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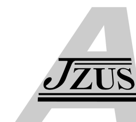

Reuse of waste frying oil for production of rhamnolipids using *Pseudomonas aeruginosa* zju.u1M*

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Abstract: In this work, rhamnolipid production was investigated using waste frying oil as the sole carbon source. By culture in shaking flasks, a naturally isolated strain synthesized rhamnolipid at concentration of 12.47 g/L and its mutant after treatment by UV light increased this productivity to 24.61 g/L. Fermentation was also conducted in a 50 L bioreactor and the productivity reached over 20 g/L. Hence, with a stable and high productive mutant strain, it could be feasible to reuse waste frying oil for rhamnolipid production on industrial scale.

Key words: Rhamnolipid, *Pseudomonas aeruginosa*, Biosurfactant, Waste frying oil
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INTRODUCTION

Because chemical surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at the interface between fluid phases of different degrees of polarity and hydrogen bonding (Mulligan, 2005), they play an important role in industrial chemicals and thus are widely used in many industries such as adhesives, flocculating, wetting and foaming agents, deemulsifiers and penetrants (Mulligan and Gibbs, 1993). Similarly, biosurfactants are a group of biological molecules with the same surface properties as their chemical counterparts and are produced by a wide variety of diverse microorganisms. Moreover, the chemical diversity of naturally produced biosurfactants offers a wider selection of surface active agents with properties closely tailored to specific applications. Unlike synthetic surfactants, microbially-produced compounds are easily biodegradable and thus particularly suited for environmental applications such as bioremediation and the dispersion of oil spills. Thus, the interest in biosurfactants has greatly increased in recent years due to their lower toxicity, biodegradable nature, and unique surface-active properties (Bognolo, 1999).

However, biosurfactants have not yet been employed extensively in industry because of economic reasons. The high cost in the production of biosurfactants, mainly determined by the high cost raw material and low product concentration, inhibited their competition with chemical surfactants. A positive strategy to reduce biosurfactant production costs is the use of inexpensive substrates or high-efficiency strain to increase its productivity. Some renewable industrial residuals have been widely used for biosurfactant production including olive oil mill effluent (Mercade *et al*., 1993), oil refinery wastes (Bednarski *et al*., 2004), distillery and whey wastes (Dubey and Juwakar, 2001), potato process effluent (Fox and Bala, 2000), soapstock (Benincasa *et al*., 2002).

On the other hand, used frying oils are generated

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in large quantities during food preparation in both household and food industries, e.g. the fast-food networks, large restaurants, dining rooms and catering establishments, etc. For example, it is reported by USDA (United States Department of Agriculture) that the world production of oils and fats was about 380 million tones in 2004~2005, 75% of which comes from plants (Mielke, 1992). The used frying oils in large quantities are hence considered as a problematic waste product contributing to the pollution of the environment. This problem is especially severe in China where frying with vegetable oil is a popular way of food preparation for the main reasons of delightful taste and specific smell of the meal. Overall, the waste frying oil disposal is a growing problem needing effective solution.

Biotransformation by microorganisms into biosurfactants may be the effective way to solve the problem above. Previous study (Haba *et al*., 2000) reported the reuse of olive and sunflower cooking oil as substrate to produce rhamnolipid at equivalent productivity of 6.75~9.25 g/L (measured as 2.7 g rhamnose/L) in shaking flasks. Rhamnolipids, mainly composed of dirhamnolipid (Rha-Rha-C_{10}-C_{10}) and monorhamnolipid (Rha-C_{10}-C_{10}) (Parra *et al*., 1989), were the most extensively used biosurfactants in biodegradation of hexadecane (Noordman *et al*., 2002), palmitic acid, stearic acid (Chayabutra *et al*., 2000), pyrene, phenantherene (Hwang and Cutright 2002), 2,4-dichlorophenol (Uysal and Turkman, 2005), and even crude oil (Urum *et al*., 2003; Yateem *et al*., 2002; Qin *et al*., 2005), due to their physicochemical and microbiological effects on the bioavailability of the contaminants. However, this rhamnolipid productivity is disappointedly too low so that the process of using waste oil for rhamnolipid production is not economic on industrial scale (Haba *et al*., 2000). So far, studies on improvement of rhamnolipid productivity and bioreactor run have not been reported. We previously used *Pseudomonas aeruginosa* ZJU isolated from crude oil field (Tang *et al*., 2007) to synthesize rhamnolipids by applying waste frying oil as the sole carbon source, but unfortunately the productivity was also lower than 8.5 g/L.

