Enhancing rhamnolipids production and exploring the mechanism of low-foaming fermentation under weakly acidic conditions

Zhijin Gong

Qufu Normal University

Ge Yang

Qufu Normal University

Chengchuan Che

Qufu Normal University

Jinfeng Liu

Qufu Normal University

Meiru Si

Qufu Normal University

Qiuhong He (✉ hqiuhong228@163.com)

Qufu Normal University <https://orcid.org/0000-0003-0513-7615>

Research

Keywords: Rhamnolipids fermentation, *Pseudomonas aeruginosa*, weakly acidic conditions, mutagenesis, zeta potential, morphology

Posted Date: September 24th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-34693/v2>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The commercial applications of rhamnolipids have seriously been impeded by the high cost of production caused by severe foaming. Effective low-foaming fermentation and mechanism study have become the most urgent requirement for the larger-scale rhamnolipids production. In this study, the low-foaming fermentation of rhamnolipids is realized by controlling fermentation at pH 5.5. Further, the rhamnolipids production is enhanced 0.9-fold by ultraviolet and ethyl methanesulfonate composite mutagenesis. To the best of our knowledge, this is the first report to enhance production of rhamnolipids by strain improvement under weakly acidic conditions. The mechanism exploration tests indicated that increasing surface tension and decreasing viscosity are conducive to reducing foaming ability of rhamnolipids fermentation. The decrease of negatively charged ions may weaken the electrostatic repulsion between charged substances adsorbed on membranes of bubbles such as rhamnolipids and cells, making liquid membranes prone to rupture. In addition, the vesicle or lamellar structures of rhamnolipids are formed under pH 5.0-6.0, weakening the foaming ability of rhamnolipids. The work reveals promising potentiality by genetic modification to enhance rhamnolipids production and is conducive to understanding the pH-associated foaming behavior as well as developing the more effective pH-associated control strategies for large-scale rhamnolipids production under weakly acidic conditions.

Background

Rhamnolipids with excellent properties, such as biodegradability, low toxicity, antimicrobial, good surface activity and emulsification [1, 2] and usually produced by *Pseudomonas aeruginosa* [3], are currently the most promising biosurfactants to apply in oil recycling [4, 5], environmental protection [6], food processing [7, 8] and biomedical industry [9, 10]. However, despite their potential, the industrial application of rhamnolipids is seriously restricted, since the cost of rhamnolipids production is approximate ten times higher than that of synthetic surfactants [11]. One of the main reasons is due to that the severe foaming during fermentation largely affects production efficiency [12-14]. Although a large quantity of fermentation strategies, such as anaerobic [15, 16] and solid fermentation [14, 17, 18] have attempted to solve this problem, submerged liquid fermentation still seems to be the sole approach for high-yield production of rhamnolipids at present [19]. Therefore, to reduce foaming behavior of submerged liquid rhamnolipids fermentation have become an urgent requirement for the large-scale production and application of rhamnolipids [13]. In this respect, some fermentation strategies, such as fermentation-defoaming tandem system [12], foam fractionation fermentation [20, 21] and foam adsorption fermentation [22, 23] have been studied, but all of these fermentation strategies are ex-situ and cause some other problems, such as increasing the cost of equipment, losing the biomass and broth as well as increasing the risk of contamination. Recently, the in-situ low-foaming fermentation with maximal rhamnolipids production is realized by controlling fermentation at pH 5.5-5.7, which provides a promising strategy to efficiently product rhamnolipids by the conventional submerged liquid fermentation [24].

However, according to the previous studies, the most of rhamnolipids fermentation by controlling pH at pH 5.0-6.0 are unsatisfactory, because the cells growth and rhamnolipids synthesis are conspicuously inhibited [25, 26]. Therefore, in this study, we firstly prove the potentiality of low-foaming rhamnolipids fermentation of *Pseudomonas aeruginosa* SW1 by evaluating foaming behavior and productivity at weakly acidic conditions. Further, the production ability of *P. aeruginosa* SW1 is enhanced by ultraviolet (UV) and ethyl methanesulfonate (EMS) composite mutagenesis under pH 5.5. Finally, the mechanism of low-foaming rhamnolipids fermentation caused by weakly acidic conditions are systematically explored by characterizing the effects of fermentation pH on viscosity, surface tension, zeta potential, foaming behavior and morphological change of rhamnolipids.

Materials And Methods

Bacterial strain and medium

A *Pseudomonas aeruginosa* SW1 stored in our laboratory was grown in nutrient broth at 37°C and 200 rpm in an orbital shaker until the optical density at 600 nm reached approximately 2.0. The broth culture then was used as the fermentation inoculum. The medium of fermentation contained: 40 g/L soybean oil; 5 g/L NaNO₃; 1.1 g/L KCl; 1.1 g/L NaCl; 4 g/L KH₂PO₄; 4.4 g/L K₂HPO₄; and 0.5 g/L MgSO₄·7H₂O. The medium was sterilized at 121 °C for 20 min for fermentation. Chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. The bean oil was purchased from Shandong Luhua group Industry Co., Ltd (Laiyang, China).

