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Effect of fatty acid substrate chain length on *Pseudomonas aeruginosa* ATCC 9027 monorhamnolipid yield and congener distribution

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ABSTRACT

Rhamnolipids are surface-active molecules produced by *Pseudomonas aeruginosa* as congener mixtures. They are considered "green" alternatives to synthetic surfactants used in industrial, remediation and pharmaceutical applications. Optimizing yield as well as controlling congener distribution are necessary steps for successful commercialization of rhamnolipids. This study used a mixture of glucose and fatty acids of different chain length (C_{12}–C_{22}) and saturation ($C_{18:1}$ and $C_{18:2}$) to produce monorhamnolipids and determine the effect of fatty acid substrates on rhamnolipid yield, percent carbon conversion and congener distribution. Results show that 1% glucose + 0.25% stearic acid (C_{18}) produced the greatest yield (2.1 g L^{-1}) compared to other glucose–fatty acid combinations (0.8–1.8 g L^{-1}). Various glucose + C_{18} ratios were then tested to optimize yield and percent substrate carbon conversion to monorhamnolipid. Results revealed a positive linear correlation between the mass percent of C_{18} used and the percent carbon conversion. A mass percent of 67% C_{18} was optimal resulting in a 44% carbon conversion and a yield of 13.7 g L^{-1} monorhamnolipid. For all fatty acid substrates tested, the $RhaC_{10}C_{10}$ was the most abundant and $RhaC_{10}C_{12:1}$ was the least abundant of the four major congeners produced. However, the relative amount of $RhaC_{10}C_8$ and $RhaC_{10}C_{12}$ congeners was dependent on several factors: in general, fatty acid substrates with relatively short chain length (C_{12} and C_{14}), unsaturated fatty acid substrate ($_{C18:2}$), and longer cultivation time resulted in a higher $RhaC_{10}C_8/RhaC_{10}C_{12}$ ratio. These findings will assist in mass production of monorhamnolipids and controlling the specific congeners produced.

1. Introduction

Rhamnolipids (RLs) are surface active molecules (biosurfactants) secreted from *Pseudomonas aeruginosa* and related species in the stationary phase. The molecule is comprised of one (mono-RL) or two (di-RL) rhamnose sugars and a lipid moiety composed of two, and more rarely, one or three β-hydroxy fatty acids [1]. The physiological function of rhamnolipids remains unclear, but there is growing interest in these materials as alternatives to synthetic surfactants. Characteristics that make these molecules interesting include biodegradability, low toxicity, and effectiveness in extreme conditions [1,2]. Additional interesting properties of these molecules have also been observed, such as antimicrobial activity [3], skin compatibility [4], and ability to complex heavy metal contaminants and enhance solubility and biodegradation of slightly soluble organic compounds [5]. These properties show that rhamnolipids have the potential to be useful in a wide range of applications, ranging from biological control, cosmetics, pharmaceuticals, and detergents, to environmental cleanup and petroleum recovery [3,5–7].

The major challenge to commercialization of rhamnolipids is the high production costs resulting from low yields and the expense of raw materials [8–10]. Many cost-effective raw materials have been examined for increasing rhamnolipid yield. Studies show that hydrophobic substrates, especially vegetable oils or oil wastes from refineries and the food industry can improve rhamnolipid yield over water-soluble substrates, e.g. glucose [8–10]. However, these raw materials are typically complex mixtures of different ratios of a variety of carbohydrates and lipids [11]. Thus, the ability of these waste materials to increase rhamnolipid yield differs, suggesting that the component composition of the waste substrates affect rhamnolipid production.

A more subtle issue with commercialization of rhamnolipids is the variability that occurs in congener distribution.

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Rhamnolipids are synthesized as complex mixtures with conserved hydrophilic head groups and variable hydrophobic tail groups [12]. The two major component congeners within the mixtures are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate, referred to as $RhaC_{10}C_{10}$ and $Rha_2C_{10}C_{10}$, respectively. Other minor congeners produced vary in the length and saturation of the β-hydroxy fatty acid units [12–14]. Even though these congeners represent a minor mass fraction of the total rhamnolipid produced, they can have remarkably different properties (e.g., solubility, surfactancy). As a result, they can influence the packing pattern of the total mixture at surface/interfaces, micelle formation, and the sensitivity to precipitation from water by counterions [15,16]. Thus, differences in congener distribution may affect the physico-chemical properties and hence application of these materials in industry and environmental cleanup [15].

