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Production and modification of bioactive biosurfactants

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Dedicated to Prof. Dr. Wolf-Dieter Deckwer on the occasion of his 65th birthday

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Abstract

The soil bacterium *Tsukamurella* spec. DSM 44370 produces a mixture of oligosaccharide lipids when cultivated on sunflower oil. In contrast cultivation with calendula oil as carbon source afforded a different product composition with overproduction of 2,3-di-*O*-acyl-ß-D-glucopyranosyl-(1-2)-ß-D-galactopyranosyl-(1-6)-4,6-di-*O*-acyl-α-D-glucopyranosyl-(1-1)-α-D-glucopyranose (GL 3) that amounted to 60 % of the whole product. GL3 and its parent tetrahexose backbone G 3 were then modified enzymatically with the lipase Novozyme 435 from *Candida antarctica* by addition of one and two oleic acid molecules to GL3 and four molecules to G 3. The new glycolipids were shown to exhibit interesting surface activities compared to commercially available products, decreasing the surface tension of water to 23 mN/m. In addition these products showed novel biological activities through the inhibition of the activation of Epstein-Barr virus early antigen.

Keywords: Tsukamurella spec.; di- and oligosaccharides; calendula oil; bioreactor cultivation; lipase; ß-galactosidase

1. Introduction

Various microorganisms are known that produce biosurfactants with a wide variety of different molecular structures. Most of these are formed by growth on hydrophobic substrates such as n-alkanes, fatty acids or plant oils [1-3]. The most common and best investigated class of biosurfactants are the glycolipids [4], which often consist of one or two sugar molecules linked to long chain aliphatic or hydroxylated aliphatic acids. Their amphiphilic structure makes them good surface active reagents that have advantages with respect to their toxicity and biodegradability over chemical detergents [5, 6].

In [7], a new bacterium of the genus *Tsukamurella*, *Tsukamurella* spec. DSM 44370, is described, which produces a mixture of di-, tri- and tetrasaccharide lipids when grown on sunflower oil (Fig. 1). These products show good surface active and antimicrobial properties [8]. In contrast to most other microbial glycolipids that consist of a constant carbohydrate and a variable fatty acid moiety, the *Tsukamurella* spec. product is a mixture of molecules containing different sugar moieties. Although some publications [7, 8] have already dealt with the improvement of their production and properties, these aspects still require optimization. In particular the directed production of particular glycolipids and their improvement by bioconversion are of great interest.

This article deals with the production of glycolipids obtained by cultivation of *Tsukamurella* spec. on unusual oils. Notably the use of calendula oil as substrate to favour the production of a specific product spectrum was investigated in detail. Additionally, the number of oligosaccharide lipids and related products were increased by chemo-enzymatic modifications. These compounds were tested for their ability to influence the surface tension of water and their modified bioactivity has been studied.

2. Materials and Methods

2.1. Bacterial cultivation, production and purification of glycolipids

Tsukamurella spec. DSM 44370 was maintained on nutrient broth agar (Difco-Laboratories, Detroit, USA), where it could be stored at 4 °C for three months and then transferred. Submerse cultivation was performed with sunflower, calendula and rapeseed oil in a mineral salt medium containing per litre: 7.44 g $(NH_4)_2SO_4$, 1.78 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g $CaCl_2 \cdot 2\ H_2O$, 100 mL 1 M Tris/HCl-buffer pH 7.5, 444 µg $ZnSO_4 \cdot 7\ H_2O$, 75 µg $CuSO_4 \cdot 5\ H_2O$, 6.3 mg $FeSO_4 \cdot 7H_2O$, 255 µg $MnSO_4 \cdot H_2O$, 672 µg $CoSO_4 \cdot 7\ H_2O$, 81 µg $NiSO_4 \cdot 6\ H_2O$, 78 µg $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 186 µg H_3BO_3, 30 µg KI and 260 mg EDTA. The oils served as carbon sources and antifoam reagents, at starting concentrations of 20 g/L. Inoculates were grown in 500 mL and 2 L shake flasks containing 100 mL and 500 mL medium, respectively, at 30 °C and 100 rpm. The inoculation volume was 10 % of the final cultivation volume.

Bioreactor cultivations were performed on 10 and 50 L scales using Biostat C and Biostat E (Braun, Melsungen, Germany). They were equipped with an intensor system, pO_2-electrode and exhaust gas analysis (Oxygor and Unor, Maihak, Hamburg, Germany). The temperature was maintained at 30 °C, with stirring at 550 rpm, aeration rate of 0.4 vvm and pH adjusted to 7.5. Variations of the cultivation conditions are described in the text.

