Production and characterization of low molecular weight sophorolipid under fed-batch culture

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HIGHLIGHTS

- The study was aimed for the production and optimization of the C12–C14 sophorolipid.
- Coconut oil (15%) and glucose (10%) were used as carbon sources.
- The maximum yield was 54 g/L, during eight day of fermentation.
- Significant surface activities, and emulsion ability were recorded.
- Molecular characterization of sophorolipid was done by LC–MS.

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ABSTRACT

The present study was designed for the production and optimization of the C12–C14 sophorolipid, using the yeast Candida bombicola ATCC-22214. The fermentation was carried under fed-batch culture conditions i.e., maintaining 15% coconut oil and 10% glucose as hydrophobic and hydrophilic carbon sources, respectively. A maximum yield 54.0 g/L (in 234 h) was achieved. A significant antimicrobial activity, surface activity, and emulsion ability were recorded. The native sophorolipid was found as enhancer of detergent efficacy of commercial detergent, tested on complex, smudge and oil contaminated clothes. Molecular weight of the C12 (605/623) and C14 (633/651) sophorolipids were determined by LC–MS which revealed it as diacetylated sophorolipid. This study is being important in terms of yield, which is better than the previously reported.

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1. Introduction

Sophorolipids are surface-active compounds synthesized by a selected number of yeast species such as Candida bombicola, Candida apicola, Candida batistae, Wickerhamiella domericiaque and Rhodotorula bogoriensis (Chen et al., 2006; Gorin et al., 1961; Konishi et al., 2008; Spencer et al., 1970; Tulloch and Spencer, 1968). In an estimate, the annual consumption of the sophorolipid is around 10 million ton per annum (Van Bogaert et al., 2007). Sophorolipids are mostly used as household/laundry detergents, but instead they also have some potential application in chemical, textile, food, paper, and cosmetics industries. Commercialization of sophorolipid based products is rapidly occupying the market share of the herbal cosmetics. The French company Soliance (http://www.groupesoliance.com) and the Korean MG Intobio Co. Ltd are producing sophorolipid-based cosmetics and skin health products. In addition to surfactant properties of sophorolipids, the anti-bacterial properties, especially against Propionibacterium acnes and Corynebacterium xerosis, make them a better emulsifying agent for cosmetics formulations. Sophorolipids also exhibit lower cytotoxicity. Other beneficial properties like anticancer, stimulation to dermal fibroblast metabolism, free radical scavenging skin desquamation and depigmentation, hair protection, sanitization make sophorolipid, an attractive component for cosmetic and pharmacodermatological products (Kim et al., 2002; Mager et al., 1987; Shao et al., 2012; Van Bogaert et al., 2007, 2011).

In last few years, various vegetable oils were screened for a higher production of sophorolipid with different bioactivities (Kim et al., 1997, 2002, 2005, 2009). Among numerous used substrates, the production variability was major constrain for industrial application of sophorolipid as a surfactant. The bioactivities of the macromolecule are not only class dependent but the size, structural anomaly, and the purity also contribute a great role (Morya et al., 2012). In fact, the sophorolipids synthesized by
C. bombicola were a mixture of molecules having differences in the structural aspects like type of fatty acids part, lactonization and the acetylation patterns (Van Bogaert et al., 2007). These variations also influence the hydrobophicity of sophorolipid molecules (Tran et al., 2012; Van Bogaert et al., 2011) In nature, the Sophorolipids were synthesized as a mixture of acidic and lactonic forms, and also showing a huge number of structural variability (Ashby et al., 2005; Kim et al., 2009).

Coconut oil contains mainly lauric acid (\(\text{CH}_3(\text{CH}_2)_{10}\text{COOH}\)) as a major fatty acid component. More than 90% of the fatty acids of coconut oil were reported as saturated fatty acids (iodine value- 7–12), thus having minimum risk of oxidative damage (Gordon and Rahman, 1991). Coconut oil is one of the richest sources of medium chain fatty acids (MCFA). It was also reported that the coconut oil contains 47.5% Lauric (C12) and 18.1% Myristic (C14) acids of the total fatty acids (Gordon and Rahman, 1991).

