Sophorolipid Biosynthesis from a Biodiesel Co-product Stream

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ABSTRACT: We applied a biodiesel co-product stream (BCS) as a fermentation feedstock for the microbial synthesis of sophorolipids (SL). The BCS was composed of 40% glycerol, 34% hexane-solubles (made up of 92% FA soaps/FAME and 6% MAG/DAG), and 26% water. Batch culture fermentations of the yeast *Candida bombicola* on pure glycerol resulted in low-level synthesis of SL (~9 g/L). HPLC associated with atmospheric pressure CI–MS (LC/APCI–MS) revealed that the SL derived from pure glycerol had 99% of the FA side chains linked to the 4″ hydroxyl group of the sophorose sugar, resulting in a lactonic structure. In contrast, the use of the BCS as feedstock increased the SL yield to 60 g/L and the open-chain form to 75% including both oleic acid and linoleic acid (along with their methyl esters) as the dominant species comprising the side chains. By favoring the open-chain structure, the SL molecules (particularly the FA side chain) can be chemically modified without the need to open a lactone ring first. The ability to use the BCS as a feedstock for SL synthesis will provide an outlet for this residual material, thus helping to stimulate growth in the biodiesel market and the use of agricultural fats and oils from which the biodiesel was synthesized.

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KEY WORDS: Biodiesel co-product stream, *Candida bombicola*, glycerol, sophorolipid.

As the world's energy sources slowly gain momentum toward sustainability, corn and oilseed crops are being examined as potential starting materials to produce cheap, renewable fuels. Presently, bioethanol and biodiesel are products of intense interest to the biofuels industry. Bioethanol is typically produced using raw materials derived from starch plants (mostly corn), sugar plants (sugar beets and sugar cane), and, more recently, from lignocellulosic material through microbial fermentation (1), whereas biodiesel (methyl or ethyl esters from animal fats and/or vegetable oils) is synthesized primarily by the chemical transesterification of TAG with methanol or ethanol, respectively (2).

Recent figures indicate that the United States produced an average of 5.5 million metric tons of animal fat and grease (47% of which is tallow) and 10.8 million metric tons of vegetable oil (77% soybean oil) annually between the years 1999 and 2001. These production numbers continue to fluctuate slightly from year to year. However, recent trends indicate a net increase in both animal fat and vegetable oil production, making it increasingly important to provide additional outlets for these commodity materials. Between the years 1998 and 2002, U.S. production of biodiesel increased from 0.2 to 32 million gallons (~0.8 to 121 million liters) and, because of its fuel properties and improved emission characteristics, production is projected to reach 350 million gallons (~1.3 billion liters) by the year 2011 (3). Such increases in biodiesel production, while providing an additional outlet for fats and oils, will result in a large co-product stream, the disposal of which must be addressed to make such large production capacities economically and environmentally feasible. The biodiesel co-product stream (BCS) is composed primarily of glycerol, FFA, and FAME, the ratio of which results from the transesterification process used to manufacture the alkyl esters and the recovery efficiency of the biodiesel. The content of BCS makes this material intriguing for use as a feedstock for the production of additional value-added products. Although some work has been reported with respect to the use of glycerol and BCS as feedstocks for bacterial poly(hydroxyalkanoate) (PHA; bacterial polyester) production (4–6), more recently work has been initiated to produce additional value-added products from these materials.

Sophorolipids (SL) are extracellular glycolipids produced by yeasts (primarily *Candida bombicola*) that are composed of a disaccharide (sophorose; 2-*O*-β-D-glucopyranosyl-β-D-glucopyranose) attached to a hydroxy fatty acyl moiety at the ω-1 or ω carbon (Fig. 1) (7,8). Typically, the 6′ and 6″ hydroxyl groups of the sophorose sugar are acetylated, and the FA chain length varies between 16 and 18 carbons (although recently we confirmed an organism that can synthesize SL containing FA side-chains of 22 and 24 carbons; (9) and may be saturated or unsaturated. In addition, the carboxylic acid group of the FA

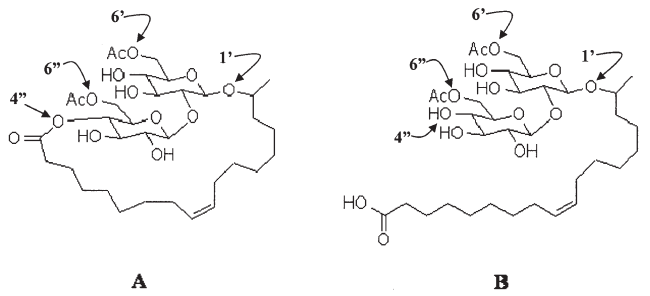

FIG. 1. Structures of 17-L-[2′-*O*-β-glucopyranosyl-β-D-glucopyranosyl)-oxy]-9-octadecenoic acid 6′,6″-diacetate sophorolipids in the (A) 1′,4″-lactone and (B) free acid forms.