For product recovery, the cultivation broth was exhaustively extracted with methyl-tert.-butyl ether (MTBE) and centrifuged at 4 °C. The organic phase was evaporated and afterwards the extract redissolved in CH_3OH/H_2O (4/1, v/v). This solution was extracted twice with cyclohexane to separate product (aqueous phase) and residual oil substrate (hydrophobic phase). The methanol/water phase containing the glycolipids was reduced by freeze-drying (vacuum, -50 °C) and the crude glycolipidic product mixtures were separated by medium pressure liquid chromatography. The stationary phase used was silica gel 60 with a mobile phase of $CHCl_3/CH_3OH/H_2O$ (65/15/2, v/v/v). A RP-C_{18} stationary phase with a liquid phase

of CH_3OH/H_2O (4/1, v/v) was used to separate compounds with identical carbohydrate moieties but differing acyl substituents.

2.2. Chemo-enzymatic modification of natural glycolipids

The chemical desacylation of GL 3 was performed by boiling a solution of 0.5 mmol in 50 ml 0.5 N NaOH for 4 h. After neutralization with 0.5 N HCl, CH_3OH was added, precipitates of NaCl were removed by filtration and fatty acids were removed by threefold extraction with MTBE. Methanol was evaporated at 250 mbar and 40 °C, and the residue was lyophilized. The crude product was purified by ion exchange chromatography (DOWEX 50WX8 and DOWEX 2XA, Serva, Heidelberg, Germany).

Enzymatic acylations using 100 mg lipase from *Candida antarctica* (Novozyme Lipase 435) were generally carried out in 100 ml flasks at 100 rpm and 55 °C in the presence of 2 g activated molecular sieve. For GL 3, 0.1 mmol microbial product was incubated in 50 ml MTBE with 0.3 mmol oleic acid for 48 h. For the carbohydrate backbone G 3, 0.1 mmol hydrolysis product was incubated in 50 ml MTBE/pyridine (1/1, v/v) with 1.0 mmol oleic acid for 48 h. Finally, in both cases the enzyme and molecular sieve were removed by filtration and washed three times with MTBE. The solvents were evaporated under reduced pressure and the crude residue extracted with methanol/water/n-hexane (3/1/1, v/v/v) to remove residual oleic acid. The methanol/water phase containing the glycolipids was reduced by freeze-drying (vacuum, -50 °C) and the crude products were subsequently purified by medium pressure liquid chromatography. The stationary phase used was silica gel 60 with a mobile phase of $CHCl_3/CH_3OH/H_2O$ (65/15/2, v/v/v).

The enzymatic hydrolysis of 0.05 mmol GL 3 using the ß-galactosidase from *Aspergillus oryzae* (75 U) was performed at 100 rpm in 10 ml 0.1 mmol phosphate buffer (pH 7.5) at 30 °C for 48 h. For downstream processing, the product and residual substrate were extracted with MTBE and separated by medium pressure liquid chromatography (stationary phase: silica gel 60, mobile phase $CHCl_3/CH_3OH/H_2O$ (65/15/2, v/v/v).

2.3. Analytical methods

Biomass was measured gravimetrically in duplicate. 10 mL culture suspension were centrifuged for 15 min at 13000 rpm, washed twice with 5 mL ethanol/butanol (1/1, v/v), and dried at 300 mbar and 75 °C. Ammonium and phosphate ion concentrations were measured semiquantitatively with analytical test strips (Merckoquant 10024 and Merckoquant 10428, Merck, Darmstadt, Germany). The content of oil substrates was measured via HPLC using a RP-18 column (ET 250/3 Nucleosil 120-5C_8, Macherey and Nagel, Düren, Germany) with $CHCl_3$/CH_3CN (30/70, v/v) as liquid phase at 1 mL/min. Detection was performed with an evaporative light scattering detector (Alltech, MKIII, Deerfield, USA).

To measure the concentration of glycolipids, 3 mL of medium broth were extracted three times with the same volume of MTBE, the phases separated by centrifugation and the organic phases analysed by thin layer chromatography (silica gel, $CHCl_3$/CH_3OH/H_2O (65/15/2, v/v) as mobile phase). Quantification was performed by densitometry with α-naphthol/sulfuric acid as detecting agent. Alternatively, the glycolipid concentration was measured in duplicate by HPLC with a Nucleosil 120-5C_{18} column (CS-Chromatographie Service, Langerwehe, Germany) with a gradient system of H_2O/CH_3OH as mobile phase. Detection was performed with an evaporative light scattering detector (Alltech, MKIII, Deerfield, USA). Surface tension was determined for aqueous solutions of the test substances with a Tensiomat (MGW Lauda, Königshofen, Germany) at 25 °C using the ring method.