Among the Sophorolipid producing organisms, C. bombicola is placed at the highest rank in terms of yield. The synchronized, de-novo and biocconversional production of sophorolipid by this organism were an additional advantage for selecting it as a producer (Kim et al., 2009). The product of the de-novo synthesis was mainly a derivative of Palmitic, Stearic, and Oleic acids (Van Bogaert et al., 2007). The bioconversion of oils for production of sophorolipids have different mechanisms then the de novo, that is glycerol of oils are being consumed by the organism as co-substrate, and the fatty acids being utilized as feedstock (Ashby et al., 2005; Kim et al., 2009). Thus, oils could serve as a feedstock as well as a nutrient source during the production of sophorolipid. A number of substrates and culture conditions were attempted and employed to achieve novel properties, and expand the applications of sophorolipid (Felse et al., 2007; Glenns and Cooper, 2006; Hu and Ju, 2001; Kim et al., 2005, 2009). All described studies were mostly carried with C18 or higher fatty acid containing substrates. The surface active properties of sophorolipid are primarily governed by fatty acid chain length, and respective modifications, i.e. position of unsaturated bond and side groups. It was reported that the long-chain length of fatty acid domain restricts the bioavailability and also diminishes the biological properties (Van Bogaert et al., 2007, 2011). Whereas the medium or small size fatty acids get easily metabolized via \(\beta\)-oxidation, which results in a lower yield of sophorolipid during the fermentation process. Thus, optimization of fermentation conditions to resolve the issue is yet a major challenge for the researchers (Van Bogaert et al., 2007). In previous work, various substrates for medium chain sophorolipid production were used but they are not applied to industrial processes because of the many control factors (Kim et al., 2005, 2009).

In 1998, Brakemeier and coworkers used secondary alcohol (C12 and C14) as a substrate along with the glucose (carbon source) as an alternative method to produce medium chain sophorolipids (Brakemeier et al., 1998). However, this method was not adopted by the industries due to a very high cost of the secondary alcohols (Van Bogaert et al., 2011). Addressing the same issue, in the present study the coconut oil was attempted as a substrate for production of medium chain sophorolipid. Designing and establishment of fermentation conditions (pH, temperature, aeration rate, agitation, etc.) were done during study. In addition to above, surface like properties, antimicrobial properties and molecular weight were also elucidated.

2. Methods

The cryopreserved (at \(-70^\circ\text{C}\)) C. bombicola (ATCC 22214) was revived in YM broth media and incubated at 25 \(^\circ\text{C}\) with 250 rpm for 24 h. This culture broth was then transferred to the production medium, and also persevered for the further cell storage. All chemicals were procured from Sigma–Aldrich. The culture media and components were purchased from Difco and Merck, respectively. Coconut oil and corn oil were obtained from Shindongbang Co. (Korea).

2.1. Culture conditions and media

The composition of production medium was kept similar to previous work (Kim et al., 2009), in brief, 100.0 g glucose, 5.0 g yeast extract, 1.0 g of KH2PO4, 0.5 g MgSO4·7H2O, 0.1 g CaCl2·2H2O, 0.1 g NaCl, 0.7 g peptone and 10% (w/v) vegetable oil in one liter of distilled water. The inoculum size used for this study was 5% (v/v) of total volume of the production medium. The fermentation was carried out in 30 L of fermenter (Kobiotech, Korea) on a working volume of 15 L. The culture conditions used for this study were as followed- temperature 30 \(^\circ\text{C}\); pH 3.5; agitation 550 rpm; aeration rate 1 vvm and duration 3–8 days. The oxygen saturation was controlled at 20% by changing the agitation speed. When necessary, the cultivation pH was adjusted by automatic/manual titration with 6 N NaOH. Samples were withdrawn from the fermentation broth at one-day intervals for further analysis.

