Efficient Production of Acid-Form Sophorolipids from Waste Glycerol and Fatty Acid Methyl Esters by *Candida floricola*

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Abstract: We discovered that *Candida floricola* ZM1502 is capable of selectively producing the promising hydrophilic biosurfactants, acid-form sophorolipids (SLs), from glycerol. However, productivity was very low (approximately 3.5 g L⁻¹) under the initial culture conditions. Here, we describe the design of culture medium for abundant production of acid-form SLs by *C. floricola* ZM1502 using waste glycerol and hydrophobic substrates in order to develop a method for SL production and disposal of waste glycerol produced by oleo-chemical industries. Urea provided the best nitrogen source for acid-form SL production from glycerol among four nitrogen sources tested [urea, NaNO₃, NH₄NO₃, and (NH₄)₂SO₄]. Among carbon sources we compared, hydrophobic substrates (soybean oil and oleic acid) led to productivities of approximately 20 g L⁻¹, indicating that hydrophobic substrates provided fatty acid moieties for SL production. Addition of olive oil and oleic acid to waste glycerol enhanced acid-form SL production to 42.1 ± 0.9 and 37.5 ± 3.4 g L⁻¹, respectively. To develop a potential industrial process, we explored other suitable hydrophobic substrates for SL production, which were obtained on site from oleo-chemical industries. Alkyl C₁₈ esters (Pastell M-182), along with waste glycerol, increased acid-form SL production to 48.0 ± 3.4 g L⁻¹ over a 7-d period. Furthermore, we demonstrated abundant production of acidic SLs at the mini-jar fermenter scale, obtaining 169 g L⁻¹ over 180 h using a fed-batch cultivation technique. Efficient acid-form SL production by *C. floricola* could have a great impact on the development of bio-industrial processes using waste glycerol as a substrate.

Key words: sophorolipid, *Candida floricola*, glycerol, high concentration cultivation, fatty acid methyl esters, vegetable oils

1 INTRODUCTION

Biosurfactants are extracellular surface-active compounds produced by microorganisms from a variety of carbon neutral feedstocks. In addition to biodegradability and biocompatibility, they exhibit versatile biological functions as well as excellent interfacial properties⁴. Thus, they have been utilized as advanced surfactants in food, cosmetics, pharmaceuticals, and in environmental applications⁵,⁶. Sophorolipids (SLs, Fig. 1) are glycolipid biosurfactants produced by yeast strains such as *Starmerella* (*Candida*) bombicola⁶, *Candida* apicola⁷, *Candida* bogoriensis⁸, and *Wickerhamiella domercqiae*⁹. SLs are produced by these yeasts in quantities greater than 100 g/L from different carbon sources, and can be efficiently recovered from culture broths, generally by precipitation⁵,⁹. Thus, these
compounds have attracted attention as practical surfactants, and a wide range of applications have been developed. Recently, SLs and their derivatives have also been reported to exhibit antimicrobial \cite{10, 11}, anticancer \cite{12}, and anti-viral \cite{10} activities.

SLs generally exist as a mixture of eight components with varying degrees of acetylation at the 6'- and 6''- positions of the sophorose moiety (Fig. 1) \cite{13, 14}. In these SLs, the major fatty acid is usually 17-hydroxyoctadecenoic acid. *Starmerella bombicola*, the best SL producer reported to date, produces primarily lactone-form SLs, with acid-form SLs as minor components. Other known SL producers also show a similar production pattern to that of *S. bombicola*.

The surface and interfacial activities of SL homologs, however, become stronger as the lactone ring is opened. For acid-form SLs, the interfacial tension is similar to that of sodium dodecyl sulfate (SDS), and the removal activity of trioleylglycerols, called washing power, is the same as for SDS, dodecyl-β-D-maltoside, and linear alkylbenzene sulfonate (LAS) \cite{15}. Recently, Gross *et al.* reported on chemical and/or enzymatic modification of SLs through esterification of the carboxyl end of the fatty acid, with the aim of improving interfacial and biological functions \cite{16, 17}. Hence, acid-form or other new types of SLs with superior hydrophilicity and/or water-solubility are highly preferable as major fermentation products compared to lactone-form SLs \cite{18}. Recently, we reported the isolation of acid-form SL-producing yeasts, *Candida batistae* \cite{19} and *Candida floricola*, which may enable efficient acid-form SL production \cite{20}.