Structural elucidation of the purified glycolipids was performed using standard nuclear magnetic resonance spectroscopic techniques. One-dimensional and two-dimensional (COSY) ^{1}H NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer with $CDCl_3$/CD_3OD (70:30) as solvent. Chemical shifts (δ) are given in ppm relative to internal tetramethylsilane and coupling constant (J) are in Hz.

2.4. Bioactivity test

The inhibition tests (anti-tumor-promoting activities), determined using a short term in vitro test for Epstein-Barr virus (EBV) activation in Raji cells (EBV genome carrying human lymphoblastoid cells; nonproducer type) induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), were performed as described in Shirahashi et al. [9]. The Raji indicator cells ($1 \cdot 10^6$ cells/mL) were incubated in 1 mL of a medium containing 4 mM n-butyric acid as an inducer, 32 pM of TPA (20 ng/mL in DMSO), and a known amount (32, 16, 3.2, or 0.32 nmol) of the test compound at 37 °C in a CO_2 incubator. After 48 h, the cell suspension was centrifuged at 1,000 rpm for 10 min, and the supernatant was removed. The activated cells were stained with high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients, and the conventional indirect immunofluorescence technique was employed for detection. The viability of the cells was assayed by the Trypan Blue staining method.

3. Results

3.1. Production of Glycolipids

Although sunflower oil is the first substrate where glycolipid production with *Tsukamurella* spec. has been reported, it is known that product formation also takes place on various other oily substrates [8]. Therefore, calendula and rapeseed oil, as unusual substrates, were used as carbon sources to test their potential for improving product formation or modifying its composition. Calendula oil is a triglyceride consisting of about 60 % of calendula acid, which is octadec-8,10-trans,12-cis-trienic acid. Rapeseed oil contains about 60 % of erucic acid (C 22:1), so both are significantly different to sunflower oil, which contains about 80 % oleic acid (C 18:1). Table 1 shows the results of fermentations performed with sunflower, rapeseed and calendula oil as carbon sources. Obviously, the highest product amounts were obtained using sunflower oil as substrate, but the highest biomass production was found in the fermentation with calendula oil. These findings illustrate that the composition of the glycolipidic products depends on the substrate used with the calendula oil cultivation clearly promoting the formation of one glycolipid, GL 3. As an example, Fig. 2 shows the course of the cultivation of *Tsukamurella* spec. with calendula oil as carbon source. The typical growth behaviour of *Tsukamurella* spec. started with a lag-phase of 20 hours, followed by exponential growth until ammonium and phosphate were consumed after 50 hours and finally a stationary phase from 50 hours until the end of the cultivation. Glycolipids were mainly produced under ammonium- and phosphate-limiting conditions after growth had finished, that is after 50 hours. The final product concentration was already reached after 160 h and remained almost unchanged until the end of the cultivation. After 150 h, a sudden increase of product concentration was observed which correlated with an increase of biomass, after growth had finished because of the limiting conditions. The two-phase cultivation with aqueous and oil phases causes a number of problems that lead to considerable deviations in the analyzed parameters that can be ascribed to sampling rather than measuring problems. These problems are not unique and occurred during similar two-phase cultivations.

Here, not only is the amount of products of interest but also their composition. The individual glycolipid concentrations (Table 1) were determined by separation of the different product molecules by HPLC. It is remarkable that cultivation with calendula oil led to a significantly different product ratio with GL 3 accounting for more than 60 % of the whole product. Fig. 3 shows the change in the concentrations of the different glycolipids during this cultivation.

3.2. Chemo-enzymatic modification of the oligosaccharide GL 3

In order to obtain new molecular species, which might show interesting novel properties with respect to their surfactant characteristics or biological activities, glycolipids can be chemo-enzymatically modified by, for example, regiospecific trans-esterification, acylation or hydrolysis. As GL 3 is the most interesting structure of the *Tsukamurella* glycolipids, it was chosen for modification experiments. It was directly acylated with oleic acid using a lipase (Novozyme Lipase 435) as catalyst to give additional mono- and bi-acylated molecules, GL 4 and GL 5, respectively (Fig. 4). For the standard batch with GL 3/oleic acid/immobilized lipase (0.1 mmol/0.3 mmol/100 mg) in 50 ml hydrophobic solvent, the reaction was optimised with respect to (optimal values in parentheses) water content (< 0.01 %), concentration of molecular sieve (40 mg/mL) and temperature (55 °C). Quantitative measurements resulted in approximately 90% GL 4/GL 5 in a ratio of 5/1. Problems with the stability in the presence of traces of acids and temperatures > 40°C after downstream processing resulted in a reduced yield (20%).