2.2. Fatty acid composition

The fatty acid composition of oils and their respective sophorolipids were determined by gas chromatography equipped with a flame ionization detector (GC–FID, HP 6890, HP, USA) using standard method reported by Kim et al. (2009).

2.3. Optimization of operational conditions and hydrophilic & lipophilic carbon sources

Optimization of the glucose consumption rate for maximum production of sophorolipid was achieved by the two-phase batch fermentation. During the growth phase (0–72 h), the standard medium was supplemented by 10 g/L of glucose to achieve appropriate cell growth (Kim et al., 2009). After optimum cell growth, the glucose gradient was supplemented (10–200 g/L) to different cultures along with 15 g/L of coconut oil during the production phase (72–96 h). The culture conditions were as follows: agitation 250 rpm, initial pH of 5, for 25 \(^\circ\text{C}\) temperature, flasks with working volume of 50 mL/250 mL in 4-days (three days for growth and one day for production) culture period. After that, culture broths were harvested and assayed for determination of residual oil, glucose, sophorolipid content, and estimation of dry biomass.

2.3.1. Residual oil determination and extraction of sophorolipid

The residual oil was separated from the culture filtrate by extraction method using n-Hexane (Shin et al., 2010). The top non-aqueous layer which contains vegetable oil was separated by separation funnel. The oil was recovered by evaporation of n-Hexane using rotary vacuum evaporator (Eyela, Japan), and then weighed. The aqueous phase was extracted three times by using same volume of ethyl acetate. The crude sophorolipid was collected by evaporation of the ethyl acetate using a rotary vacuum evaporator (Eyela, Japan), and then weighed.

2.3.2. Determination of residual glucose and dry cell weight

After extraction of sophorolipid and residual oil, the rest of the broth was subjected to evaporation in a rotary vacuum evaporator (Eyela, Japan), to remove the traces of solvents. This aqueous broth was centrifuged at 7000g for 15 min. Residual glucose was estimated from the supernatant, while the pellet was dried and weighted (Kim et al., 2005) for the biomass estimation. The glucose level was examined by Accu-Check (Loche) for feeding the deficient glucose during cultivation immediately and reconfirmed by
HPLC (Aminex HPX-87H column; mobile phase, 0.01 N of H2SO4, 1.0 mL/min).

2.4. Feeding rate controlled fed-batch cultivation

The fed-batch cultivation was carried out in a 30 L Industrial fermenter (Kobiotech, Korea) on a working volume of 15 L. The steam sterilized and properly stabilized media was inoculated in a ratio of 1:100 (Inoculum:Media). The initial fermentation condition was 30 °C temperature with an aeration rate of 0.3 vvm at an agitation rate of 130 rpm for three days (Growth phase). The oxygen saturation was controlled at 30% by changing the agitation speed. After three days of growth phase, the final operating conditions were – temperature 27 °C with an aeration rate of 1.0 vvm at an agitation rate of 250 rpm for thirteen days. The samples were regularly withdrawn from the fermentation broth and analyzed to determine the variables. The feeding media had 10% glucose and 15% coconut oil as a final concentration during the sophorolipid production. The initial 10% of coconut oil supplementation controlled the foam. During the feeding pH was adjusted to 3.5 by adding 6 N NaOH, to control the same.

2.5. Measurements of surface-active properties and emulsification capacity

The surface tension of crude sophorolipid was measured by the du Nuoy method (Surface Tensimat, model 21, Fisher Scientific, Pittsburgh, PA, USA), using platinum–iridium ring (with a circumference of 5.935 cm) (Hyun-Soo et al., 2005; Ma et al., 2012). The surface tension of different concentrations of sophorolipid from 0 to 300 ppm in distilled water was measured. This solution was incubated for 30 min prior to use for measurement. Critical micelle concentration (CMC) at which the micelle begins to form, CMC corresponds to the point in a limitation of minimum surface tension, was determined by the same tensionmeter at 25 °C. The CMC and minimum surface tension values were obtained from the relationship curve between sophorolipid concentrations and their corresponding surface tension (Ma et al., 2012).

