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Stimulatory effects of biosurfactant produced by *Pseudomonas aeruginosa* BSZ-07 on rice straw decomposing

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Abstract

Biosurfactant, produced by *Pseudomonas aeruginosa* BSZ-07, was added to the rice straw decomposing process to enhance the production of reducing sugars. Observed by Fourier Transform InfraRed (FT-IR) and Nuclear Magnetic Resonance (NMR) analysis, the purified biosurfactant was considered as a mixture of RL1 and RL2, which are two different types of rhamnolipids. Two different adding methods, adding the purified rhamnolipid and the on-site production of it were compared. The results showed that 0.5 g/L was the optimum concentration for adding purified rhamnolipid and the optimum temperature for on-site production was 30°C for the first 48 h and 34°C for the next 48 h. Under the optimum conditions, these two adding methods could improve the production of reducing sugar to 2.730 and 2.504 g/L, which was 22.30% and 12.20% higher than that of the rhamnolipid-free sample, respectively, which indicated that both of them were more effective than any other kind of surfactant discussed in this article. As the on-site production of rhamnolipid could omit the purification process, thus reducing the production cost effectively, it seemed to be a prospective adding method of the biosurfactant for enhancing rice straw decomposing.

Key words: biosurfactant; rhamnolipid; on-site; rice straw decomposing

Introduction

In recent years, environmental issues such as substitution of methyl tert-butyl ether (MTBE) and reduction of carbon dioxide emission by blending bioethanol into gasoline, provide crop residues, such as rice straw, a new way to be utilized (Jørgensen and Olsson, 2006). Through an enzymatic hydrolysis subprocess, rice straw, rich in cellulose, can be converted to reducing sugars, which can subsequently be fermented to target products such as ethanol (Lawford and Rousseau, 2003), lactic acid (Hawary *et al*., 2001), single cell protein (Solomon *et al*., 2000), and hydrogen (Taguchi *et al*., 1996) by suitable microorganisms (Zhu *et al*., 2006).

The hydrolysis of cellulose to reducing sugar can be catalyzed by a group of enzymes, collectively termed as cellulase. However, the bottleneck in the enzymatic hydrolysis of cellulose is the significant enzyme deactivation. The partially irreversible adsorption of cellulase on cellulose is usually proposed as a responsible mechanism (Wu and Ju, 1998; Eriksson *et al*., 2002). Many reports have shown that surfactants can modify the cellulose surface property and minimize irreversible binding, thus promoting the production of cellulase (Reese and Manguire, 1969; Pardo, 1996) and enhancing the enzymatic hydrolysis of cellulose (Helle *et al*., 1993; Eriksson *et al*., 2002).

Surfactants, both chemically-synthesized surfactants and biosurfactants, are extensively used in bioremediation, pharmaceutical, cosmetic, fine chemical, and food industries (Banat *et al*., 2000; Thanomsub *et al*., 2007) because of their surface activity. Although, chemically-synthesized surfactants are not biodegradable and can be toxic to the environment, biosurfactants have attracted a lot more attention because of their specificity, biodegradability, and biocompatibility (Mulligan, 2005). Biosurfactants are amphiphilic compounds produced by a number of microorganisms, including bacteria, yeasts, and fungi. On the basis of the types of biosurfactant producing microbial species and the nature of their chemical structures, biosurfactants can be categorized as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids, and neutral lipids (Benincasa *et al*., 2002).

It has been reported that addition of biosurfactants, particularly rhamnolipid, effectively improves the cellulase activity as well as preserves them for being recycled in the course of cellulose decomposing (Kaya *et al*., 1995; Park *et al*., 1992). Liu *et al*. (2006) discovered that rhamnolipid at 0.018% (*W/W*) can noticeably increase the production of xylanase in solid substrate fermentation (SSF), which is 119.6% higher than that of the control. However, the major hurdles for commercial application of the biosurfactant are low yield and high production cost (Wei *et al*., 2005). Therefore, of late, many researchers have devoted time to

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developing an efficient biosurfactant producer and a cost-effective production bioprocess.

In the current study, *Trichoderma reesei* ZM4-F3 and *Pseudomonas aeruginosa* BSZ-07, preserved in the laboratory, are used for rice straw decomposing and biosurfactant producing respectively. The stimulatory effects derived from adding biosurfactant to the rice straw saccharification hydrolytic medium was mainly studied in current work. The two different methods of adding biosurfactants have been compared. One is by purifying the biosurfactant from fermentation broth of *Pseudomonas aeruginosa* BSZ-07 first, and then adding it to the rice straw decomposing process, which is used in many observations. Another is by making *Trichoderma reesei* ZM4-F3 and *Pseudomonas aeruginosa* BSZ-07 work together, which is a new bioprocess that has been ignored by many researchers. This on-site production of the biosurfactant can omit the purification of biosurfactants, thus reducing the production cost effectively. Moreover, to find out the mechanism of the stimulatory effects from adding this biosurfactant to rice straw decomposing aftertime, the component of the biosurfactant produced by *Pseudomonas aeruginosa* BSZ-07 has been observed by Fourier Transform Infra Red (FT-IR) and NMR analysis.