Previous studies have shown that rhamnolipid congener distribution can be affected by the producing isolate and cultivation conditions, e.g., carbon source, temperature, and cultivation time [12,14,16]. When using oils or oil wastes as carbon source, *P. aeruginosa* catabolizes the fatty acid components through β-oxidation [17]. Beta-oxidation enzymes, e.g. FadD, FadA and FadB, have been reported to respond differently to different chain-length fatty acids [18,19]. Our recent work demonstrated that specific intermediates of β-oxidation can be used as precursors for rhamnolipid biosynthesis [20]. The response and selectivity of enzymes for a substrate of given chain length may influence the ratio or the chain length of the intermediates of β-oxidation that can be diverted to rhamnolipid biosynthetic pathway. In turn, this could affect the final ratio between rhamnolipid congeners.

The objective of this work was to further investigate the effect of different fatty acid substrates on rhamnolipid production. Glucose and different chain length (C_{12}–C_{22}) and saturation ($C_{18:1}$ and $C_{18:2}$) fatty acids were used as sole or co-carbon sources for rhamnolipid production by *P. aeruginosa* ATCC 9027. The resulting rhamnolipids were harvested and partially purified to determine yield, percent carbon conversion (the portion of substrate carbon converted to rhamnolipid carbon), and major congener distribution. Selected samples with differing congener distributions were further purified to evaluate the effect on surface activity.

2. Materials and methods

2.1. Culture conditions

P. aeruginosa ATCC 9027, which produces only monorhamnolipids [21,22], was obtained from the American Type Culture Collection (Rockville, MD, USA) and stored in glycerol freezer stock at −80 °C. A PTYG (peptone, yeast extract, glucose, Difco) plate was streaked from freezer stock and used to inoculate a preculture composed of $NH_4H_2PO_4$ (0.3%), K_2HPO_4 (0.2%), glucose (0.2%), $FeSO_4{\cdot}7H_2O$ (0.5 mg L^{-1} Fe), and $MgSO_4{\cdot}7H_2O$ (0.1%). The preculture was incubated at 37 °C for 24 h with constant shaking at 200 rpm and then 1 mL was inoculated into 100 mL growth culture containing phosphate buffered mineral salts medium (MSM) composed of solutions A and B. Solution A contained per liter: 2.5 g $NaNO_3$; 0.4 g $MgSO_4{\cdot}7H_2O$; 1.0 g NaCl; 1.0 g KCl; 0.05 g $CaCl_2{\cdot}2H_2O$, and 5 mL H_3PO_4 (85.0%). Solution B contained per liter: 0.5 g $FeSO_4{\cdot}7H_2O$; 1.5 g $ZnSO_4{\cdot}7H_2O$; 1.5 g $MnSO_4{\cdot}H_2O$; 0.3 g H_3BO_3; 0.15 g $CoCl_2{\cdot}6H_2O$; 0.15 g $CuSO_4{\cdot}5H_2O$; and 0.10 g $Na_2MoO_4{\cdot}2H_2O$. One milliliter of solution B was added into 1 L of solution A. The final MSM pH was adjusted to 7.0 with KOH pellets.

Two major experiments were performed to test various glucose–fatty acid substrate combinations. Experiment 1 (Table 1) tested the effect of various fatty acids as a co-substrate with glucose. Preliminary experiments using the drop collapse assay [23] to measure the surface tension of the cell-free spent medium showed that the rhamnolipid concentration in the treatments supplemented with 1.25% glucose alone and 1% glucose + 0.25% fatty acids reached a plateau around 72 h and showed a slight decrease after 120 h of cultivation. Therefore rhamnolipids were harvested after 3 days. Experiment 2 tested the effect of varying glucose/stearic acid substrate ratios. The substrate combinations and corresponding cultivation times tested are detailed in Table 1. Note that the incubation times were increased for treatments that had increased amounts of substrate.

All fatty acids were purchased from Aldrich Chemical Co. and have 99% purity. Samples (100 μL) were taken at regular intervals and plated on TSA plates (tryptic soy agar, Difco) for enumeration. All treatments were repeated in triplicate and each experiment performed a minimum of two times.