The dispersion power was determined by standardized protocol (Ma et al., 2012). The foaming power was measured by the Ross and Miles method according to international standard ISO 696 at 50 °C (Urum and Pekdemir, 2004). Detergency was determined by standard procedure (Sin et al., 2010), in brief the JIS (compound scum on cotton) soiled with oleic acid, triolein, cholesterol oleate, paraffin wax, squalene, cholesterol, gelatin, clay, carbon black; smudged scum on cotton (CFT AS 10) with pigment, oil, milk; and oily scum on cotton (WFK 10D) with pigment, sebum, were used. The reflectance of the fabrics was measured using the standard procedure (Sin et al., 2010; Srivastava et al., 2006). The rinsing power was calculated as followed:

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\text{rinsing power } P(\%) = \frac{(R - R_o)}{(65 - R_o)} \times 100
\]

where \(R_o\) stands for reflectivity before washing, \(R\) stands for reflectivity after washing and \(P\) stands for relative rinsing power. Emulsification capacity of the produced crude sophorolipid to soybean oil was determined by standard method, briefly, 2.0 mL of 0.1% (w/v) of solution sophorolipid was mixed with 2.0 mL soybean oil in presence of 2.0 mL phosphate buffer (200 mM, pH 7.0). This mixture was vertexes for one minute. The emulsifying capability was monitor at 10 min interval by measuring the absorbance at 540 nm (Shin et al., 2010).

2.6. Antimicrobial activity

Anti-bacterial properties of sophorolipid against Staphylococcus aureus and Escherichia coli were determined according to Shin et al. (2010). DF-100 (the grape seed extract) was used as a standard (Defera et al., 2009).

2.7. HPLC and LC–MS analysis of sophorolipid

The extracted sophorolipid derived from coconut oil was dissolved in ethanol and applied to further analysis by analytical HPLC (Waters, Japan) with a 4.6 mm × 150 mm Spherisorb ODS2 column (Waters Inc). Acetonitrile/water was used as the mobile phase at an acetonitrile gradient from 40% to 60% in 15 min followed by an acetonitrile gradient from 60% to 80% in 35 min at a flow rate of 0.5 mL/min. The injection volume was 25 μL, and the eluent was monitored with UV detector at 195 nm.

The LC–MS system (HP 1100 series with UV-Visible diode-array detector-Bruker’s Esquire electrospray MSn analyzer) was used to separate and identify the structures of individual sophorolipid component. The mobile phase used was 8/2(v/v) acetonitrile/H2O. The sophorolipid separated by HPLC was passed through the mass spectrometer (Hu and Ju, 2001; Tran et al., 2012). The molecular ions were collected in an ion trap and the mass/charge (m/z) values were detected. The ions in the ion trap could be isolated and further fragmentized by collision with helium molecules. The molecular structure was depicted from LC–MS data as described in literatures (Hu and Ju, 2001; Tran et al., 2012).

2.8. Statistical analysis

The experiments were carried out in three replicates and results are presented as the average with standard deviation (SD) represented by error bars in graphs.

3. Results and discussion

3.1. Analysis of fatty acid residues

Results from GC–FID showed that the coconut oil used for this study contained 49.83% C12 and 17.55% C14, similar to previous report (Gordon and Rahman, 1991). Sophorolipid derived from coconut oil contained 53.27% C12 and 21.54% C14. While in corn oil and its sophorolipid, which were used as a reference, C12 and C14 fatty acids were absent (Table 1). As the results suggested that the sophorolipids obtained from coconut oil were mainly from Lauric and Myristic acid derivatives. Proportion of C12 sophorolipid from coconut oil was higher enough to purify with better yield. However, the earlier method using n-alkane and glucose as a substrate for the production of specific type of sophorolipid was quite costly with the lower yield (Hommel et al., 1994; Jones and Howe 1968; Van Bogaert et al., 2007).