So we screened new candidate strains from the polluted area in order to improve the productivity. This work aims to improve the performance of the newly isolated biosurfactant-producing microorganisms using frying oil as the sole carbon source and further carry out the fermentation in bioreactor to establish a possible process for economical production of rhamnolipids on industrial scale.

MATERIAL AND METHOD

Isolation of candidate strains and random mutagenesis

Heavily oil-contaminated soil samples collected from the sewer sludge on either the public catering or refinery location, were transferred to laboratory at an ambient temperature and subsequently stored at 4 °C. Soil samples (50 g) were suspended in flasks with 200 ml of sterile phosphate-buffered saline (PBS) stirred. Then the supernatant was inoculated into cetyltrimethyl-ammonium bromide (CTAB) methylene blue agar plates with 3% (m/v) glycerol as the sole carbon source to detect anionic rhamnolipids produced by *Pseudomonas aeruginosa* (Siegmund and Wagner, 1991). After the plates had been incubated at 36 °C for 7 d, the colonies on CTAB plates with large dark blue halos on a light blueplate background were selected as candidate high rhamnolipid producers. The selected strain was then transferred to a shaking flask with 60 ml basal medium supplied with 4% (v/v) waste frying oil. Culture broth (3%, v/v) was used for subculture every 3 d.

For UV mutagenesis, 5 ml of the culture broth subcultured for 3 d was inoculated into sterilized 60-mm culture plates. Then, the culture plates were set 10 cm away under a 15 W-power UV light and exposed to UV radiation at a wavelength of 253.7 nm for 15, 30, 45, 60, 75 and 90 s, respectively. After that, the culture solution in each plate was diluted with 10 ml sterilized 0.85% NaCl and 0.5 ml of the dilution was plated onto CTAB plates (Siegmund and Wagner, 1991). After 4 days' culture in an incubator (37 °C), the culture plates exposed to UV light for 60 s were selected to screen the highly productive mutants, because of its suitable lethal ratio which was around 80%. Each colony with the large dark blue halos on a light blue background was subsequently transferred into shaking flasks with 60 ml basal medium supplied with 4% (v/v) waste frying oil for further quantitative evaluation of its rhamnolipid productivity.

Growth condition

Experiments were carried out in 250 ml baffled Erlenmeyer flasks containing 60 ml medium. The composition of the basal medium was (g/L): $NaNO_3$, 4.0; NaCl, 1.0; KCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; KH_2PO_4, 3.0; $Na_2HPO_4 \cdot 12H_2O$, 3.0; $MgSO_4$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.001, and 2 ml of a trace element solution containing (g/L): $FeCl_3 \cdot 6H_2O$, 0.08; $ZnSO_4 \cdot 7H_2O$, 0.75; $CoCl_2 \cdot 6H_2O$, 0.08; $CuSO_4 \cdot 5H_2O$, 0.075; $MnSO_4 \cdot H_2O$, 0.75; H_3BO_3, 0.15; $Na_2MoO_4 \cdot 2H_2O$, 0.05. Then, used frying oil was added and the final pH of the medium was adjusted to 6.8. The subsequent microbial cultures were conducted at 35 °C on a reciprocal rotary shaker (140 r/min). Microorganisms were preserved at −20 °C with 10% glycerol.

Waste frying oils were kindly offered by the public catering in Zhejiang University. The waste frying oils with a dark-brown color due to the existence of free fatty acids were initially precipitated for 48 h and then the major part of waste frying oils in the middle were collected as carbon source for microbial cultivation after the upper foam and the bottom solid parts were discarded.

Bioreactor experiments condition

Fermenter running was performed with a 50-L aerated stirring fermenter (Bioengineering Institute of Jiangsu University of Science and Technology, China) with a working volume of 25 L, operating with a foam recycling system. The culture medium was inoculated with a 24 h inoculum. The reactor was aerated at 0.6 vvm (volume/volume/min) for the first 48 h and at 0.5 vvm after 48 h culture. In this experiment, the carbon source used was waste frying oil and the media composition had the same growth condition as described before. The temperature was controlled to around 35 °C and the stirring speed was set at 300 r/min. The pH was regulated at 6.8 by adding 0.67 mol/L H_3PO_4 or 2 mol/L KOH. In addition, a foam recycling system was connected to the air outlet for security reasons.