Fermentation

The seed culture was inoculated at 1:10 into medium of fermentation. In 250 ml Erlenmeyer flasks, fermentation was conducted at 37 °C with orbital shaking at 200 rpm for 96 h. In the fermenter, a 7.5 L fermenter produced by Shanghai Baoxing Bio-Engineering Co, Ltd. (Shanghai, China) with 4 L working volume was employed to the fermentation. Four different fermentation strategies were used as described in Fig 6. The temperature of fermentation was maintained at 37 °C. The pH was controlled by addition of 3M HCl or ammonia. Agitation was fixed at 300 rpm/min and aeration flux was set to 3 L/min.

Rhamnolipids concentration and biomass

The production of rhamnolipids in the fermentation broth were quantified by the colorimetric determination of sugars moiety with orcinol [14]. The culture broth of rhamnolipids was initially centrifuged (10 000 g, 10 min) to separate the cells from the supernatant. 0.5 ml of supernatant was extracted thrice using 1 ml of chloroform/ethanol (2:1, v/v). The organic phase was collected and evaporated to dryness, the rhamnolipids residue was then dissolved in deionized water. Approximately 2.7 ml of a solution containing 0.19% orcinol (in 53% H₂SO₄) was then added to 0.3 ml of each sample. After heating for 30 min at 80°C, the samples were cooled in an ice-water bath for 2 min, and the samples' absorbance at 421 nm was measured. Rhamnolipids were quantified in triplicate and rhamnolipids concentration was calculated by multiplying the amount of rhamnose by 3.4 [27]. The dry weight method

was employed to measure biomass. Error bars in the figure were standard deviations from triplicate experiments.

Rhamnolipids purification

The rhamnolipids purification was performed according to wang's method with minor modification [28]. Briefly, the culture medium was centrifuged at 10000 rpm at 4°C for 10 min. The supernatant was precipitated by acidifying culture supernatant to pH 2.0 with 6 M hydrochloric solution and kept at 4°C overnight, then recovered by centrifugation at 10,000 rpm at 4°C for 1 h and dissolved in deionized water. Two volumes of chloroform:ethanol (2:1, v/v) were added to rhamnolipids solution and shaken 1 h for extraction at room temperature. The organic phase was evaporated to dryness to remove the solvent at 40 °C. After solvent evaporation, the rhamnolipids residue was obtained.

Foaming properties

The fermentation broth of rhamnolipids (22 g/L, 30 ml) from 250 ml Elenmeyer flask were controlled at pH 4, pH 5, pH 6, pH 7 and pH 8 by 3M HCl and ammonia. Among them, the pH 7 and pH 8 were control groups for neutral pH and weak alkaline pH, respectively. Then they were whipped with a rotor-stator disperser (AM400W-H, Angni Instruments Ltd, Shanghai, China) at 1000 rpm for 2 min at 37 °C, after whipped, the foam was poured into a 100 mL graduated cylinder immediately. Foaming ability (FA) was determined by comparing the foam volume at 1 min to the initial liquid volume of samples. The foam volume of fermentation broth of rhamnolipids at 5 min was measured to evaluate the accumulation ability of foam.

$$FA (\%) = V_0 / 30 \times 100 \quad (1)$$

where V_0 is the foam volume at 1 min.

Foaming microstructure observation by optical microscopy

According to the method of Xiong's report [29] with slight modification, the microstructures of foams from fermentation broth of rhamnolipids at pH 4-8 were visualized using a microscope (CX43, Olympus). The samples (10 μ L) were placed on microscopy glass slides. Foams were observed after being stored at 37 °C for 1 and 5 min. The magnification was 10 (objective) $\times$ 10 (eyepiece).

Composite mutagenesis by UV and EMS.

For UV mutagenesis, the collected cells (3000 r/min for 10 min) were resuspended in nutrient broth at approximately 1×10^4 cells mL⁻¹. Then the cells were exposed to UV for 5, 10, 20, 30, 40, and 50 s using a 30 W lamp (245 nm, Shengxing ZW30S19W) at a distance of 30 cm. The irradiated cells suspension was diluted 100-fold with sterilized saline, and then 0.1 mL of dilution was spread on the blue agar plates [30] (the nutrient broth agar plates added 0.2 g cetyltrimethylammonium bromide (CTAB) and 0.005 g methylene blue, the value of pH was adjusted to 5.5 with 3 M HCl and 1M NaOH). The plates were

incubated at 37 °C for 4 days. All these manipulations were conducted in darkness to avoid possible photoreactivation. Strains with large blue circle were isolated for the further EMS mutagenesis. For EMS mutagenesis, the collected cells (10000 r/min for 5 min) were resuspended in sterilized saline at approximately 1×10^6 cells ml^{-1} . Cells were subjected to 5 ml 2% (v/v) EMS over 0.5 h in a dark room and then EMS was inactivated by addition of sterile sodium thiosulfate to a final concentration of 5%. Cells were pelleted at 10000 r/min and washed three times with sterilized saline and resuspended in nutrient broth at approximately 1×10^6 cells ml^{-1} . The cells suspension of 0.1 ml was spread on the blue agar plates and were incubated at 37 °C for 4 days.