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may be lactonized (generally preferred) to the disaccharide ring at carbon 4″ or remain as the free acid in the open-chain form.

The amphiphilic nature of SL imparts surfactant-type properties to them and has allowed their use as additives in shampoo, body washes, and detergents (10,11), in cosmetic products (12), and in the lubricant industry. In addition, their unique structure has increased interest in their use as a source of specialty chemicals such as sophorose and hydroxylated FA (13). These compounds are nontoxic, biodegradable and are produced in large quantities by *C. bombicola*, thus making them an attractive target for the feedstock use of glycerol and BCS. Modification of the FA portion of the SL structure can alter their physical and chemical properties, such as critical micelle concentration (CMC), surface-active properties, and detergent properties. To a certain extent, structural variation can be achieved by changing the lipidic carbon source, which alters the SL FA content (14,15) and may influence the acetylation pattern of the SL (15–17). Recent studies showed that SL also can be customized through the use of chemoenzymatic reactions to produce molecules such as glucose lipids (13) and SL with FA chains of varying functionality (18,19). Because SL are naturally synthesized with a preference for the lactonic form, the lactone ring must be opened prior to introducing a chemical modification at the carboxylic group of the FA. While this procedure is not difficult, it does cause the deacetylation of the sophorose sugar and imparts an additional modest cost to the alteration of the FA side-chain. In this paper, we report the use of glycerol and BCS as substrates for the production of SL by *C. bombicola* and show the advantages of using BCS as the feedstock in the production of open-chain SL, thus eliminating the lactone ring-opening step necessary for FA modification.

MATERIALS AND METHODS

Strain information and inoculum preparation. Candida bombicola ATCC 22214 was obtained from the American Type Culture Collection (Manassas, VA). The inocula for all of the SL production cultures were prepared in Candida Growth Media (CGM), which consisted of glucose (10% wt/vol), yeast extract (1% wt/vol), and urea (0.1% wt/vol) (7) and were grown at 26°C with shaking at 250 rpm. Frozen stock cultures were prepared by inoculating 100 mL of CGM (contained in a 250-mL Erlenmeyer flask) with a loopful of *C. bombicola* and grown for 24 h. Ten milliliters of this culture was then inoculated into 500 mL of CGM in a 1-L Erlenmeyer flask and the culture allowed to grow for an additional 48 h. The culture was then divided into 200-mL volumes and the cells were aseptically collected by centrifugation (16,000 × *g*; 10 min; 4°C). Fifty milliliters of a sterile aqueous glycerol solution (10% v/v) was then used to resuspend each cell pellet, and the cell suspensions were frozen in a dry ice/acetone bath and stored at −80°C until needed.

SL production. Production of SL was performed in 2-L volumes in a 2.5-L capacity bench-top fermenter (BioFloIII Batch/Continuous Bioreactor, New Brunswick Scientific, Edison, NJ). The SL production medium contained the following (in g/L): glycerol (99% pure, Sigma-Aldrich Chemical Co., St. Louis, MO) or BCS (Ocean Air Environmental, Lakeland FL; derived from a soybean oil-based reaction), 100; yeast extract, 10; and urea, 1. The pH was brought to 6.0 with concentrated HCl, and the media was sterilized by autoclaving. The glycerol or BCS was added as the carbon source for the fermentations in place of the glucose present in the CGM described above. A single 50-mL frozen stock culture was thawed and used to inoculate the 2 L of media. Each fermentation was carried out at 26°C, 700 rpm, 2 L air/min, and an initial pH of 6.0. After 24 h, an additional 100 mL of glycerol or BCS was added to the culture and the pH set to maintain at 3.5. At the 48-h timepoint, an additional 100 mL of glycerol or BCS was added to the culture, and the fermentations were allowed to continue for an additional 5 d.

