μV min^{-1} and 215, 437 μV min^{-1} for $Al_2(SO_4)_3$ and $(NH_4)_2SO_4$ treatment, respectively.

The recovery strategies using salting-out method showed an increment in the peak area due to the high removal of the residual impurities, which led to 75.5% and 78.57% purity for $Al_2(SO_4)_3$ and $(NH_4)_2SO_4$ treatment, respectively. The chromatogram of the $(NH_4)_2SO_4$ treatment is shown in *Figure 4*. It also supported the earlier report in which the average peak area for hydrolysed SL produced from oleic acid started at 2870 mV min^{-1} and decreased to 546 mV min^{-1} after the removal of precipitated calcium soap (Fleurackers, 2006).

When SL structure was elucidated through a mass spectrometer, seven peaks were observed and a similar report was obtained for the centrifuged and salting-out methods in recovering SL samples. However, there was a difference in molecular mass for a peak appearing at RT 37.4 min, in which centrifuged and solvent-extracted sample exhibited 736 g mol^{-1} while salting-out sample exhibited 690 g mol^{-1}. From the calculation, the proportion of separated lactonic and acidic forms on salt treatment of SL was 64% and 36%, respectively. The seven peaks represented five types of SL compound (*Table 2*), in which the dominant structure was diacetylated lactonic SL with m/z 687 (688 g mol^{-1}) (*Figure 5*).

In total, five structures belonging to SL were revealed due to their molecular weight obtained as reported by Yang *et al.* (2012). The successful purification of lactonic compounds on both salt treated SL also indicates that this recovery method can be used to substitute the conventional recovery process. This was supported by the fact that

TABLE 2. THE MOLECULAR MASS AND CONTENT OF EXTRACTED PALM-BASED SOPHOROLIPID (SL) THROUGH NON-SOLVENT EXTRACTION VIA SALTING OUT METHOD

Sophorolipid type	Average retention time (min)	M.W.	Intensity (mV min^{-1})*	Lipid type	Acetyl number	SL type
1	14.1	706	1 025	C18:1	2	Free acid
2	19.0	646	994	C18:1	1	Lactone
3,4	26.3	662	2 436	C18:2	1	Free acid
5,6	30.6	688	3 692	C18:1	2	Lactone
7	37.3	690	1 483	C18:0	2	Lactone

Note: * The relative peak area was the mean value of intensity obtained on salting-out of SL with $Al_2(SO_4)_3$ and $(NH_4)_2SO_4$.