Analytical methods

After the culture samples were centrifuged at 500 g for 10 min, the precipitates were used for determining the concentration of cell biomass while the cell-free supernatants were used for the analysis of waste frying oil and rhamnolipids. The cell concentrations were determined by measuring absorbance of the samples at 660 nm (OD_{660}) which was converted to dry cell weight concentration by a standard curve of dry cell weight vs OD_{660} (Moon *et al*., 2002). The surface tension of the cell-free culture broth was measured using a Bayer Tensionmeter (Bayer, Germany) according to the Du Noüy ring method. Rhamnolipids were determined using the modified method (Tang *et al*., 2007).

Frying oil content was determined by a spectrometrical method (Folch *et al*., 1957). In brief, a cell-free culture broth (20 μl) was added to 0.2 ml of sulfuric acid and heated for 10 min. After cooling, 1 ml vanillin reagent was added to the 5 ml test tube, mixed and allowed the reddish color to develop for at least 5 min. The color was stable from 5 to 30 min and then faded gradually. The reactants are read directly by a spectrophotometer at 525 nm against a reagent blank.

Rhamnolipid characterization

Rhamnolipid was extracted from the cultures by the method previously reported (Sim *et al*., 1997). Crude extracts were analyzed by thin layer chromatography (TLC) on silica gel G plates. Chromatograms were developed with chloroform/methanol/acetic acid (65/15/2) and visualized with an anthrone solution in sulfuric acid.

RESULTS AND DISCUSSION

Isolation of strain and its characteristics on rhamnolipid production

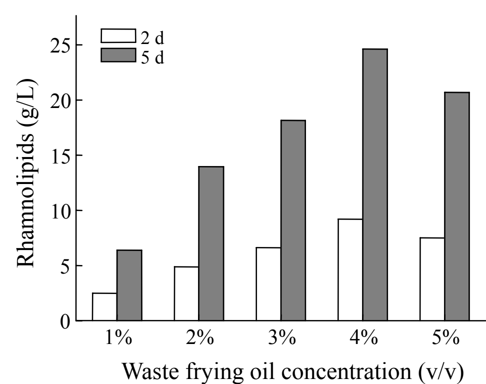
More than 40 isolates on the CTAB plates were examined for both Gram reaction and cell morphology while rhamnolipid producing strains were identified by the dark-blue halos on CTAB agar plates. Ten rod-shaped strains with the largest dark-blue halos around CTAB plates and showing Gram reaction were further transferred for culture in shaking flasks supplemented with 4% (v/v) waste frying oil and their corresponding rhamnolipid productivities at 5 days' culture were quantified as shown in Table 1. Among these ten isolates, the microbial named as zju.u1 produced the highest biosurfactant yield of 12.47 g/L while the second highest producer was zju.o1 with a yield of 11.61 g/L. The most productive strain of

Table 1 The rhamnolipid productivity at 5 days' incubation and Gram reaction of the isolated strains

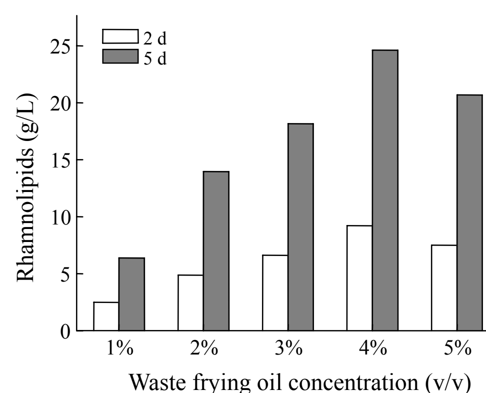
Isolate	Source of isolation	Ramnolipids concentration (g/L)	Gram reaction
zju.e1	Sludge at oil refinery plant	8.46	Negative
zju.e2	Sludge at oil refinery plant	7.74	Negative
zju.o1	Sewer sludge at university catering	11.61	Negative
zju.o2	Sewer sludge at university catering	10.25	Negative
zju.o3	Sewer sludge at university catering	9.97	Negative
zju.o4	Sewer sludge at university catering	9.24	Negative
zju.u1	Sewer water at university catering	12.47	Negative
zju.u2	Sewer water at university catering	9.83	Negative
zju.u3	Sewer water at university catering	10.47	Negative
zju.b1	Activated sludge in aeration tank	10.25	Negative

zju.u1 was hence selected for subsequent studies due to its potential for producing high yield of biosurfactant. Morphological observations and a battery of conventional taxonomic tests were performed to provide a generic description of zju.u1 later identified as *Pseudomonas aeruginosa*.