SL recovery and purification. To isolate the SL, the entire culture (cells and broth) was divided into six portions and lyophilized to dryness (~2 d). The dried residues were placed into six 1-L Erlenmeyer flasks, and each portion was extracted with 500 mL of ethyl acetate by shaking at 250 rpm, 30°C for 5 d. The extraction mixture was filtered through Whatman No. 2 filter paper, and the solid residues were rinsed an additional two times with ethyl acetate (500 mL each time) to maximize SL recovery. The combined filtrate was concentrated by evaporation and added to 1 L of a mixture of hexane/petroleum ether (90:10 vol/vol) to precipitate out the pure SL. After vacuum drying in a desiccator, the purified SL were accurately weighed to obtain the product yield.

BCS composition. BCS was assayed for glycerol, FA soaps (as FFA), and FAME. Glycerol content (in mg/dL) was determined using the Glycerol-SL assay (Diagnostic Chemicals Limited, Charlottetown, Prince Edward Island, Canada) according to manufacturer's specifications. FFA and FAME content were determined gravimetrically as follows: The alkaline BCS (100 g) was acidified to pH 1 with concentrated HCl and extracted with three 50-mL volumes of hexane. The hexane layers were combined into a tared flask, evaporated under nitrogen, and dried *in vacuo* (635 mm Hg) for 24 h to a constant weight. The flask was then reweighed, and the amount of hexane-solubles (FFA and FAME) was calculated by difference from the starting weight. The total concentration of FFA and FAME was determined by dissolving a known amount of hexane-solubles back into hexane and using an established HPLC procedure (20).

FFA/FAME compositional analysis. The HPLC procedure just described was used to determine the total amount and ratio of FFA and FAME in the BCS, and the specific FFA and FAME present in the hexane-soluble fraction of BCS were determined by GC/MS. Samples were dissolved in hexane at a concentration of 10 mg/mL and silylated by reacting 10 μL of each sample with 250 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA; Pierce Biotechnology Inc., Rockford, IL) and 200 μL of pyridine. The mixtures were heated at 70°C for 30 min and allowed to cool to room temperature. Finally, an additional 150 μL of hexane was added to each sample, and the samples were analyzed by GC/MS. Samples (1 μL) were injected into a Hewlett-Packard (HP; Wilmington, DE) gas chromatograph

(GC) model 5890 Series II Plus equipped with a capillary inlet and an HP Mass Selective Detector model 5972 Series set to scan from *m/z* 40 to 550 at a rate of 1.5 scans/s. The capillary column (30 m × 0.25 mm) was coated with 0.25 mm of 5% cross-linked phenyl methyl silicone (HP-5MS). The oven temperature was programmed from 80 (1 min) to 230°C (10 min) at 10°C/min. The injector port temperature was 250°C in the splitless mode, and the detector transfer line was at 280°C.

SL analysis. The SL content was determined as described previously (14). In short, the SL mixtures were separated by HPLC with a Waters 2690 Separation Module (Waters Co. Milford, MA) using a 15 cm × 2.1 mm Symmetry C18 3.5-µm column. The linear gradient elution used was as follows: water/acetonitrile (0.5% formic acid)/acetonitrile (50:10:40), hold for 5 min, to a final composition of acetonitrile (0.5% formic acid)/acetonitrile (10:90) over 25 min with a total running time of 40 min. The flow rate was 0.25 mL/min. The effluent was connected to a Micromass ZMD mass spectrometer with an atmospheric pressure CI (APCI) probe (Waters Co.) set to the positive mode to scan from *m/z* 200 to 900 at 2 s per scan. Corona pin voltage was tuned to 3.8 kV, sample cone 20 V, and extraction cone 2 V for detection of fragments and molecular ions.

RESULTS AND DISCUSSION

Past experiments have shown that high SL yields from *C. bombicola* were achieved through the co-substrate fermentation of glucose and a FA source (in the form of FFA or TAG) (21,22). BCS is a renewable, inexpensive (current estimates indicate a selling price of about 15¢/lb or ~33¢/kg) raw material and is composed primarily of glycerol (which is known to be a good substrate for microbial growth), FFA, and FAME. Because of its content and in an attempt to find a new outlet for BCS, we tested a single BCS from soybean oil-based biodiesel production as a substrate for the synthesis of SL.

In 1995 Deshpande and Daniels calculated a cost of $2.69/kg for the production of SL (assuming yields of 100 g/L) *via* fermentation using corn syrup (@ $315.00/ton) and yellow grease (@ $330.00/ton) as substrates (23). Subsequently, Rau *et al*. (24) estimated the cost to produce SL at $1.00—$3.00/kg (24). These reports indicate that the production of SL by fermentation could be competitive with synthetically produced surfactants, which typically sell for approximately $2.00/kg. One of the economic strategies for biosurfactant production includes the choice of inexpensive raw materials. By substituting BCS as the substrate material, production costs for SL should diminish provided yields are maintained. In addition, this added outlet for BCS would eliminate the costs involved in isolating and purifying the glycerol from residual FA and esters.