Surface tension, viscosity and zeta potential

The fermentation broth of rhamnolipids from 7.5 L fermenter was adjusted to pH 4-7 by utilizing 3M HCl and ammonia. The surface tension was measured by the Wilhelmy plate method [31], using a tensiometer (SFZL-E, Innuo Precision Instruments Ltd, Shanghai, China). The Wilhelmy plate was immersed in 20 mL of aqueous phase at 37 °C. The viscosity was measured with a viscometer (NDJ-9S, Pingxuan Scientific Instrument CO., Ltd. Shanghai, China) operating in 60 rpm. The zeta potential was measured using a Zetasizer Nano ZSP (Malvern Instruments Ltd, Malvern, UK).

Rhamnolipids morphology

The morphologies of rhamnolipids residue were observed with transmission electron microscopy (TEM). A drop of the test rhamnolipids solution sample was placed onto a copper grid and dried naturally, then was stained 2 min with a 1% phosphomolybdic acid aqueous solution. The excess phosphomolybdic acid aqueous solution was removed by absorbing the drop with a piece of filter paper and the grid was dried naturally. The samples were imaged under a transmission electron microscope (JEM-2100Plus, JEOL).

Results And Discussion

Evaluation of foaming properties in pH 4-8

As shown in Fig. 1A, the FAs of fermentation broth at pH 4, pH 5, pH 6, pH 7 and pH 8 were 32%, 41%, 42%, 146% and 174%, respectively (the corresponding visual foam volume image at Fig. 1C). After being stored for 5 min, there are less accumulation of foam at pH 4, pH 5 and pH 6 (Fig. 1B). These indicated that the foaming behavior of fermentation broth of rhamnolipids could be obviously suppressed at pH 4, pH 5 and pH 6. The time evolutions of bubbles formed by fermentation broth of rhamnolipids were shown in Fig. 2. The fermentation broth of rhamnolipids at pH 4-6 exhibited lesser multiple bubbles layers and smaller size of bubble at 1 min, which indicated that the fermentation broth of rhamnolipids at pH 4-6 had a poor foaming ability. A main property of aqueous foams is that there is out-of-equilibrium systems, and they evolve in time by gravitational drainage, coarsening and film rupture [32]. In response to gravity the liquid flows out of the foam and this process is called drainage [33]. Drainage reduces the liquid fraction in the foam and increases film surface area of the bubbles, causing an easier gas diffusion

between bubbles. In foam, bubbles grow because of the gas diffusion from one bubble to the next and the coalescence of bubbles when films are breaking. Foams are coarsen because of pressure differences between bubbles of different sizes [29]. These result in an increase in the mean bubble size. The bubbles growth eventually causes film rupture. From the Fig. 2, after being stored for 5 min, a lot of bubbles have ruptured and only a few small sizes bubbles existed in the field of vision at pH 4-6, but the fermentation broth of rhamnolipids at pH 7-8 still had more multiple bubbles layers and bigger size of bubble. These suggested that the ability of bubbles to prevent drainage or coarsening at pH 7-8 was stronger than these at pH 4-6 and the foam was easy to be accumulated. The results of time evolution of air bubbles also provided a better understanding for high foaming behavior of fermentation of rhamnolipids.

Based on the above results, the foaming behavior of fermentation broth of rhamnolipids was typically pH dependency and could be conspicuously suppressed by weakly acidic conditions.

Fermentation of rhamnolipids at pH 5.5 and pH 6.0

Owing to the metabolic activities of the microorganism, the pH value of fermentation of rhamnolipids by *Pseudomonas aeruginosa* would be increased from weak acid to weak alkaline (in this study, the value pH of was increased from 6.6 to 8.2, Fig. S1) [2, 34]. Base on above results, the foaming behavior could be enhanced at alkaline environments caused by metabolic of microorganism. Therefore, in order to reduce the foaming behavior of fermentation of rhamnolipids, the fermentation was firstly conducted at pH 5.5. As shown in Fig. 3A, the fermentation exhibited a low-foaming behavior and could be conducted for 120 h or longer, which was conspicuously longer than the fermentation time of no control pH in Fig. S1. In addition, although the concentration of rhamnolipids and biomass increased through the entire fermentation, the maximum rhamnolipids concentration (8.1 g/L) and biomass (6.5 g/L) were obviously lower than that of Erlenmeyer flask fermentation (22 g/L and 12.9 g/L, Fig. S1). These results suggested that the foaming behavior, rhamnolipids synthesis and cells growth were simultaneously inhibited at pH 5.5.