To investigate if the initial carbon substrate of the waste frying oil (4%, v/v) is under the preferred condition, the rhamnolipid productivity of the zju.u1 was evaluated by adding waste oil at concentrations ranging from 2% (v/v) to 5% (v/v). The rhamnolipid productivities at two culture periods (2 d and 5 d) are listed in Fig.1a. The rhamnolipid production increased significantly at both time points of 2 d and 5 d with the increase of the substrate concentration from 1% (v/v) to 4% (v/v) but decreased drastically at concentration of 5% (v/v). At the waste frying oil concentration of 4% (v/v) oil, the zju.u1 gave the highest biosurfactant yield of 5.45 g/L at 2 d and 12.54 g/L at 5 d, indicating the waste oil previously used at a concentration of 4% (v/v) is preferable. Compared with the culture of *Pseudomonas aeruginosa* using fresh vegetable oil as the sole carbon source at the suitable concentration of 125 g/L (Giani *et al*., 1997), the optimal waste oil concentration in this paper is much lower. This could be due to the existence of toxic substance in waste frying oil as it was reported that cooking oil changes its composition and contains more than 30% (v/v) of polar compounds after frying at a rather high temperature (Kock *et al*., 1996).



(a)



(b)

Fig.1 The various rhamnolipid productivity of naturally-isolated strain zju.u1 (a) and zju.u1M (b) at different waste frying oil concentrations at 2 and 5 d of culture in shaking flasks

Mutant of zju.u1 and its characteristics

In order to get a mutant of zju.u1 with higher productivity of rhamnolipid, mutagenization by UV was employed in this paper. In each run of mutagenesis, the strain with best performance on producing rhamnolipids was selected as starting strains for the subsequent UV mutagenization. After such three subsequent runs of mutagenic action, a productive strain mutant named as zju.u1M was obtained with the rhamnolipid productivity around

18~24 g/L, almost double in comparison with the initial natural strain.

The crude rhamnolipids with a purity of around 90% (v/v), extracted by chloroform and methanol, were analyzed by TLC and the standard rhamnolipids with a known composition (kindly provided by the Technical University of Braunschweig, Germany) were used as controls which were characterized by the combination of NMR spectroscopic and mass-spectrometric techniques in GBF. The TLC analysis identified the rhamnolipids as composed of two components just like the standard one: L-α-rhamnopyransyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-$C_{10}C_{10}$) with Rf about 0.483 and 2-O-α-rhamnpyranosyl-β-hydroxydecanoyl-β-hydroxyde-canoate (Rha-Rha-$C_{10}C_{10}$) with Rf about 0.655 as shown in Table 2.

Table 2 Rf1 and Rf2 of the standard sample and the crude rhamnolipids

Rhamnolipid sample	Rf1	Rf2
Standard sample	0.655	0.483
Crude extract	0.655	0.476

The optimal oil concentration was further evaluated following the same procedure in Fig.1a and the results are shown in Fig.1b. Comparison of the two figures in Fig.1 showed that the mutagenization did not alter its optimal frying oil concentration although the productivity of rhamnolipid increased by one fold. Moreover, the mutant was quite stable for at least 10 subcultures in producing rhamnolipids as shown in Fig.2.

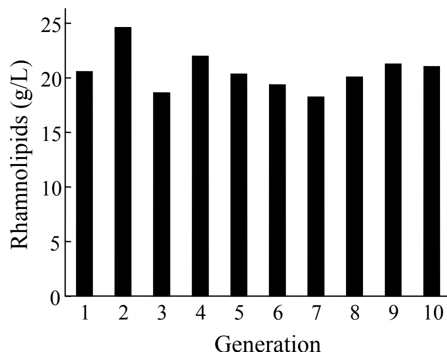


Fig.2 Rhamnolipids production of metagenized strain at different generations after 5 days' culture in shaking flasks with use of waste frying oil at 4% (v/v)