We postulated that the presence of a methyl group on the carboxyl end of the FA would hinder lactonization of the SL and result in an increased concentration of open-chain structures. This would permit further postsynthetic modification of the FA side chain without the need to open the lactone ring. Compositional analysis revealed that the BCS used in this study was composed of 40% glycerol, 34% hexane-soluble compounds (made up of 92% FFA/FAME and 6% MAG and DAG), and 26% water. GC/MS analysis of the hexane-soluble fraction indicated the presence of substantially higher amounts of FFA compared with FAME [FFA (mol%): $C_{14:0}$, 1; $C_{16:0}$, 18; $C_{18:0}$, 13; $C_{18:1}$, 25; $C_{18:2}$, 25. FAME (mol%): $C_{16:0}$, 2; $C_{18:0}$, 1; $C_{18:1}$, 7; $C_{18:2}$, 7]. The higher concentrations of FFA compared with FAME (on a mol% basis) in the BCS indicated that, although the recovery rate of the biodiesel from the BCS may have been less than optimal, the incomplete conversion of the starting oil to methyl esters probably caused the large hexane-soluble fraction in the BCS. In addition, the ratio of oleic acid to linoleic acid in the BCS reflected the soybean-oil origin of the biodiesel.

Candida bombicola grew and produced SL from both pure glycerol and BCS. Specifically, pure glycerol resulted in low yields of SL (~9 g/L) whereas the use of BCS increased the SL yields to 60 g/L. This difference was not unexpected owing to the higher osmotic stress created in the pure glycerol fermentations and the lack of a FA source. The fermentations used in this study consisted of an initial 10% (wt/vol) carbon source concentration prior to inoculation. This translated into approximately 4% (wt/vol) glycerol in the BCS fermentations compared with 10% (wt/vol) glycerol in the pure glycerol fermentations. This large discrepancy is enough to affect the productivity of the culture. In fact, the cell yields from the pure glycerol fermentations were 8.7 ± 2.1 g/L compared with 42.5 ± 3.0 g/L from the BCS fermentations, indicating that higher glycerol concentrations did indeed have a deleterious effect on the growth of *C. bombicola*. In addition, glycerol is typically transported across the cell membrane by facilitated diffusion over a concentration gradient and is sequentially converted to dihydroxyacetone phosphate (DHAP), a common intermediate in substrate-level phosphorylation, by the action of glycerol kinase and glycerol phosphate dehydrogenase, respectively. This conversion is ATP-dependent and is therefore energy-intensive for the cell. DHAP can then be enzymatically isomerized to glyceraldehyde-3-phosphate (G3P), and together DHAP and G3P can be converted to glucose through the gluconeogenic pathway and subsequently to sophorose. Alternatively, G3P may be converted to pyruvate for subsequent conversion to acetyl CoA (the vital metabolic precursor for FA biosynthesis). Because the organism does not have an exogenous source of FA, survival is predicated on the ability of the organism to synthesize FA from glycerol for use as membrane components. This, along with the energy-intensive nature of FA biosynthesis, does not allow for the accumulation of large concentrations of excess FA that could be assimilated into large quantities of SL. On the other hand, approximately one-third of the BCS was made up of FFA and/or FAME. While the glycerol fraction of the BCS could be used for cellular growth, the hexane-soluble portion could be used for SL biosynthesis, thus allowing larger synthetic capabilities for SL from BCS.

We previously reported an LC/APCI–MS method that allowed for the separation and characterization of SL (14). The mass spectrum generated by APCI for either the lactone or free acid form of SL had a protonated molecular ion $(M + H)^+$ fol-

