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Research Paper

Maximize rhamnolipid production with low foaming and high yield

Maysam Sodagari[1], Krutika Invally, Lu-Kwang Ju*

Department of Chemical and Biomolecular Engineering, The University of Akron, Akron, OH 44325-3906, United States

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ABSTRACT

Rhamnolipids are well-known microbial surfactants with many potential applications. Their production cost, however, remains high due to the severe foaming tendency in aerobic fermentation and the relatively low productivity and yield. In this study, we assessed the boundaries set by these constraints after optimization of basic parameters such as dissolved oxygen concentration (DO), pH and carbon sources. DO 10% and pH 5.5-5.7 were found optimal; cell growth and/or rhamnolipid production were slower at lower DO (5%) or pH (5.0) while foaming became hard to control at higher DO (30%) or pH (6.0 and 6.5). Although the *Pseudomonas aeruginosa* strain used was selected for its high rhamnolipid production from glycerol as substrate, soybean oil was still found to be a better substrate that increased specific rhamnolipid productivity to 25.8 mg/g cells-h from the glycerol-supported maximum of 8.9 mg/g cells-h. In addition, the foam volume was approximately halved by using soybean oil instead of glycerol as substrate. Analysis by liquid chromatography coupled with mass spectrometry revealed that rhamnolipid compositions from the two carbon sources were also very different, with primarily (82%) monorhamnolipids from soybean oil and more (64%) dirhamnolipids from glycerol. The optimized fermentation produced 42 g/l rhamnolipids at a yield of approximately 47% and a volumetric productivity of 220 mg/l-h. These values are among the highest reported.

1. Introduction

Biosurfactants can be more sustainable alternatives to synthetic surfactants [1,2]. Rhamnolipids are a group of well-known glycolipid biosurfactants, most commonly produced by *Pseudomonas aeruginosa* as a mixture of congeners [3]. They consist of a sugar moiety of one or two linked rhamnose residues and a lipid moiety of one or two linked β-hydroxyl fatty acid residues [4]. Rhamnolipids are biodegradable and less harmful to the environment [5]. They have potential uses in pharmaceutical, cosmetic, pesticide, anti-fouling, anti-microbial and bioremediation applications [6–11]. However, rhamnolipids are rather challenging to produce inexpensively [12]. Aerobic rhamnolipid fermentation faces high foaming tendency [13–16] and low volumetric productivity and yield as major obstacles [17,18]. Large amounts of antifoams may need to be added to control foaming. The foaming would also restrict the extent of aeration and, as a result, limit the maximum cell concentration employable in the process. The useful reactor volume may be reduced to provide larger headspace for foam management. Extreme foaming also increases the risks of contamination and spillage.

Several fermentation parameters were manipulated in this work to minimize broth foaming and evaluate their effects on rhamnolipid production. One important factor to consider was the dissolved oxygen concentration (DO). Maintaining the fermentation at a lower DO can avoid the use of excessive aeration and, in turn, reduce foaming. Implementing this approach requires knowing the low DO limit without negative effects on cell growth and rhamnolipid production. Kronemberger et al. studied the effect of rather high DO (14%, 57% and 86%) on rhamnolipid production by *P. aeruginosa* PA1 fermentation [16]. No effect on cell growth was expected at these high DO levels; maximum cell concentrations reached were similar at about 3 g/l. Rhamnolipid productivity was reported at about 30 mg/l-h at 57% and 86% DO and about 25 mg/l-h at 14% DO. The maximum rhamnolipid concentration reached was low at only 6 g/l. There was a need to more closely examine the effect of lower DO levels on rhamnolipid fermentation. In this current study, cell growth and rhamnolipid productivity were compared at three DO levels: 5%, 10% and 30%.