3.2. Optimization of operational conditions and hydrophilic & lipophilic carbon sources

During the two-phase batch fermentation, it was observed that the production was optimal at the glucose concentration of 100 ± 5 g/L, and it was maintained about 100 g/L throughout the experimentation. The regular monitoring and supplementation of glucose ensured the optimal production of the sophorolipid. Theoretical neutralization of fatty acid originated from coconut oil was calibrated by 6 N NaOH. The pH for the growth phase was maintained about 5 ± 0.1, to achieve the optimum growth. During the production phase the pH, was found to be dropped heavily, and
it was optimized that 3.5pH was appropriate for the production, thus the running pH was maintained at 3.5, using 6.0 N NaOH. After 48 h of incubation, 16 g/L dry cell mass was obtained. However, it was observed that the production pattern of sophorolipid followed the trend of our previous lab work, i.e., production of secondary metabolite was started from the mid of exponential phase (Shin et al., 2010). 140 g/L concentration of coconut oil was added in final production media, from initial 10 g/L to final 140 g/L was attained in 264 h at the rate of 14 g/day/L. During the production phase, sophorolipid was produced continuously. The 54 g/L of extracellular sophorolipid was obtained, and the cultivation of *C. bombicola* was terminated after eight days (Fig. 1). Presence of both the lipophilic and hydrophilic carbon sources in the production medium insures the higher yield of sophorolipid (Van Bogaert et al., 2007; Kim et al., 2009). Glucose was being identified as the better hydrophilic carbon source among the screened carbohydrates (Kim et al., 2005, 2009; Van Bogaert et al., 2007). It was reported, the glucose as an essential feedstock for sophorolipid production, thus it influencing the production (Sarubbo et al., 2007; Ashby et al., 2005; Kim et al., 2005), most of the cases the optimal value of glucose concentration was reported nearly 100 g/L of the media (Van Bogaert et al., 2011).

### 3.3 Feeding rate controlled fed-batch cultivation

This study was designed to have maximum possible production of sophorolipid in a fed-batch mode using coconut oil, and glucose. Fig. 1 showing, fed-batch cultivation with an initial glucose concentration of 10 g/L and the coconut oil of 10 g/L. In comparison to pulse feeding, the feeding-rate-controlled fed-batch cultivation was found to be almost two times, to the stationary phase. As a result, a 54 g/L of the crude sophorolipid was collected, after an eight days of the fermentation. It was not possible to carry the fermentation for longer than the eight days as the viscosity of the culture was very high.

The controlled feeding of the coconut oil was one of the majors constrain for the production and protection from the metabolism. The consumption of coconut oil was correlated with the adjustment of pH of media which was adjusted by 6 N NaOH. The optimum operating concentration of the suitable carbon sources, i.e., glucose and coconut oil, was determined. This optimization was also re-validation of our previous work (Shin et al., 2010), and thus based on the coefficient calculation, a better control method for maintaining optimum concentrations of lipophilic substrates (coconut oil) was being suggested. The coconut oil was reported as not a good substrate for the production of sophorolipid, as it contains mainly C12 and C14 fatty acid thus, easily metabolized during fermentation process. Thus optimization of process was major challenge to get maximum production. The literatures also suggest the surface-active properties were usually inversely proportional to the chain length of the fatty acid (Van Bogaert et al., 2007; Felse et al., 2007; Kim et al., 2009).