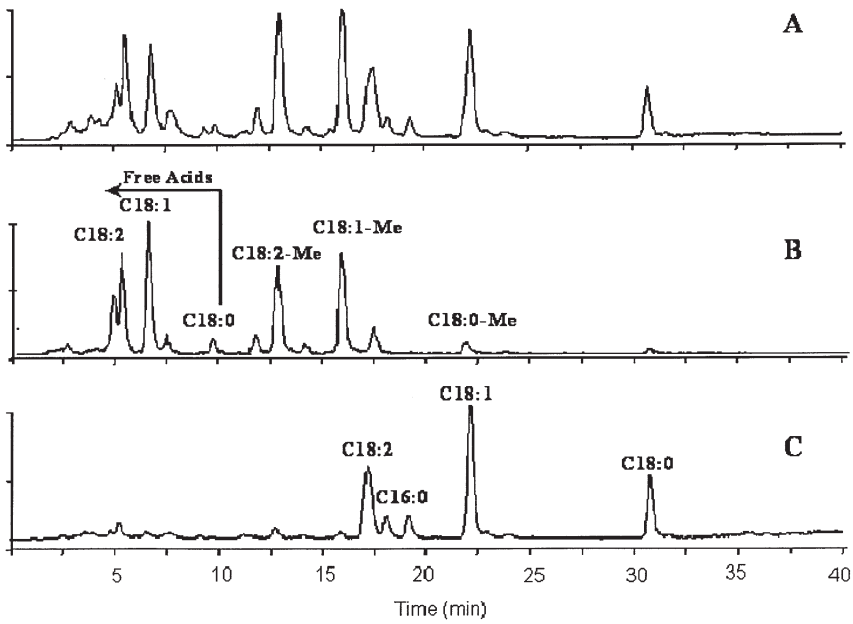

FIG. 2. LC/atmospheric pressure CI-MS (LC/APCI-MS) analysis of the sophorolipids (SL) produced by *Candida bombicola* when grown on a biodiesel co-product stream. (A) Total current ion chromatogram, (B) resulting chromatogram showing the nonlactonized products after plotting the ion mass of 409, and (C) chromatogram of the elution of the lactone SL forms after plotting the ions corresponding to the mass range of 600–700 amu.

lowed by a series of ions with decreasing intensity corresponding to the sequential loss of H_2O molecules. The SL spectrum typically shows a characteristic mass loss of 204 amu from the molecular ion, which corresponds to a C_6 acetylated hexose ring. The lack of acetyl groups on the sophorose ring is reflected by this ion. Both SL forms give a fragment ion for the protonated hydroxyl FA, which is at *m/z* 299 for the $C_{18:1}$ SL, providing evidence of the FA moiety. This ion is more intense in the free acid form than the lactone. Also, the nonlactonized free acid form presents a characteristic ion at *m/z* 409 corresponding to the disaccharide fragment. Since the *m/z* 409 ion is independent of the FA moiety and has a negligible intensity in the lactonized form, the extracted ion chromatogram resulting from this ion allows the identification of the nonlactonized form eluting from the column.

The FA moiety on the SL structure can be influenced by the FA content of the oil used as the carbon source, but in general the lactone form with a $C_{18:1}$ FA moiety is secreted as a major product (14). When BCS was used as the carbon source, the LC/APCI–MS chromatogram had the elution profile shown in Figure 2A, which is more complex than those previously observed. Extraction of the ion at *m/z* 409 from the total ion current chromatogram indicated that nonlactonized forms of the FA were responsible for the different chromatographic profile. The peaks eluting in the first 10-min window had spectra consistent with FA, as labeled in Figure 2B. The second, more intense peak, eluting at about 16 min (Fig. 2B), had the APCI spectrum shown in Figure 3. The spectrum had a base peak at *m/z* 313 consistent with a methylated C_{18} hydroxy FA ion fragment, followed by double bond formation at the hydroxy group and the loss of the methoxy group to generate the ions at *m/z* 295 and 263, respectively. The ions at *m/z* 721 and 743 confirmed the $[M + H]^+$ and $[M + Na]^+$, respectively, corresponding to a SL structure bearing a methylated $C_{18:1}$ FA that generates the ion at *m/z* 703 after the loss of water. The loss of mass 204 from the molecular ion to generate the ion at *m/z* 517 is consistent with the loss of an acetylated hexose ring. The chromatographic peaks eluting between 10 and 22 min had spectra that are consistent with nonlactonized methyl ester forms and are labeled accordingly in Figure 2B. Plotting the mass chromatogram in the range of 600–700 amu produced the chromatogram shown in Figure 2C, whose spectra of the individual peaks were consistent with lactone forms and are labeled according to the FA moiety.