Another factor studied in this work was the carbon-source substrate. The strain used in this study was isolated from soil samples taken near a biodiesel plant and selected for its ability to grow and produce rhamnolipids using glycerol as substrate [19]. In this current study, soybean oil and glycerol were compared for their effects on foaming tendency, cell growth, and rhamnolipid production. It is known that oils can be used as antifoaming agents in fermentation processes [20,21]. There

* Corresponding author.
E-mail addresses: m.sodagari@gmail.com (M. Sodagari), LukeJu@uakron.edu (L.-K. Ju).
[1] Current affiliation: Grifols Diagnostic Solutions, Emeryville, CA 94608, United States.

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Table 1
Summary of fermentation conditions and the results obtained.

No.	C source	N (g/l)	DO (%)	pH*	Max cell conc. (g/l)	Average specific productivity (mg/g-h)	Yield (%)	Foam-to-liquid vol
F1	Glycerol	2.70	5	6.7	8.9 ± 0.3	8.9 ± 5.2	10.0	< 25%
F2		1.35	10		6.0 ± 0.1	8.1 ± 0.5	8.6	25%–50%
F3		1.35	30		6.3 ± 0.0	8.9 ± 3.1	10.5	> 50%
F4	Soy bean oil	1.35	10	6.7	10.1 ± 0.9	10.5 ± 5.9	35.0	< 25%
F5		1.35		5.1	1.8 ± 0.0	ND[a]	ND[a]	0%
F6		1.35		5.5	8.0 ± 0.7	12.5 ± 3.5	ND[b]	0%
F7		1.35		5.7	10.1 ± 0.8	14.4 ± 6.0	ND[b]	0%
F8		1.35		6.0	8.0 ± 0.4	12.8 ± 7.8	ND[b]	< 25%
F9		2.55		7.0 to 5.7	13.5 ± 0.3	25.8 ± 8.0	47.3	< 25%

* pH control band width was narrowed for more precise control in later fermentations: ± 0.2 for F1-F4, ± 0.1 for F5, and ± 0.05 for F6-F9.
[a] Due to very low cell concentrations in F5, rhamnolipid concentrations were not measured.
[b] F6, F7 and F8 were stopped before the added soybean oil was completely consumed; therefore, the final yield (from the consumed carbon source) could not be calculated.

have also been reports on rhamnolipid production from hydrophobic substrates [22–25]. The effectiveness of rhamnolipids in emulsifying/solubilizing hydrophobic substrates to avail them for microbial consumption has been considered as one of the important physiological roles of the biosurfactants [26]. In addition to the potential effects on broth foaming and rhamnolipid productivity, soybean oil was also investigated in this study for its effect on the composition of rhamnolipid mixture produced, compared to that from glycerol-based fermentation.

The pH effect was also studied. For some fermentations, maximum foaming was reported to occur at pH close to the isoelectric points of proteins, when those were most responsible for the foaming of fermentation broths [21,27]. Also, presence of charged surfactants, such as the anionic rhamnolipids at near neutral pH, could increase foaming because the bubbles were stabilized against coalescence due to the electrostatic repulsion effect imparted by the surfactants adsorbed on bubble surfaces [28]. In a previous study, we showed that negatively charged *P. aeruginosa* cells were the main foaming agent in the rhamnolipid fermentation and that the foaming could be decreased by about 80% if pH was lowered from 6.7 to 5.0 [29]. It was however unknown how lower pH would affect cell growth and rhamnolipid production. In this study, the rhamnolipid fermentation was performed at different pH values to investigate these pH effects on cell growth and rhamnolipid productivity.

Overall the objectives of this study were to address the constraint of severe foaming in aerobic rhamnolipid production and to assess the maximal rhamnolipid productivity and yield achievable through optimization of basic operating parameters such as dissolved oxygen concentration (DO), pH and carbon sources in the fermentation. Although a conventional bioprocess engineering study, the results are important as baseline values for meaningful development of advanced, unique rhamnolipid production processes in the future. The change of rhamnolipid mixture composition with the substrate used, i.e., glycerol or soybean oil, was also examined by using the high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS).