For further development of the acid-form SL production process, we focused on glycerol as a substrate for acid-form SLs, because this material is a nontoxic, edible, biodegradable compound mostly derived from natural sources such as vegetable oils and animal fats, and is created as a by-product of the growing oleochemical industry which produces soaps, fatty acids, waxes, and surfactants \cite{21}. Additionally, glycerol is a by-product of biodiesel production, which has increased dramatically over the last 10 years, resulting in a large in excess of glycerol, especially in Europe \cite{22, 23}, since the pH of that glycerol is strongly alkaline, and neutralization is necessary. This indicates that glycerol is an attractive feedstock for production of useful chemicals. Moreover, glycerol, as a fermentative substrate for biosurfactant production, can be converted to acid-form SLs by *C. floricola* \cite{20}. The bioconversion from glycerol to glycolipids, however, suffers from low production yields \cite{20}. Improvement of the glycolipid yields is necessary for application of this process.

In the present study, we aimed to generate a high yield of acid-form SLs from waste glycerol using hydrophobic substrates to improve acid-form SL production. Here, we describe the enhancement of acid-form SL production by optimizing media components and the production of abundant acid-form SLs in the optimal medium using a fed-batch cultivation technique.

### 2 EXPERIMENTAL

#### 2.1 Materials

Waste glycerol, palm oil, palm kernel oil, coconut oil, and fatty acid methyl esters (FAMEs) were obtained from Lion Co. Ltd. Waste glycerol (350 mL) was diluted with an equal amount of de-ionized water, and with treated charcoal (200 g) overnight, and then filtered through a filter paper (Whatman No. 5) before use. All other materials were purchased from Wako pure chemical Co. Ltd.

#### 2.2 Microorganism

*Candida floricola* ZM1502, which can selectively produce acid-form SLs from glycerol, was used in this study \cite{20}. The strain ZM1502 has been deposited in the International Patent Organism Depositary, National Institute of Technology and Evaluation (IPOD-NITE), Japan, as FERM P-21133.
2.3 Cultivation conditions

To prepare glycerol stocks, *C. floricola* ZM1502 was cultivated in YM medium at 28°C and 250 rpm for 1 d, then stored at −80°C after addition of 100 g L⁻¹ of glycerol. These glycerol stocks were used for all experiments in this study.

Glycerol stocks were inoculated in 30 mL of YM medium, and pre-cultured at 28°C and 250 rpm for 1 d. The pre-culture broth (0.6 mL) was inoculated into 30 mL of production medium (100 g L⁻¹ glycerol, 3 g L⁻¹ urea, 0.5 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄, 1 g L⁻¹ yeast extract) with 50 g L⁻¹ hydrophobic substrate in 300-mL Erlenmeyer flasks. Urea was filtered separately for sterilization. Hydrophobic substrates used were soybean oil, oleic acid, palm oil, palm kernel oil, coconut oil, and various chain length FAMEs (Pastell M-8, M-10, M-12, M-16, and M-182; Lion Co. Ltd., Tokyo, Japan). Acid-form SL production was performed at 28°C and 250 rpm for 1 week. For high-concentration cultivation using a fed batch method, 30 mL of the pre-culture broth was introduced into 2 L of the initial medium included 200 g L⁻¹ glycerol, 6 g L⁻¹ urea, 12 g L⁻¹ KH₂PO₄, 2 g L⁻¹ K₂HPO₄, 1.5 g L⁻¹ MgSO₄, 7H₂O, 2 g L⁻¹ yeast extract, and 50 g L⁻¹ alkyl C₁₈ esters (Pastell M-182).

Twenty g L⁻¹ of yeast extract and 50 g L⁻¹ of the C₁₈ esters were fed in to avoid shortage of the C₁₈ esters or minor vitamins and minerals, and residual esters were monitored using thin-layer chromatography (TLC). The fermenter (TBR2-1, SakuraSeiki Co., Tokyo Japan) was operated at 28°C, 800 rpm, with 1vvm of airflow, and without pH control.