Rhamnolipids produced by zju.u1M growing on 4% (v/v) waste frying oil could reduce the interfacial tension of the cell free culture broth to 37.3 mN/m as shown in Fig.3. Critical micelle concentration (CMC), a parameter used as an indirect measurement of surfactant concentration, was determined by measuring the surface tension of serial dilutions of the cell free culture broth or crude rhamnolipid extracts in distilled water at pH 7.0. With rhamnolipids used as the sole biosurfactant in cell-free culture broth containing about 24 g/L of rhamnolipid, the CMC of the rhamnolipid was determined by measuring surface tensions of a serial dilution of culture broth. It is interesting to notice that the CMC of the culture broth in the cell-free culture broth was diluted 320 fold, in other words, the nominal CMC of rhamnolipid was 63.3 mg/L (Fig.3a) while the CMC of the rhamnolipid in crude extracts was 160 mg/L (Fig.3b). The CMC of nominal rhamnolipid in cell-free culture broth being lower than the CMC of rhamnolipid in crude extract could be explained by the fact that rhamnolipid is not the sole biosurfactant because the other accumulated proteins (Sabra *et al.*, 2002), glycolipids or polysaccharide (Turovsky *et al.*, 2005) in culture broth may also facilitate the decreasing surface tension.

Rhamnolipid fermentation in bioreactor

To investigate the feasibility of rhamnolipid production using waste frying oil, the mutant strain was transferred to culture in a bioreactor with a total volume of 50 L at an agitation speed of 300 r/min. The waste frying oil was supplemented in the

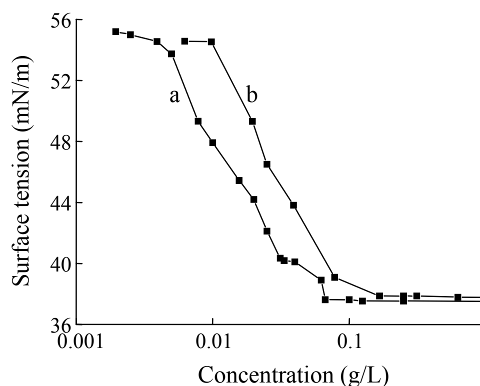


Fig.3 The surface tensions of the serially diluted solutions of the cell free culture broth (a) and the crude extract (b)

medium at a concentration of 4% (v/v), or 35 g/L according to the density of the waste oil of 0.875 g/ml. According to Fig.4, bacteria increased in the initial 24 h and then reached a stationary phase while the oil substrate decreased drastically before 48 h. Most rhamnolipids were accumulated after the growth phase. At the end of the growth phase, rhmnolipids productivity reached 6.13 g/L. At the end of the stationary phase, productivity increased to 19.53 g/L.

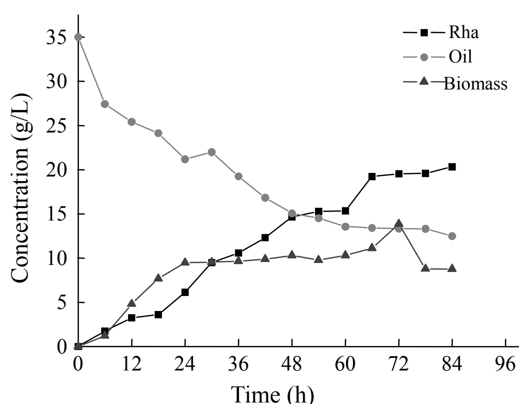


Fig.4 Time variation of growth, rhamnolipid production and substrate consumption during cultivation of *Pseudomonas aeruginosa* zju.u1M in a 50 L tank using an agitation speed of 300 r/min

From an ecological point of view, rhamnolipids offer significant advantages over traditional, chemically manufactured detergents. No hazardous chemicals, no high temperatures or pressures are required during production and the whole process is based on renewable materials. Furthermore, rhamnolipids are readily and fully biodegradable and display an aquatic toxicity at a factor less than that of commonly used detergents such as alkylbenzen sulphonate. Use of waste materials yields an ecological advantage and thus increases the already excellent environment-friendly potential of rhamnolipids for their use in washing and cleaning. Considering that the rhamnolipid yield in this paper approaches to that of 20 g/L using fresh vegetable oil as the sole carbon source (Sim *et al*., 1997), this process of rhamnolipid production using waste frying oil is therefore an economical alternative. To enhance the possibility of rhamnolipid production using waste frying oil on industrial scale, optimization of both medium composition and feeding strategies would be greatly expected to increase rhamnolipid productivity.

In conclusion, this paper for the first time reported high rhamnolipid productivity of over 20 g/L using waste frying oil as the sole carbon source and its feasibility in the operational process of bioreactor. Hence, due to the decreased environmental pressure and increased economy in producing biosurfactants using low cost waste products, waste frying oil could be applicable in industrial scale as the sole carbon source.

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