To improve the production of rhamnolipids, the pH of fermentation was adjusted to 6.0. Anti-foam agent was added at 1 ml/L broth at 60, 72 and 86 h of the fermentation. The production of rhamnolipids and biomass were plotted in Fig. 3B. It was observed that the increase of pH could promote cells growth and rhamnolipids synthesis. The maximum production of rhamnolipids (12.5 g/L) and biomass (8.7 g/L) were 1.5-fold and 1.3-fold higher than that of pH 5.5, respectively. However, severe foam occurred during fermentation (Fig. 3B) and a small amount of foam was overflowed. Given that overflowed cultivation was unacceptable in fermentation [35]. Therefore, the fermentation of rhamnolipids should be conducted below pH 6.0. In addition, the fermentation results of pH 5.5 and pH 6.0 further indicated that the foaming behavior of rhamnolipids fermentation was typical pH-sensitive and 0.5 change of pH value will cause a conspicuous decline of foaming ability.

Improved the production of rhamnolipids by mutagenesis at pH 5.5

In order to improve the production of rhamnolipids, the *P. aeruginosa* SW1 was mutated by UV and EMS, and then screened by the blue agar plates. The rhamnolipids produced from strain colony can form blue circle around the colony in the blue agar plates [30]. The blue circle diameter/colony diameter (BC) is proportional to the rhamnolipids concentration produced by strain. For UV mutagenesis, to obtain the mutant strains with increased rhamnolipids production, 20 colonies with large blue circles were screened and the values of BC were calculated. As shown in table 1, compared with control strain 0, all the mutant strains showed higher values of BC. Strain 3 showed the highest value of BC with 16 mm blue circle diameters and 7 mm colony diameters. Strain 8 and strain 15 also showed significant enhanced production capacity with the high values of BC. Therefore, strain 3, strain 8 and strain 15 were isolated for the EMS mutagenesis. For EMS mutagenesis, strain 3, strain 8 and strain 15 were mutated separately according to the method abovementioned in "Mutagenesis" and 20 colonies with large blue circles were screened and the values of BC were calculated. As shown in table 1, strain 7 showed the highest BC value followed by strain 18, strain 15, strain 19 and strain 8, which indicated that the rhamnolipids production may have a significant increase by mutagenesis in these strains. Therefore, strain 7, strain 8, strain 15, strain 18 and strain 19 were isolated for further studies.

Table 1
BC values of strains in culture plate after UV and EMS composite mutagenesis

UV				EMS			
Code	R1(mm)	R2 (mm)	BC	Code	R1(mm)	R2 (mm)	BC
0	6.0	4.0	1.5				
1	10.0	6.5	1.5	1	14.5	7.0	2.1
2	9.5	5.0	1.9	2	19.0	7.5	2.5
3	16.0	7.0	2.3	3	15.0	6.0	2.5
4	7.5	4.0	1.9	4	19.0	9.0	2.1
5	9.0	5.0	1.8	5	15.5	7.0	2.2
6	16.5	9.0	1.8	6	14.0	6.5	2.2
7	9.5	5.5	1.7	7	21.0	7.0	3.0
8	13.0	6.0	2.2	8	16.0	6.0	2.7
9	8.5	5.0	1.7	9	17.0	7.0	2.4
10	8.0	4.5	1.8	10	15.0	6.0	2.5
11	9.0	5.5	1.6	11	18.0	7.5	2.4
12	11.5	6.5	1.8	12	17.0	6.5	2.6
13	8.0	5.0	1.6	13	19.0	7.5	2.5
14	12.5	6.0	2.1	14	18.0	8.0	2.3
15	14.5	6.5	2.2	15	18	6.5	2.8
16	14.5	8.5	1.7	16	19.0	7.5	2.5
17	9.5	5.0	1.9	17	17.0	6.5	2.6
18	13.0	7.5	1.7	18	20.0	7.0	2.9
19	9.0	5.0	1.8	19	17.0	6.0	2.8
20	13.0	7.5	1.7	20	18.0	7.0	2.6
R1: Blue circle diameters; R2: Colony diameters; BC: R1/ R2. Code "0" is the control strain without mutation induction treatment.							