2.4 Fatty acid compositions analysis

To examine the fatty acid compositions of hydrophilic substrates, gas chromatography-mass spectrometry (GC-MS) analysis was conducted as previously reported²⁰. Methyl ester derivatives of fatty acids were prepared by mixing hydrophilic substrate or purified SL derivative (10 mg) with 1 mL of 5% HCl-methanol reagent (Tokyo Kasei Kogyo, Tokyo, Japan). After heating at 80°C for three hours, the reaction was quenched with water (1 mL), and the methyl ester derivatives were extracted with n-hexane (2 mL) and then analyzed by GC-MS (Agilent 5973 system, Agilent Technologies, Inc. Palo Alto, CA) using a TC-Wax column (GL-science Ltd., Tokyo, Japan) with the temperature programmed to rise from 80°C (after holding for 4 min) to 250°C at 10°C min⁻¹.

2.5 HPLC analysis for quantification of SLs

Total glycolipid production was determined by measuring the dry weight of glycolipids separated using a published method²⁰. The ratio of acid-form SLs to lactone-form SLs was determined using high-performance liquid chromatography (HPLC) on an HPLC system (SSPC; Tosoh, Tokyo, Japan) equipped with a silica gel column (Inertsil SIL-100A 5 μm, 4.6 mm × 250 mm; GL Science, Japan) and a low-temperature evaporative light scattering detector (ELSD-LT; Shimadzu, Kyoto, Japan). HPLC followed a gradient solvent program with varying proportions of chloroform and methanol (from 100:0 to 0:100) at a flow rate of 1 mL min⁻¹. Standard SL samples were obtained from olive oil by *S. bombicola* according to a published method²⁰. All measurements reported here were calculated as means from at least three independent experiments.

2.6 Dry cell weight

To estimate dry cell weight, SLs and residual hydrophobic substrates were removed from culture broth using ethyl acetate. Then, the culture broth was washed twice with methanol to remove residual ethyl acetate. Cells were suspended into water of the same volume as the culture broth. The suspensions were dried at 105°C overnight, then weighed.

All measurements reported here were calculated from at least three independent experiments.

3 RESULTS AND DISCUSSION

3.1 Effects of nitrogen sources on acid-form SL production

In previous research, the nitrogen source exhibited significant effects on SL productivity. Therefore, we first examined the effect of various nitrogen sources on acid-form SL production by *C. floricola* ZM1502. Urea, ammonium nitrate, sodium nitrate, potassium nitrate, and ammonium sulfate were used as nitrogen sources. The production yields using each nitrogen source are shown in Fig. 2. Urea increased acid-form SL production approximately 20% compared to ammonium nitrate, which was used in screening medium in previous research²⁰. On the other hand, sodium nitrate, potassium nitrate, and ammonium sulfate decreased acidic SL accumulation by 30% compared to ammonium nitrate. According to these results, urea was the best of the nitrogen sources tested. In previous studies, urea was often used as nitrogen source for SL fermentation by *S. bombicola*²⁸, ²⁵-²⁷. Davila *et al.* reported that the amount of nitrogen present, including ammonium ion in the broth, is a growth-limiting factor²⁸. Deshpande *et al.* used corn-steepliquor as a nitrogen source for SL fermentation²⁰. Chen *et al.* used ammonium sulfate as the nitrogen source in SL production medium for *W. domercqiae*²⁹.

In our results, strain ZM1502 appeared to utilize a broad range of nitrogen sources for acid-form SL production, which may also be true of other SL-producing yeasts. Considering the need for efficiency in industrial scale production, low-cost natural nitrogen sources such as corn steep liquor need to be explored. In the following experiments, urea was provisionally used as the nitrogen source.
3.2 Enhancement of acid-form SL production by supplemental hydrophilic substrates

To examine the effect of carbon source on acid-form SL production, soybean oil and oleic acid were added at 50 g L\(^{-1}\) as hydrophobic substrates, and glucose and glycerol were added at 100 g L\(^{-1}\) as hydrophilic substrates.