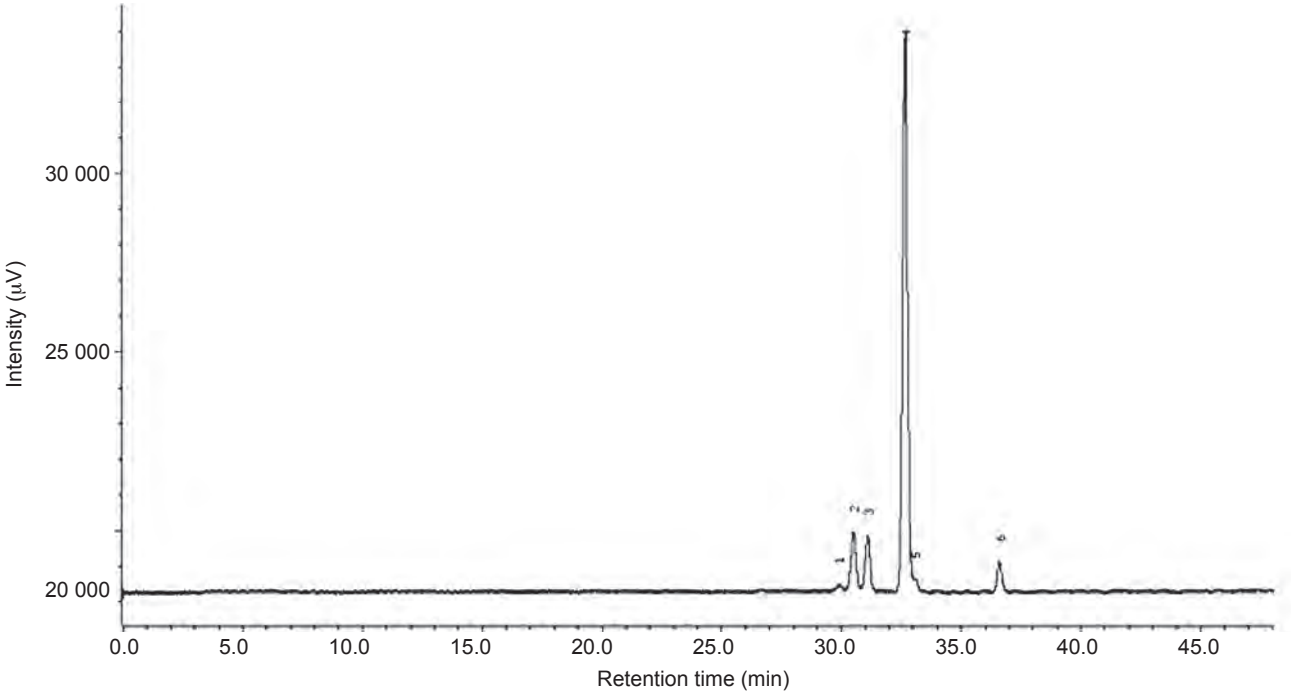

Figure 4. High performance liquid chromatography (HPLC) chromatogram of salt-treated SL with $(NH_4)_2SO_4$.

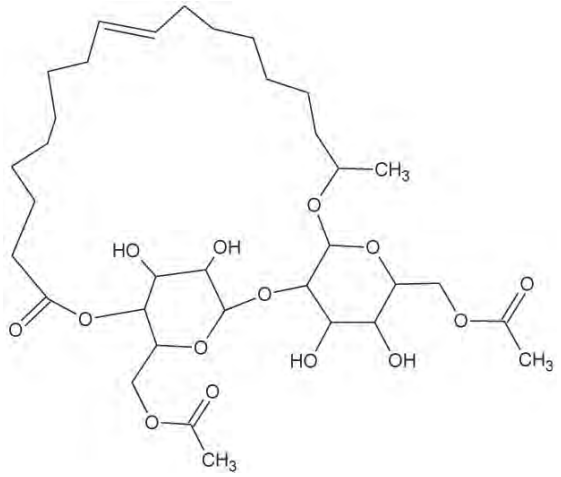

Figure 5. The dominant structure of palm-based sophorolipid (SL) was diacetylated lactonic SL with m/z 687.

buffer played an important role in discarding the acidic compounds especially the monoacetylated acidic form. Buffer exchange can be performed by increasing the rate of buffer volume change or cycles to alter the pH of the aqueous system gradually until the desired condition is reached (Hu and Ju, 2001a). An earlier study also described the advantage of employing ammonium sulphate in culture medium, as the ammonium ions were able to inhibit the production of lower polarity SL (Ma *et al*., 2011). The use of potassium phosphate buffer has also been reported to enhance the lactones structure since it neutralised the acid compounds by raising the pH and hence shifting the acidic peak to the left into lactones peak (Develter and Fleurackers, 2008).

CONCLUSION

This study found that firstly, RBD palm olein is a suitable secondary carbon source producing a high yield of SL biosurfactant using *S. bombicola*; and secondly, the use of $Al_2(SO_4)_3$ and $(NH_4)_2SO_4$ salts is successful in recovering SL with a high production yield, thereby making this method suitable to replace the conventional extraction methods of using solvents. Furthermore, this solvent-less purification method will lead to a greener process not only in terms of environmental-friendliness but also in economic values, and would possibly reduce the overall production cost of SL biosurfactant. In addition, the qualitative and quantitative characterisation of the recovered SL revealed that palm-based SL product also contains a mixture of lactonic: acidic content similar to the commercial and other sources of SL. Finally, the salt-treated products have the highest purity contents as compared to other extraction processes due to the major peaks obtained revealed as the lactonic compounds.

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