The high production performance of strains obtained by mutagenesis may be instability because of the mutation reversion often occurring during subculture [36]. To evaluate the stability of production, strain 7, strain 8, strain 15, strain 18 and strain19 were inoculated at a 7.5 L fermenter with 2 L initial fermentation

medium respectively. After that, 1 L of fermentation liquid was removed and simultaneously 1 L of fresh fermentation medium was added by timer control digital pump every 48 h for continuous subculture. The pH 5.5 was controlled by addition of 3M HCl or ammonia. Agitation was fixed at 300 rpm/min and aeration flux was set to 2 L/min. The productions of rhamnolipids were detected at 240, 288 and 336 h, respectively. As shown in Fig. S2, after 336 h of continuous subculture, all the 5 strains showed excellent production stability and rhamnolipids production exceed 11 g/L. The strain 7 still showed the highest productions of rhamnolipids (13.6 g/L) followed by strain 15 (12.9 g/L) and strain 18 (12 g/L).

Further, the strain 7 and strain 15 were conducted to the fermentation according to the method of fermentation involved in “materials and method”. As shown in Fig. 2, after 120 h of fermentation, the production of rhamnolipids and biomass of strain 7 reached 15.4 g/L and 10.3 g/L, which were 1.9-fold and 1.6-fold of the original strain, respectively. Corresponding, the production of rhamnolipids (14.7 g/L) and biomass (10.1 g/L) of strain 15 were about 1.8-fold and 1.6-fold higher than that of the original strain. The fermentation still exhibited low-foaming behavior through whole process of fermentation. These results suggested that enhancing rhamnolipids production by mutagenesis was a promising strategy to realize low-foaming rhamnolipids fermentation under weakly acid conditions. In addition, other methods for strain improvement like metabolic engineering and synthetic biology techniques [37, 38] should be included in future studies for improving rhamnolipids productivity under weakly acid conditions.

Characterizing the effects of pH 5-6 on foaming of rhamnolipids fermentation

According to these results abovementioned that fermentation at pH 5.5 was a suitable strategy to realize low-foaming production of rhamnolipids by *P. aeruginosa* SW1. In addition, according to the previous reports, the pH 5-6 were also considered to be suitable range to carry out the low-foaming fermentation of rhamnolipids [25, 26, 35]. Therefore, in order to explore the mechanism of low-foaming caused by weak acid pH, we used a weak-alkaline condition (pH 8) as the control group to systematically evaluate the effects of rhamnolipids fermentation broths and residues on foaming behavior at pH 5.0, pH 5.5, and pH 6.0.

Effects of pH 5-6 on viscosity and surface tension

Generally speaking, the foaming ability of solution is related to its surface tension and film viscosity [35]. The formation of bubbles is the process of increasing film surface area, and the increase in film surface area means that the energy of the system of bubbles also is increased correspondingly [39]. Therefore, from a thermodynamic point of view, the high surface tension of fermentation broth is obviously not conducive to foaming, because high surface tension will increase the surface energy of bubbles and producing the same volume of foam do more work. In addition, the liquid film of bubbles formed by low viscosity solution have low viscosity and strength [40, 41], which are also conducive to accelerating the film rupture of bubbles. Therefore, according to the Fig. 5A and Fig. 5B, in comparison to that at pH 8, the fermentation broth of rhamnolipids at pH 5-6 exhibited lower viscosity and higher surface tension, which were conducive to suppressing the severe foaming in rhamnolipids fermentation.

Effects of pH 5-6 on zeta potential and foaming behavior of rhamnolipids

The foaming ability (Fig. 5C) and zeta potential (Fig. 5D) of solution of rhamnolipids residue were simultaneously reduced with the decline of pH. Specially, there were obvious molecules aggregation of rhamnolipids and less foam at pH 5.0 (Fig. S3). Given that the trend of the change of foaming behavior was accordance with the zeta potential, it was considered that the decrease of foaming could be also partly attributed to the decline in charge potential of rhamnolipids. Because rhamnolipids containing carboxyl group has negatively charge and smaller fractions of rhamnolipids will be present as negatively charged ions in aqueous solution [42, 43]. The decrease of negatively charged ions can weaken the electrostatic repulsion between rhamnolipids and make the rhamnolipids molecules easy to aggregate and further impede the foaming ability [11, 44]. On the other hand, the decrease of negatively charged ions can also weaken the electrostatic repulsion of the charged cells adsorbed inner and outer membranes of bubbles and make the liquid membrane prone to drain, thin and rupture [45].

Effects of pH 5-6 on morphology of rhamnolipids

According to previous studies, the reported pKa of rhamnolipids are about 4.3-5.6, so rhamnolipids molecules may present vesicles and/or lamellar structures while at pH 5.5 [46, 47]. To verify the rhamnolipids structure at pH 5-6, TEM was used to examine the morphology of rhamnolipids microstructure. Fig. 6 shown the conversion of rhamnolipids molecular aggregates is typical pH-sensitive. The vesicles structure predominates at pH 5.0, while the larger lamellar were presented at pH 5.5. This was consistent with obvious molecules aggregation of rhamnolipids shown in pH 5.0 (Fig. S3). The size of lamellar significant became small at pH 6.0, but no lamellar or vesicles was observed at pH 8.0. Compared with the foaming behavior of rhamnolipids presented in Fig. 5C, it may be that formation of such vesicles or lamellar structures significantly suppressed the foaming ability of rhamnolipids.