The acid-form SL concentrations and dry cell weights of *C. floricola* ZM1502 are shown in Table 1. Comparing glycerol and glucose as sole carbon sources, the SL yield and cell growth with glycerol were half of those with glucose. This result suggests that glycerol is not a suitable sole carbon source for acid-form SL production by strain ZM1502. Glycerol performed poorly compared to glucose, because the yeast consumes one more mole of NADH in the reaction of glycerol-3-phosphate dehydrogenase for assimilation of one mole of glycerol compared to assimilation of glucose\(^{30}\). Using solely hydrophobic substrates led to low production yields, because the acyl portion of the SL is formed through a *de novo* fatty acid synthesis pathway. Comparing glucose and the hydrophilic substrates olive oil and oleic acids, these hydrophobic compounds increased SL production to 16.2 ± 1.7 and 22.5 ± 3.9 g L\(^{-1}\), respectively. Cell growth decreased slightly with hydrophobic substrates compared to glucose. These results indicate that hydrophobic substrates increased the specific productivity of SLs, because the SL component of the acyl chain can be provided directly from the hydrophobic compounds, as well as from conventional SL-producing yeasts\(^{25, 30}\). With the addition of both glucose and hydrophobic substrates, SL yields increased drastically to around 28.7–36.1 g L\(^{-1}\), and cell growth increased to approximately 13 g L\(^{-1}\). Under these conditions, the hydrophilic and hydrophobic parts of SLs would be efficiently provided by glucose and hydrophobic substrates, respectively.

Surprisingly, glycerol and hydrophobic substrates further increased SL yields to 37.5–42.1 g L\(^{-1}\), although pure glycerol produced only 5 g L\(^{-1}\) of SL. Moreover, cell growth in these treatments increased to the same level as that with glucose and hydrophobic substrates. This phenomenon can be explained by considering the redox balance in the cell. Assimilation of hydrophobic substrates would lead to accumulation of large amounts of FADH in the mitochondria through acyl CoA dehydrogenase (EC 1.3.99.3) and the \(\beta\)-oxidation pathway of fatty acids. Then, the FADH accumulated in the mitochondria may directly stimulate mitochondrial flavoprotein-linked glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) coupled with respiration chain reactions and the alternative pathway from glycerol.

![Fig. 2](image.png)

**Fig. 2** Effect of various nitrogen sources on acid-form SL production by *C. floricola* ZM1502. Each nitrogen source was added at 3.0 g L\(^{-1}\). Cultivations were performed at 28°C and 250 rpm for 1 week. Error bars show the standard deviations.

**Table 1** Comparison of hydrophilic and hydrophobic substrates for acid-form SL production, (A), and cell growth, (B).

| (A) SL production | Hydrophobic Substrate (50 g L\(^{-1}\)) | Hydrophilic substrates (100 g L\(^{-1}\)) |
|-------------------|--------------------------------------|----------------------------------------|
|                   |                                      | -                                      |
|                   | -                                    | 10.2 ± 0.18                            |
|                   | -                                    | 5.0 ± 1.1                              |
| Soybean oil       | 16.2 ± 1.7                           | 28.7 ± 5.0                            |
| Oleic acid        | 22.5 ± 3.9                           | 36.1 ± 0.5                            |

| (B) Dry cell weight | Hydrophobic Substrate (50 g L\(^{-1}\)) | Hydrophilic substrates (100 g L\(^{-1}\)) |
|---------------------|--------------------------------------|----------------------------------------|
|                     | -                                    | 10.2 ± 0.11                            |
|                     | -                                    | 4.13 ± 0.08                            |
| Soybean oil         | 7.52 ± 0.12                          | 13.2 ± 0.59                            |
| Oleic acid          | 6.35 ± 0.19                          | 14.3 ± 1.00                            |
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3-phosphate to dihydroxyacetone phosphate\(^{31}\), but not NAD\(^+\) dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), in the cytosol\(^{30}\). The stimulated glycerol assimilation would result in high cell growth and SL yields. On the contrary, a previous report indicates that the flavoprotein-linked glycerol-3-phosphate-dehydrogenase deleted mutant of *Candida boidinii*, accumulates glycerol in the culture broth using NAD\(^+\)-dependent glycerol-3-phosphate dehydrogenase\(^{32}\).