When the yeast was grown with glycerol as the carbon source, the product analysis revealed the formation of the lactone forms as the main products with negligible amounts of nonlactonized SL as shown in Figure 4. In an effort to maximize the open-chain conformation of SL derived from glycerol based fermentations, FAME in the form of biodiesel were added to the media at a concentration of 12.5 g/L, thus mimicking the same ratio of glycerol to hexane-soluble fraction present in the BCS. These fermentations resulted in SL yields of 43 g/L. On LC/APCI-MS analysis, it was evident that the inclusion of biodiesel produced a chromatogram with peaks having elution times and spectra consistent with the chromatogram obtained for the BCS (Fig. 2). The total composition of the SL derived from pure glycerol, BCS, and glycerol + biodiesel are

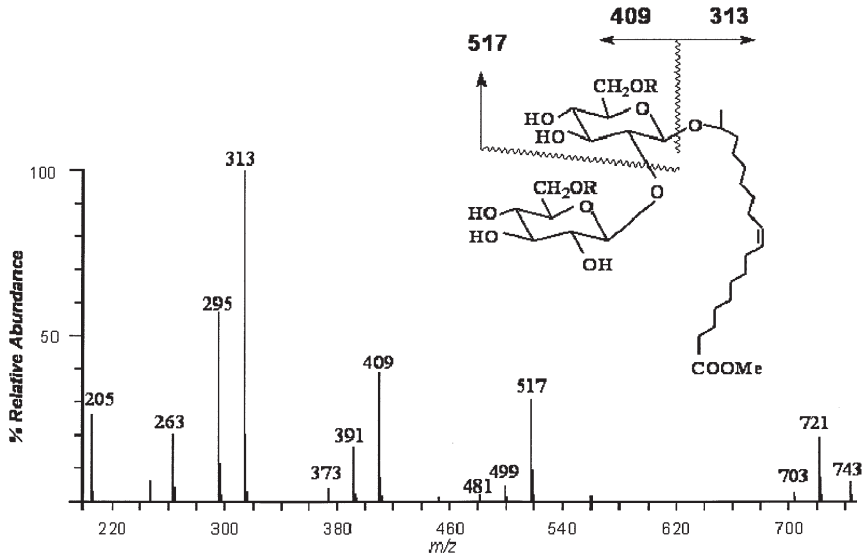

FIG. 3. APCI spectrum of the peak eluting at approximately 16 min in Figure 2A (R = either an acetyl group or proton). For abbreviation see Figure 2.

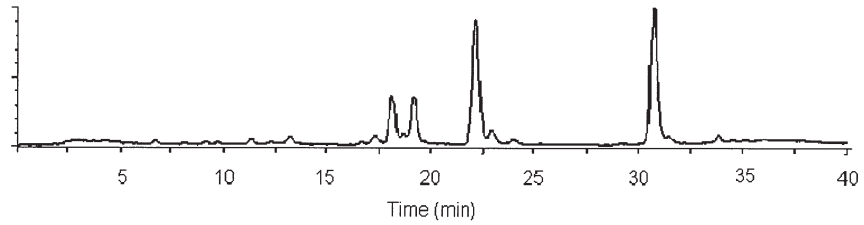

FIG. 4. LC/APCI-MS analysis of the SL produced by *C. bombicola* when grown on pure glycerol showing the formation of lactonized SL as the main products. For abbreviations see Figure 2.

given in Table 1. Results showed that there was no benefit to spiking the fermentation media with biodiesel over the use of BCS. In both cases the open-chain conformation was the most prevalent, accounting for 75% of the SL synthesized from BCS vs. 69% of the SL synthesized from glycerol + biodiesel with oleic acid and linoleic acid predominating. In comparison, the SL derived from pure glycerol were 99% lactone with the majority of the FA either palmitic, stearic, or oleic.

TABLE 1
The Structural Forms of Sophorolipids Produced by *Candida bombicola* from Glycerol, the Biodiesel Co-product Stream (BCS), and Biodiesel

	FA structural form (mol%)		
Substrate	FFA	FAME	Lactone[a]
Glycerol	1	—	99
			(C16:0, 23%)[b]
			(C18:0, 33%)
			(C18:1, 34%)
BCS	32	43	25
	(C18:1, 16%)	(C18:1, 20%)	(C18:1, 11%)
	(C18:2, 15%)	(C18:2, 17%)	
Glycerol + biodiesel	24	45	31
	(C18:1, 13%)	(C18:1, 20%)	(C18:0, 10%)
	(C18:2, 10%)	(C18:2, 18%)	(C18:1, 12%)

[a]Lactone = 1′,4″ lactonization of the FA.
[b]Parenthetical values correspond to specific FA that are in the majority in the designated structural form and the concentrations (in mol%) of those FA.

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