In condition, according to the previous findings, there are a paradox that rhamnolipids has been previously characterized as having low foam ability, but its fermentation presented severely foaming [48, 49]. The reason may be explained by this study that the pH of rhamnolipids fermentation by *P. aeruginosa* is generally carried out in weakly alkaline conditions caused by the alkaline substances of microbial metabolism (Fig. S1), based on the foaming properties of rhamnolipids in 3.4.2 and 3.4.3, which are conducive to enhancing the foaming ability of rhamnolipids during fermentation. However, aqueous solution of rhamnolipids is a weak acid (the value of pH was about 4.5 in our study) due to the existence of the carboxylic acid moiety [11, 47], which make rhamnolipids molecules low foaming. Therefore, it was advised that rhamnolipids solution used in foaming agents, emulsifiers, oil recovery and other fields should be adjusted to neutral or alkaline.

Conclusions

The foaming behavior of fermentation broth of rhamnolipids produced by *P. aeruginosa* SW1 was confirmed to be typical pH dependency and the low-foaming fermentation of rhamnolipids could be realized at pH 5.5. Effective low-foaming fermentation of rhamnolipids was realized by UV and EMS

composite mutagenesis at pH 5.5 and the mechanism of low-foaming fermentation of rhamnolipids caused by weakly acid pH were also systematically characterized. The work provided a guidance for further strain improvement of *P. aeruginosa* and developing a more effective pH-associated control strategies for large-scale low-foaming production of rhamnolipids under weakly acidic conditions.

Abbreviations

UV, ultraviolet; EMS, ethyl methanesulfonate; FA, Foaming ability; TEM, transmission electron microscopy; BC, Blue circle diameters/Colony diameters; CTAB, cetyltrimethylammonium bromide.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by grant from Natural Science Foundation of Shandong Province (grant number ZR2019PB015).

Authors' contributions

ZG, CC, JL, MS and QH performed the experiments. GY supervised the project. ZG and QH drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Natural Science Foundation of Shandong Province (ZR2019PB015). We also would like to thank professor QinHong Wang, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, for providing guidance and experimental materials.

References

1. Varjani SJ, Upasani VN. Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. *Bioresour Technol.* 2017; 232:389-397.
2. Zhu L, Yang X, Xue C, Chen Y, Qu L, Lu W. Enhanced rhamnolipids production by *Pseudomonas aeruginosa* based on a pH stage-controlled fed-batch fermentation process. *Bioresour Technol.* 2012; 117:208-213.
3. Tan YN, Li Q. Microbial production of rhamnolipids using sugars as carbon sources. *Microb Cell Fact.* 2018; 17:1-13.
4. Zhao F, Zhang J, Shi R, Han S, Ma F, Zhang Y. Production of biosurfactant by a *Pseudomonas aeruginosa* isolate and its applicability to in situ microbial enhanced oil recovery under anoxic conditions. *RSC Adv.* 2015; 5:36044-36050.
5. Zhao F, Zhou J, Han S, Ma F, Zhang Y, Zhang J. Medium factors on anaerobic production of rhamnolipids by *Pseudomonas aeruginosa* SG and a simplifying medium for in situ microbial enhanced oil recovery applications. *World J Microbiol Biotechnol.* 2016; 32:54-54.
6. Sun S, Wang Y, Zang T, Wei J, Wu H, Wei C, Qiu G, Li F. A biosurfactant-producing *Pseudomonas aeruginosa* S5 isolated from coking wastewater and its application for bioremediation of polycyclic aromatic hydrocarbons. *Bioresour Technol.* 2019; 281:421-428.
7. Nitschke M, Costa SG. Biosurfactants in food industry. *Trends Food Sci Tech.* 2007; 18:252-259.
8. Kiran GS, Priyadharsini S, Sajayan A, Priyadharsini GB, Poulouse N, Selvin J. Production of lipopeptide biosurfactant by a marine nesterenkonia sp. and its application in food industry. *Fact Microbiol.* 2017; 8:1138-1138.
9. Yi G, Son J, Yoo J, Park C, Koo H. Rhamnolipid nanoparticles for in vivo drug delivery and photodynamic therapy. *Nanomed Nanotechnol Biol Med.* 2019; 19:12-21.
10. Chen J, Wu Q, Hua Y, Chen J, Zhang H, Wang H. Potential applications of biosurfactant rhamnolipids in agriculture and biomedicine. *Appl Microbiol Biotechnol.* 2017; 101:8309-8319.
11. Sodagari M, Ju LK. Cells Were a More Important Foaming factor than free rhamnolipids in fermentation of *Pseudomonas aeruginosa* E03-40 for high rhamnolipid production. *J Surfactants Deterg.* 2014; 17:573-582.
12. Gong Z, Peng Y, Wang Q. Rhamnolipid production, characterization and fermentation scale-up by *Pseudomonas aeruginosa* with plant oils. *Biotechnol Lett.* 2015; 37:2033-2038.
13. Long X, Sha R, Meng Q, Zhang G. Mechanism study on the severe foaming of rhamnolipid in fermentation. *J Surfactants Deterg.* 2016; 19:833-840.
14. Gong Z, He Q, Che C, Liu J, Yang G. Optimization and scale-up of the production of rhamnolipid by *Pseudomonas aeruginosa* in solid-state fermentation using high-density polyurethane foam as an inert support. *Bioprocess Biosystems Eng.* 2020; 43:385-392.
15. Zhao F, Shi R, Zhao J, Li G, Bai X, Han S, Zhang Y. Heterologous production of *Pseudomonas aeruginosa* rhamnolipid under anaerobic conditions for microbial enhanced oil recovery. *J Appl Microbiol.* 2015; 118:379-389.