### 3.4 Surface active properties and emulsifying capacity

The results of surface active properties and emulsifying capacity of the sophorolipid derived from coconut oil were presented in Fig. 2. In both the cases, Linear Alkylbenzen Sulfonate (LAS) and sugar ester were used as references. Both the sophorolipid from coconut oil and corn oil exhibited relatively better foamability as compared to the LAS and sugar ester. Their foaming stability properties were also very low as measured by the foaming heights after

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**Fig. 1.** Fermentation conditions in feeding rate controlled fed-batch cultivation of *C. bombicola* ATCC-22214 for the production of sophorolipid from coconut oil. *DCW = Dry Cell Weight.*
5 min. Foaming properties of sophorolipids decreased with the increased carbon length in the fatty acid chain (Shin et al., 2010). Sophorolipids from corn oil exhibited slightly higher foaming properties than the coconut oil derived sophorolipid. Moreover, it well known that the foamability of a surfactant solution increases at low surfactant concentrations, until the critical micelle concentration (CMC) was reached (Montufar et al., 2011). Therefore calculating CMC and Min S.T. were being important, and the Surface tension decreased rapidly from about 70 to Min. S.T. of 25–35 mN/m with the increase in concentration of sophorolipid, Fig. 2(A). The Min S.T. of coconut oil was slightly lowered than the corn oil derived one. That indicated the hydrophobicity of the coconut derived sophorolipid was higher that the corn oil one, though it was smaller in size. CMC values of sophorolipid varied from 20 to 35 mg/L, and both the sophorolipid showed markedly lower CMC in comparison standard surfactant (data not shown). The dispersion ability of sophorolipid was found to be better than the LAS (Fig. 2B). In compare to corn oil derived sophorolipid the coconut oil sophorolipid showed lowered dispersion ability (Fig. 2B). These results were in agreement with the previous (Shin et al., 2010; Montufar et al., 2011), the reason for which was probably increased polarity stemming from the unsaturation of the non-polar moiety (Montufar et al., 2011).

3.5. Antimicrobial activity

The data of the antimicrobial efficacy of produced sophorolipid on two reference bacteria i.e., E. coli and S. aureus have been presented in fig. 3(A and B). Results showed that the sophorolipid obtained from coconut oil showed better ability of emulsification then used standards (LAS and Sugar ester). The Emulsification activity of surface active molecule was not only determined by the surface tension, but also depends on direct interaction of the hydrophobic moiety of the molecule with hydrophobic substrates (Shin et al., 2010; Montufar et al., 2011).

3.6. HPLC and LCMS analysis of sophorolipid

The results from LC–MS data of complex mixture of sophorolipid showed in supplementary data. A complex mixture of sophorolipids (e.g. with the fatty acid moiety of C12 and C14, m/z = 623
and 651, respectively) was formed in the fermentation using coconut and glucose as the C-source. The calculated value of mass suggested that R1 and R2 (Fig. S1, inset) might be COCH₃ group. The LC–MS spectrum, together with the structures reported in the literature strongly suggested that the molecules were the diacetylated sophorolipids (Fig. S1). The more detailed analysis of the forms of sophorolipid was revealed by HPLC analysis, and the results showed several peaks. The sophorolipid produced from coconut oil was complex and contained both lactonic and acidic sophorolipids (Table 1). The HPLC data was similar to previous reports, i.e., peaks between 4 and 7 min as the combined acidic sophorolipid and peaks that between 10 and 20 min as combined lactonic sophorolipid (Casas and García-Ochoa 1999; Hu and Ju, 2001; Tran et al., 2012). The peak area of the lactonic sophorolipid was significantly larger. Due to higher proportion of lactonic form in the partially purified sophorolipid showed higher hydrophobic properties, although the chain length was shorter. In addition to lactonization the acetylation also contributed to higher hydrophobicity of the molecules. As described in the literature that the lactonization and acetylation of sophorolipid depend on fermentation time (Hu and Ju, 2001).

4. Conclusion

Production of medium chain fatty acid derived sophorolipid is an ever challenging task. To resolve the issue, coconut oil was used and the yield was about 54 g/L, with a relatively higher proportion of C12 sophorolipid. The relative cost was much lower in compare to use of secondary alcohol as a substrate. Applicability of this method to produce the sophorolipid using correlation correction can be employed for the different kind of sophorolipids production. The biochemical and biological properties along with surface activities had been analyzed. The primary screening results suggested that the sophorolipid from coconut oil can create a huge opportunity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.05.094.

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