2. Materials and methods

2.1. Bacterial culture preparation

P. aeruginosa E03-40 was used in this study. This strain was isolated from soil samples collected from a biodiesel plant near Ralston, Iowa, USA. Multiple strains were isolated for growth and rhamnolipid production in glycerol-based media. The strain E03-40 was selected for its high rhamnolipid producing ability and low synthesis of other metabolites [19]. For inoculum preparation, the seed culture was first activated in 10 ml Tryptic Soy Broth (TSB, 30 g/l) medium for 8 h at 34 °C under 280 rpm shaking. It was then added to 90 ml TSB and grown similarly for 20 h before being used to inoculate the fermentor experiment. More details are available elsewhere [29].

2.2. Fermentation

Many fermentation experiments were done in this work. The fermentation conditions used in some of them are summarized in Table 1. All the experiments were carried out in 2-l fermentors (BIOFLO 110, New Brunswick Scientific) containing 1-l initial media. Temperature was maintained at 32 °C. The fermentation broth was agitated at 600 rpm with two 6-blade Rushton turbine impellers. Pure oxygen was introduced near the bottom of fermentor. The DO control value was typically set at 10% (air saturation) but the actual value fluctuated in the range of 5%–20% due to automatic adjustment of the oxygen flow rate. Three fermentations (F1, F2 and F3 in Table 1) were controlled with DO set points at 5% (1%–10%), 10% (5%–15%) and 30% (15%–45%) to study the effect of DO levels. The extents of DO fluctuation during these fermentations are shown in Fig. 1.

By addition of 1 N NaOH or H_2SO_4, pH was controlled at 6.7 ± 0.2 in all but 5 experiments: F5, F6, F7, F8 and F9. Fermentation F5 was made at pH 5.1 ± 0.1, F6 at 5.50 ± 0.05, F7 at 5.70 ± 0.05, and F8 at 6.00 ± 0.05, all with the initial medium pH already adjusted to those control values. These 4 experiments were designed for observing the effect of lower pH values on cell growth, rhamnolipid production and broth foaming. F9 was made for demonstrating the optimized operation: initial pH was 7.0 and pH was allowed to drop naturally along cell growth till 5.70 before being controlled at this set point. The higher initial pH enabled faster cell growth.

Glycerol was used as the carbon source in three of the experiments (F1, F2 and F3); soybean oil was used as the carbon source in the others (F4, F5, F6, F7, F8 and F9). The initial medium had 20 g/l glycerol or 37 g/l soybean oil. More glycerol or soybean oil was added as needed during the fermentation. In addition to the carbon source, the medium for all but 2 (F1 and F9) studies contained 3 g/l KH_2PO_4, 2.86 g/l NH_4Cl, 2.5 g/l yeast extract, 2.5 g/l peptone, 0.75 g/l NaCl, 0.45 g/l $MgSO_4{\cdot}7H_2O$, 0.05 g/l $FeSO_4{\cdot}7H_2O$, 0.015 g/l $CaCl_2{\cdot}2H_2O$, 0.015 g/l $MnCl_2{\cdot}4H_2O$, and 1 ml of a trace element solution. The trace element solution was composed of 0.75 g/l $MnSO_4{\cdot}H_2O$, 0.75 g/l $ZnSO_4{\cdot}7H_2O$, 0.15 g/l H_3BO_3, 0.08 g/l $FeCl_3{\cdot}6H_2O$, 0.08 g/l $CoCl_2{\cdot}6H_2O$, 0.075 g/l $CuSO_4{\cdot}5H_2O$ and 0.05 g/l Na_2MoO_4. The fermentation medium was designed to induce stationary phase by restricting the N-source concentrations. The other medium components were at least about 4 times in excess while the N-source was the limiting component which determined the maximum cell concentration attainable in every fermentation run.

The medium used in F1, a low DO (5%) fermentation, had 2X concentrations of all components. The intention was to see how much higher the maximum cell concentration could reach without severe foaming in this system. The medium used in F9, a demonstration fermentation for optimized conditions, contained 7.46 g/l NH_4Cl (corresponding to about 2000 mg/l N) instead of 2.86 g/l. This change was also made for growing the cells to a higher concentration. The