16. Pinzon NM, Cook AG, Ju L. Continuous rhamnolipid production using denitrifying *Pseudomonas aeruginosa* cells in hollow-fiber bioreactor. *Biotechnol Prog.* 2013; 29:352-358.
17. Camiliosneto D, Bugay C, Santanafilho AP, Joslin T, De Souza LM, Sassaki GL, Mitchell DA, Krieger N. Production of rhamnolipids in solid-state cultivation using a mixture of sugarcane bagasse and corn bran supplemented with glycerol and soybean oil. *Appl Microbiol Biotechnol.* 2011; 89:1395-1403.
18. Neto DC, Meira JA, De Araujo JM, Mitchell DA, Krieger N. Optimization of the production of rhamnolipids by *Pseudomonas aeruginosa* UFPEDA 614 in solid-state culture. *Appl Microbiol Biotechnol.* 2008; 81:441-448.
19. Long X, Shen C, He N, Zhang G, Meng Q. Enhanced rhamnolipids production via efficient foam-control using stop valve as a foam breaker. *Bioresour Technol.* 2017; 224:536-543.
20. Heyd M, Franzreb M, Berensmeier S. Continuous rhamnolipid production with integrated product removal by foam fractionation and magnetic separation of immobilized *Pseudomonas aeruginosa*. *Biotechnol Prog.* 2011; 27:706-716.
21. Willenbacher J, Zwick M, Mohr T, Schmid F, Syltatk C, Hausmann R. Evaluation of different *Bacillus* strains in respect of their ability to produce Surfactin in a model fermentation process with integrated foam fractionation. *Appl Microbiol Biotechnol.* 2014; 98:9623-9632.
22. Anic I, Apolonia I, Franco P, Wichmann R. Production of rhamnolipids by integrated foam adsorption in a bioreactor system. *AMB Express.* 2018; 8:122.
23. Anic I, Nath A, Franco P, Wichmann R. Foam adsorption as an ex situ capture step for surfactants produced by fermentation. *J Biotechnol.* 2017; 258:181-189.
24. Sodagari M, Invally K, Ju L. Maximize rhamnolipid production with low foaming and high yield. *Enzyme Microb Technol.* 2018; 110:79-86.
25. Wu J, Ju L. Extracellular particles of polymeric material formed in n-hexadecane fermentation by *Pseudomonas aeruginosa*. *J Biotechnol.* 1998; 59:193-202.
26. Sodagari M, Ju LK. Cells were a more important foaming factor than free rhamnolipids in fermentation of *Pseudomonas aeruginosa* E03-40 for high rhamnolipid production. *J Surfactants Deterg.* 2014; 17:573-582.
27. Wang Q, Fang X, Bai B, Liang X, Shuler PJ, Goddard III WA, Tang Y. Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. *Biotechnol Bioeng.* 2007; 98:842-853.
28. Wang Q, Fang X, Bai B, Liang X, Shuler PJ, Goddard WA, 3rd, Tang Y. Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. *Biotechnol Bioeng.* 2007; 98:842-853.
29. Xiong T, Xiong W, Ge M, Xia J, Li B, Chen Y. Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Res Int.* 2018; 109:260-267.
30. Siegmund I, Wagner F. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnol Tech.* 1991; 5:265-268.