On the other hand, glucose may cause catabolite repression to inhibit the assimilation of fatty acids, or may lead to repression of lipase, the enzyme that reacts in the first step of uptake of the fatty acid portion. Therefore, the hydrophobic substrates and glycerol are preferable over glucose for efficient acid-form SL production by strain ZM1502.

3.3 Exploring suitable hydrophilic substrates

To utilize acid-form SL-production in industrial processes such as methyl ester production, we explored industrial hydrophobic substrates that could be obtained on site in oleo-chemical industries. Hence, we used 10\% (w/v) charcoal-treated waste glycerol, a by-product of industrial FAME production in this experiment. Vegetable oils (palm oil, palm kernel oil, and coconut oil) at 5\% (w/v) and fractional-distilled FAMEs made from palm oil were used as additional hydrophobic substrates. The yields of acid-form SLs and dry cell weights are shown in Fig. 3. Furthermore,

![Effect of various hydrophobic substrates on acid-form SL production by *C. floricola* ZM1502. Each hydrophobic substrate was added at 50 g L\(^{-1}\) with 100 g L\(^{-1}\) of glycerol in the production medium. Cultivations were performed at 28°C and 250 rpm for 7 d. Error bars show the standard deviations.](image)

Table 2 shows the fatty acid compositions of these substrates. Among vegetable oils, palm oil produced the largest quantity of acidic SLs, up to 42.7 ± 0.1 g L\(^{-1}\). On the other hand, palm kernel oil and coconut oil enhanced pro-

![Acidic SLs (g/L) Dry cell weight](image)

Table 2  Fatty acid component of hydrophilic substrates.

| Fatty acids | Vegetable oils | FAMEs |
|-------------|----------------|-------|
| Oleic acid  | Soybean oil    |       |
| C8:0        | –              | –     |
| C10:0       | –              | –     |
| C11:0       | –              | –     |
| C12:0       | –              | –     |
| C13:0       | –              | –     |
| C14:0       | –              | –     |
| C15:0       | –              | –     |
| C16:0       | 4.5            | 12.6  |
| C16:1       | 10.2           | –     |
| C17:0       | –              | –     |
| C18:0       | 1.5            | 5.0   |
| C18:1       | 64.0           | 25.9  |
| C18:2       | 7.7            | 49.8  |
| C18:3       | –              | 6.7   |
| C20:0       | –              | –     |
| C20:1       | –              | –     |
| Palm        | 1.4            | –     |
| Palm kernel | 1.6            | –     |
| Coconut     | 14.5           | –     |
| M-8         | 100            | –     |
| M-10        | –              | –     |
| M-12        | –              | –     |
| M-16        | –              | –     |
| M-18        | –              | –     |
| M-182       | –              | –     |
duction to 32.6 ± 0.8 and 23.2 ± 1.6 g L⁻¹, respectively. These production yields correspond to the content of C₁₈-length fatty acids in the oils. In the case of FAMEs, C₁₈ esters drastically enhanced acidic SL production to 48.0 ± 2.4 g L⁻¹. The C₁₂-length esters, including 99.2% of the methyl palmitate, crystallized at cultivation temperature (28°C), and were hardly consumed for SL production or cell growth. C₁₂ esters enhanced SL production to 21.0 ± 3.4 g L⁻¹, as did coconut oil. According to GC-MS analysis, these compounds possess mainly C₁₂ length fatty acids (Table 2). The C₈ and C₁₀ esters did not enhance SL production. C₆ esters appeared to negatively affect SL production, because the compound markedly inhibited SL production as well as cell growth. Ogawa et al. reported that coconut oil slightly enhanced production of SLs by S. bombicola compared to olive, rapeseed, and soybean oils (33). For C. floricola, short chain FAMEs have toxic effects on the yeast. These results indicated that C₁₈-chain length fatty acid moieties of hydrophobic substrates greatly enhanced acidic SL production by C. floricola ZM1502. Starmerella bombicola also strongly enhanced its SL production when given C₁₈-length FAMEs (33); SL production was approximately 50 g L⁻¹ with C₁₈-length fatty acids and 20 g L⁻¹ with C₁₂-length fatty acids. From these results, the pattern of acid-form SL formation from various hydrophobic substrates by C. floricola corresponded to that of S. bombicola. Thus, C₁₈-length FAMEs should be suitable for acid-form SL production using waste glycerol.