In order to significantly change the native lipid moiety of the glycolipid, GL 3 was initially hydrolysed under alkaline treatment to give 92% G3, the carbohydrate backbone. An alternative route using various lipases and esterases in aqueous buffer systems was unsuccessful. Subsequently, G 3 was acylated with oleic acid using the lipase catalyzed reaction. However, it was crucial to find a solvent in which G 3 is soluble and which allows the target reaction to proceed. A mixture of MTBE/pyridine 1/1 was found to be appropriate and afforded a 20% conversion after 48 h. One major and two minor spots were identified by TLC (data not shown) and structure elucidation by nuclear magnetic resonance

spectroscopic techniques of the major product, GL 6, indicated a tetrasubstituted G 3 (Fig. 4).

Finally, the actions of various commercially available glycosidases were tested for hydrolysis of the glycosidic linkages between the sugar units of GL 3. Only ß-galactosidase from *Aspergillus oryzae* was effective in releasing the galactose to give a 70% yield of the well-known GL 2 (Fig. 1), one of the natural *Tsukamurella* oligosaccharide lipids.

3.3. Structure elucidation of glycolipids GL 4-6

The structure of the three main glycolipids, GL 1-3, produced by *Tsukamurella* had already been established by a combination of 1D and 2D NMR spectroscopy, and positive- and negative-ion electrospray mass spectrometry, which allowed unambiguous identification of the respective acyl systems through MS-MS analyses [7]. Here comparison of the ^{1}H NMR data of GL 4-6 (Tables 2 and 3), derived from 1D and 2D (COSY) spectra, with those previously recorded for GL 3 afforded an unambiguous structure elucidation. For GL 4 the characteristic low field shifts of H-6A and H-6B (4.28-4.18 ppm) compared to those of GL 3 (3.81/3.71 ppm) of sugar unit D were indicative of acylation at C-6. The additional signals and their intensities in the ^{1}H spectrum, compared to GL 3, were characteristic of one oleic acyl group with signals at 5.37 ppm characteristic of the cis double bond (Table 3). The unambiguous identification of GL 5 was not possible as this product was unstable. However it showed a different, less polar R_f value to that of GL 4 and a similar ^{1}H NMR spectrum. We assume that an extra acyl group is present at C-6 of sugar unit C.

The ^{1}H NMR data of the sugar units GL 6 (Table 2) were very similar to those of GL 3 showing low field ^{1}H chemical shifts for the protons of the acylated carbons C-4 and C-6 of sugar unit A and C2 and C-3 of sugar unit C. Again the characteristic signals and their intensities identified the cis oleic acyl substituents (Table 3).

3.4. Surface active properties

As glycolipids consist of a hydrophilic sugar moiety and hydrophobic fatty acid moieties, nearly all show surface active properties [10]. They were quantified by their critical micelle concentration (cmc) as well as their ability to lower the surface tension of water, which under standard conditions has a value of 72 mN/m. Table 4 shows the characteristic values of *Tsukamurella* spec. glycolipids and their derivatives together with those of the commercially available alkylpolyglycoside *APG 1200 Plantaren*® (Cognis, Düsseldorf, Germany) for comparison.

3.5. Bioactive properties of oligosaccharide lipids and oligosaccharides

The natural products GL 1 and GL 3 showed effective anti-tumor-promoting activities by inhibition of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced activation of Epstein-Barr virus early antigen (Table 5). For instance, 32 nmol ($1.1 \cdot 10^{-2}$ mg/mL in DMSO; 1,000 % ratio to TPA) of GL 1 led to a 90.8 % inhibition of TPA-induced activation of EBV-EA (residual activation: 9.2 %). G 3 showed the best results with an inhibition of 97.4 % (residual activation: 2.6 %).