31. O'Sullivan J, Arellano M, Pichot R, Norton I. The effect of ultrasound treatment on the structural, physical and emulsifying properties of dairy proteins. *Food Hydrocolloids*. 2014; 42:386-396.
32. Saint-Jalmes A, Langevin D. Time evolution of aqueous foams: drainage and coarsening. *J Phys-Condens Mat*. 2002; 14:9397.
33. Guo F, Li Y, Xu H-X, Zhao G-Q, He X-J. Size-controllable synthesis of calcium carbonate nanoparticles using aqueous foam films as templates. *Mat Lett*. 2007; 61:4937-4939.
34. Chen S-Y, Wei Y-H, Chang J-S. Repeated pH-stat fed-batch fermentation for rhamnolipid production with indigenous *Pseudomonas aeruginosa* S2. *Appl Microbiol Biotechnol*. 2007; 76:67-74.
35. Jiang J, Zu Y, Li X, Meng Q, Long X. Recent progress towards industrial rhamnolipids fermentation: Process optimization and foam control. *Bioresour Technol*. 2020; 298:1-10.
36. Kanazir DT, Hartman PE, Savic DJ. Ultraviolet-induced reversions of *Salmonella* his frameshift mutations. *J Bacteriol*. 1970; 101:649-651.
37. Gao X, Jiang L, Zhu L, Xu Q, Xu X, Huang H. Tailoring of global transcription sigma D factor by random mutagenesis to improve *Escherichia coli* tolerance towards low-pHs. *J Biotechnol*. 2016; 224:55-63.
38. Noh MH, Lim HG, Woo SH, Song J, Jung GY. Production of itaconic acid from acetate by engineering acid-tolerant *Escherichia coli* W. *Biotechnol Bioeng*. 2018; 115:729-738.
39. Cai C, Liu H, Xi X, Jia M, Yin H. Bubble growth model in uniformly superheated binary liquid mixture. *Int J Heat Mass Tran*. 2018; 127:629-638.
40. Cicutti C, Valdez M, Perez T, Donayo R, Petroni J. Analysis of slag foaming during the operation of an industrial converter. *Lat Am Appl Res*. 2002; 32:237-240.
41. Yuan H, Liu Z, Ren J. Preparation, characterization, and foaming behavior of poly (lactic acid)/poly (butylene adipate-co-butylene terephthalate) blend. *Polym Eng Sci*. 2009; 49:1004-1012.
42. Helvacı Ş, Peker S, Özdemir G. Effect of electrolytes on the surface behavior of rhamnolipids R1 and R2. *Colloids Surf B Biointerfaces*. 2004; 35:225-233.
43. Raza ZA, Khalid ZM, Khan MS, Banat IM, Rehman A, Naeem A, Saddique MT. Surface properties and sub-surface aggregate assimilation of rhamnolipid surfactants in different aqueous systems. *Biotechnol Lett*. 2010; 32:811-816.
44. Lebrón-Paler A, Pemberton JE, Becker BA, Otto WH, Larive CK, Maier RM. Determination of the acid dissociation constant of the biosurfactant monorhamnolipid in aqueous solution by potentiometric and spectroscopic methods. *Anal chem*. 2006; 78:7649-7658.
45. Farahat M, Hirajima T, Sasaki K, Doi K. Adhesion of *Escherichia coli* onto quartz, hematite and corundum: Extended DLVO theory and flotation behavior. *Colloids Surf B Biointerfaces*. 2009; 74:140-149.
46. Lebronpaler A, Pemberton JE, Becker BA, Otto WH, Larive CK, Maier RM. Determination of the acid dissociation constant of the biosurfactant monorhamnolipid in aqueous solution by potentiometric and spectroscopic methods. *Anal chem*. 2006; 78:7649-7658.

47. Ishigami Y, Gama Y, Nagahora H, Yamaguchi M, Nakahara H, Kamata T. The pH-sensitive conversion of molecular aggregates of rhamnolipid biosurfactant. *Chem Lett.* 1987; 16:763-766.
48. Urum K, Pekdemir T, Ross D, Grigson S. Crude oil contaminated soil washing in air sparging assisted stirred tank reactor using biosurfactants. *Chemosphere.* 2005; 60:334-343.
49. Urum K, Pekdemir T. Evaluation of biosurfactants for crude oil contaminated soil washing. *Chemosphere.* 2004; 57:1139-1150.

Figures

Figure 1

FA of fermentation broth of rhamnolipids (A), foam volume at 5 min (B) and visual foam volume image after being stored at 1 and 5 min (C).

Figure 2

Time evolution of air bubbles within foams formed by rhamnolipids fermentation broth from pH 4 to pH 8. All images were obtained at the same magnification.

(A)

(B)

Figure 3

Curves of rhamnolipids production and biomass, and digital images of fermentation in 7.5 L fermenter. (A): the fermentation was controlled at pH 5.5; (B): the fermentation was controlled at pH 6.0.

Figure 4

Curves of rhamnolipids production and biomass of strain 7 (A) and strain15 (B) at pH 5.5.

Figure 5

Characterizing the effects of pH 5-6 on surface tension (A) and viscosity (B) of rhamnolipids fermentation broth, and FA (C) and zeta potential (D) of rhamnolipids solution.

Figure 6

TEM micrographs of rhamnolipids at pH 5-6. A: pH 5.0, B: pH 5.5, C: pH 6.0 and D: pH 8.0.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementary.docx](#)