### 3.4 Fatty acid composition of acidic SLs

SLs produced by S. bombicola are well known to have varying fatty acid compositions relative to those of the hydrophobic substrates in the production medium (35, 36). To determine the fatty acid composition of acid-form SLs produced from waste glycerol and C₁₈-length FAMEs by C. floricola, we performed GC-MS analysis for two different acid-form SLs (Table 3). In this analysis, C₁₈-length FAMEs markedly increased a component of 17-hydroxy oleic acid (18:1), which corresponds to the composition of the hydrophobic substrate, compared to pure glycerol as the sole carbon source (Table 2). This result indicates that the fatty acid moieties of hydrophobic substrates may be provided directly to the hydrophobic moieties of acid-form SLs, as is the case for S. bombicola (36, 37). This implies that the fatty acid composition of the present acid-form SLs may also be controlled by the different hydrophobic substrates with varying fatty acid compositions.

### 3.5 High-concentration cultivation

To develop an acid-form SL production method, we performed high-concentration cultivation in a jar fermenter using a fed-batch cultivation technique. According to preliminary tests, oxygen supply rates and residual hydrophobic substrate concentrations were significant limiting factors in batch cultivation, and the oxygen supply rate depended on the stirring rate (data not shown). Based on the results of preliminary tests, the initial and feeding medium components were designed for effective SL production. The stirring rate was set to 950 rpm to avoid oxygen limitation. Figure 4 shows the time series values of acid-form SL concentration, dry cell weight, pH, and residual glycerol. Dry cell weights increased to approximately 40 g L⁻¹ for 5 d, and were maintained at that level after growth. Acid-form SLs gradually increased for 4 d in the early stage of cultivation, then drastically increased thereafter, when the cell growth curve was in the plateau phase. The product concentration finally reached 169 g L⁻¹ after 7.5 d (180 h).

![Fig. 4 Time course of high cell concentration cultivation. Symbols: closed circles, acid-form SLs; open circles, dry cell weight; open triangles, pH; closed triangles, glycerol. Error bars show the standard deviations. Arrows show the time of feeding with 50 g L⁻¹ of C18 esters and 2 g L⁻¹ of yeast extract.](image-url)
The maximum volumetric productivity of acidic SLs was 22.6 g L⁻¹ d⁻¹. Glycerol was consumed completely within 7 d. In addition, the pH decreased to around 3.5. When agitation and aeration were stopped, the SLs precipitated as black liquid crystal phase on the bottom of the fermenter; the aeration was stopped, the SLs precipitated as acid-form SLs from waste glycerol and industrial hydrophobic substrates.

Felse et al. described the SL production yield as a mixture of the lactone- and acid-forms by the yeast S. bombicola to be around 50 g L⁻¹, and they improved the fermentation method using fed-batch cultivation to increase the SL mixture yield to 120 g L⁻¹. Furthermore, Davila et al. demonstrated 317 g L⁻¹ of SL mixture production via fed-batch cultivation. On the other hand, Ashby et al. reported that S. bombicola ATCC22214 produced 46 ± 4 g L⁻¹ of SLs, of which 71% were acidic SLs, from methyl esterified soy oil. From our results, C. florica has great potential for enhanced production yield of acid-form SLs with further development of fermentation methods, as does S. bombicola.

4 CONCLUSION

In conclusion, we successfully produced abundant acidic SLs using waste glycerol and fatty acid C₁₆-esters as industrial hydrophobic substrates for C. florica ZM1502. The production yield reached 169 g L⁻¹ in laboratory-scale experiments, and this yield offers great potential for commercial production. Our results impact the development of biosurfactant production as well as bio-production methods using waste glycerol as attractive feedstocks.

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