4. Discussion

It is common knowledge that product formation in bioprocesses strongly depends upon the substrates used. This has also been shown for several organisms capable of producing biosurfactants [6], such as sophorose lipids [11, 12], mannosylerythritol lipids [13, 14], rhamnolipids [15, 16] and others [17]. The choice of substrates not only influences characteristics such as biomass production, yield and productivity, but can also result in new products or different product composition. Therefore, we have used rapeseed (containing mostly C 22:1 fatty acids) and calendula oil (C 18:3) as unusual oil substrates instead of sunflower oil (C 18:1) [7] for the production of glycolipids in the cultivation of *Tsukamurella* spec. Although this did not result in higher productivities or yields, the product composition was changed significantly in the case of calendula oil. While cultivations on other plant oils always resulted in nearly equal concentrations of all three glycolipids (GL 1 to GL 3), cultivation with calendula oil favours the production of GL 3 which is more than 60 % of the whole product. This overproduction of GL 3 cannot be explained by different fatty acid compositions of the oil substrates, for GL 2 and GL 3 differ in their sugar backbone structures and not in their fatty acid residues. The formation of one specific glycolipid as the most abundant product with calendula oil could be useful if a particular product composition or a single component is of special interest and is one of the reasons why GL 3 was used here for further modification studies.

Compared to the literature on the biochemical transfer of sugars on fatty acids to afford a variety of glycolipids [18], the number of attempts to increase or decrease the functionality of natural glycolipids is relatively low. In [18], sophorolipids from *Candida bombicola* have received particular attention and have been used to produce derivatives. For instance, several alkyl esters and amides have been synthesized chemically or enzymatically using lipases [19-21]. Another interesting paper reports the lipase-catalyzed synthesis of a new type of sophorolipid that has been obtained by esterifying the primary alcohol at C6 of galactopyranose with 18:1 or 18:0 fatty acid moieties of the sophorolipids [22]. Using glycosidases such as the fungal hesperidinase and naringinase, it has been possible to

cleave the glycosidic bonds between specific sugar units in sophorolipids and rhamnolipids [23, 24]. All these modifications have been performed with regard to their potential use for technical or pharmaceutical applications.

To further diversify the glycolipids of *Tsukamurella* spec. and thus create possibly interesting derivatives, the native products were modified by enzymatic catalysis using the lipase Novozyme 435. Native GL 3 could rather easily be acylated with oleic acid and afforded 90% GL 4 and GL 5 under optimised conditions in a ratio of about 5:1. Both products were difficult to isolate however, as they were sensitive to traces of acids and increased temperature. Compared to the four native acylation positions in GL 3, the additional acylation positions (or the fatty acid chain length) appear to be energetically unfavourable.

Compared to this, the lipase-catalyzed acylation of G 3, the sugar backbone of GL 3, with oleic acid resulted in one major glycolipid, GL 6, and two side products. GL 6 resembles native GL 3 both in the extent of acylation and the position of the four substituents. As there are no naturally occurring molecules with higher or lower acylation levels, this state seems to be the energetically most favoured.

As GL 3 [2,3-di-*O*-acyl-ß-D-glucopyranosyl-(1-2)-ß-D-galactopyranosyl-(1-6)-4,6-di-*O*-acyl-α-D-glucopyranosyl-(1-1)-α-D-glucopyranose] contains two ß-anomeric centres, ß-glucosidase from almonds and ß-galactosidase from *Aspergillus oryzae* were tested for regiospecific hydrolysis. No catalysis was observed for the ß-glucosidase, possibly because of steric hindrance of the acyl substituents in glucose ring C. In contrast ß-galactosidase yielded galactose and GL 2, a compound that was also produced directly during the *Tsukamurella* cultivation.

With regard to surfactant activities, GL 4 did not perform as well as GL 2 or GL 3. Although it could reduce the surface tension of water to 23 mN/m like GL 2, it did so at a cmc of 200 mg/L instead of 100 mg/L measured for GL 2. Nevertheless, it still proved to be more effective than the commercial product *APG 1200 Plantaren*® [8], which reduces the surface tension of water only to 27 mN/m.

The antimicrobial properties of glycolipids have often been reported. Very recently, defined rhamnolipid mixtures and cellobiose lipids have shown good inhibition behaviour against gram-positive bacteria (e.g., *Staphylococcus aureus*) and phytopathogenic fungal species [16, 25-27]. In contrast the number of studies describing anti-tumor-promoting activity is smaller. In this regard GL 1 and GL 3 showed poor responses compared to either the synthetic galactoglycerolipids with branched and unsaturated acyl chains [28-30] or the natural azaphilones and uncommon amino acids from red-mold rice [31], some of which showed 95 % to 100 % inhibition of induction at 10^3 mol ratio/TPA and therefore appear to be potent cancer chemopreventive agents as anti-tumor promoters. On the other hand, specific natural glycoglycerolipids [32] and natural lupane- and oleanane-type triterpenoids [33] are effective at comparable concentrations. In this context the 97.4 % inhibition at 10^3 mol ratio/TPA of G 3, obtained by alkaline hydrolysis of GL 3, seems to possess an obvious potential as an anti-tumor promoter.

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