2.2. Rhamnolipid extraction

After cultivation, rhamnolipids were harvested as previously described [21]. Briefly, cells were removed from culture broth by centrifugation at 10,000 rpm for 10 min. Rhamnolipids were precipitated out from supernatant by acidification to pH 2.0, and recovered by centrifugation, subjected to chloroform–methanol (9:1) extraction three times, concentrated by rotoevaporation, transferred to a preweighed plastic centrifuge tube, and dried by air. The mass of rhamnolipids was recorded.

2.3. Rhamnolipid congener distribution analysis

Rhamnolipid congener distribution was determined using a Waters 600E dual pump high performance liquid chromatography (HPLC) (Milford, MA, USA) with an evaporative light scattering detector (Polymer Labs Model PL-EMD 960, Varian, Inc., Palo Alto, CA, USA) and a Waters XTerra 3.9 mm × 150 mm, 5 μm particle size, RP_{C18} column with a 3.9 mm × 20 mm, 5 μm particle size, RP_{C18} guard column. A highly purified monorhamnolipid standard (99.8% pure), obtained from the same bacterial strain, was used for comparing the peak retention times of congeners. Subsamples for HPLC analysis were prepared by resuspending 3 mg rhamnolipid in 500 μL methanol. Twenty microliter subsamples were injected into the HPLC manually. An isocratic mobile phase of 60:40:0.1 (v/v), acetonitrile, H_2O and acetic acid was used at a flow rate of 1.0 mL min^{-1} for 25 min [24]. Air temperature was set at 70 °C and nitrogen flow at 4.0 L min^{-1}. Data acquisition was carried out using PeakSimple Chromatography single channel data system (model 359), and the peaks were identified and integrated using the PeakSimple Chromatography software (SRI Instruments, Torrance, CA, USA). The mass fraction of individual congeners was determined by integrating the peak area of the congener and divided by the summed total area of all congener peaks [25]. The molar fraction of individual congener was calculated from the mass fraction.

2.4. Rhamnolipid yield quantification

The yield of rhamnolipid from 1% glucose + 0.25% fatty acid treatments (Table 1, Experiment 1) was adjusted using a specific sample purity factor derived from the previously described HPLC analysis using the equation: $Yield_{adjusted} = Yield_{extracted} \times$ purity factor. This purity factor was calculated from the sum of the areas of all major congener (mass fraction > 1%) peaks divided by the summed total area of all peaks [24].

The yield of rhamnolipid from glucose + 1% or 2% stearic acid treatments (Table 1, Experiment 2) was adjusted using the same equation as above. However, the purity factor was derived from HPLC analysis using a different isocratic mobile phase [6:3:1 (v/v) acetonitrile, water and tetrahydrofuran with 0.1% acetic acid]. This mobile phase was developed for this study and allowed identification of all major congener peaks as well as the residual stearic acid peak, if any, present in these rhamnolipid samples.

2.5. Substrate carbon conversion to rhamnolipid

A calculation was performed to determine the percent conversion of substrate carbon to rhamnolipid. This calculation allows for comparison of carbon conversion efficiency when different initial substrate mass concentrations are tested. Specifically, a series of glucose/stearic acid combinations were tested to determine the effect of percent carbon conversion. Percent carbon conversion (R) was calculated using Eq. (1):

$$R = \frac{Mass_{RL}}{M_{RL}} \times (\#C_{RL}) \times \frac{1}{\mathrm{Mass}_{glucose}/M_{glucose} \times (\#C_{glucose}) + \mathrm{Mass}_{C18}/M_{C18} \times (\#C_{C18})} \times 100\% \quad (1)$$

where M = molar mass, #C = number of carbon atoms in the molecule, RL = rhamnolipid, C18 = stearic acid. Note that rhamnolipids are mixtures. The molar mass and #C for rhamnolipids used in this calculation were those for the most abundant congener $RhaC_{10}C_{10}$ (mass percentage ~80%), which are 504 g mol^{-1} and 26, respectively.

2.6. Rhamnolipid purification

Rhamnolipids with different congener distribution were further purified by column chromatography as previously described [22] to use in surface tension measurement. Briefly, a silica gel (60 Å pore size) was packed in a glass column and a 6:6:6:1:1 (v/v) solvent mixture of hexane, dichloromethane, ethyl acetate, chloroform, and methanol with 0.1% acetic acid was used to elute the rhamnolipids. Fractions for the presence of rhamnolipids were tested with anthrone reagent dissolved in H_2SO_4. Fractions containing rhamnolipids were combined, rotoevaporated, transferred to a plastic vial, and dried by air.