1 Materials and methods

1.1 Materials

Chemically-synthesized surfactants, including octylphenol (ethyleneglycol)$_{9,6}$ ether (Triton X-100), sodium dodecylsulphate (SDS), poly(oxyethylene) 20 sorbitan-monolaurate (Tween 20), and poly oxyethylene 80 sorbitan-monolaurate (Tween 80), were all purchased from Sigma, USA.

1.2 Microorganisms

Trichoderma reesei ZM4-F3 and *Pseudomonas aeruginosa* BSZ-07, which were screened from *Trichoderma reesei* ZM-4 through compound UV/DES alternate mutagenesis, and soils that were contaminated by gasoline, respectively, were conserved in the laboratory. The filter enzyme activity (FPA) of *Trichoderma reesei* ZM4-F3 was 12.92 U/ml. The surface tension of fermentation broth could be reduced from 70.3 to 34.2 mN/m by *Pseudomonas aeruginosa* BSZ-07. These two strains were maintained at 4°C on slants of PDA and LB, respectively, both with regular subculturing every 3–4 weeks.

1.3 Enzymatic hydrolysis

Chopped rice straw was pretreated with 2% NaOH at 85°C for 1 h before enzymatic hydrolysis. Then, the pretreated rice straw was decomposed by *Trichoderma reesei* ZM4-F3 at 30°C for 4 d in a shaking bed (200 r/min). After fermentation, the culture broth was centrifuged at 8,000 r/min for 10 min and the supernatant was analyzed for reducing sugar production. All the experiments were performed in triplicate and the average values were represented.

1.4 Biosurfactant production and purification

To prepare the inoculum, the spores on the LB slant were suspended in 2 ml medium (10^6 spores/ml) and then pipetted into a 250-ml Erlenmeyer flask containing 50 ml of inoculum growth medium, followed by incubation in a shaking bed (200 r/min) at 35°C. The medium was a 1,000 ml solution with 3 g beef extract, 1 g $NaNO_3$, 1 g $(NH_4)_2SO_4$, 1.2 g Na_2HPO_4, 1 g KH_2PO_4, 0.01 g $MgSO_4{\cdot}7H_2O$, and 0.002 g $CaCl_2$. The initial pH value of the medium was adjusted to 6.5–7.0 before being autoclaved at 121°C for 15 min. After a 24-h growth, the medium was used as the inoculum for biosurfactant production. Then, 5 ml of exponential inoculum was inoculated into a 250-ml Erlenmeyer flask containing 100 ml of fermentation medium. The composition of fermentation medium was as follows: 2 g glucose, 0.1 g yeast extract, 0.5 g NH_4NO_3, 2 ml machine oil, 1 g KH_2PO_4, 1 g Na_2HPO_4, 0.02 g $MgSO_4{\cdot}7H_2O$, and 0.5 ml Mandels trace element solution. The initial pH of the medium was adjusted to 4.8 before being autoclaved at 121°C for 15 min. The culture in the Erlenmeyer flask was incubated at 35°C for 48 h in a shaking bed (200 r/min).

The purification of the biosurfactant followed the acidic sedimentation method (Zhang and Miller, 1994) with a little modification. After 48 h fermentation, cells were removed twice from the fermentation medium by centrifugation at 8,000 r/min for 20 min at 4°C. The supernatant was adjusted to pH 2.0 with 6 mol/L HCl, left overnight at 4°C and then centrifuged at 10,000 r/min for 30 min at 4°C. Then, the pellet was resuspended in distilled water, through adding 1 mol/L NaOH to adjust pH to 7.0. The solid obtained after frozen lyophilization was the coarse biosurfactant product. To further purify the biosurfactant, it was extracted by chloroform and methanol in the ratio of 2:1 (*V/V*). Ultimately, the solvent layer was pooled and concentrated under vacuum using a rotoevaporator at 65°C, thus obtaining an aqueous solution of pure biosurfactant, which was then frozen at –80°C and lyophilized.

1.5 Analytical methods

Reducing sugars, produced by *Trichoderma reesei* ZM4-F3 in the course of rice straw hydrolysis, were determined by the DNS method (Miller, 1959).

Purified biosurfactant was subjected to analysis by NMR and FT-IR. Both ^{1}H NMR and ^{13}C NMR spectra were obtained with a nuclear magnetic resonance machine (MERCURY PLUS 400, 400 MHz, Varian, Inc., USA). FT-IR analysis was performed using Fourier Transform Infrared Spectrometer (Paragon 1000, Perkin Elmer, Inc., USA).

2 Results and discussion

2.1 Biosurfactant purification and analysis

The biosurfactant was produced by *Pseudomonas aeruginosa* BSZ-07 and then purified. As seen in Fig.1, the purified biosurfactant was a brown loose solid, and its yield was 5.3 g in 1 L culture broth.

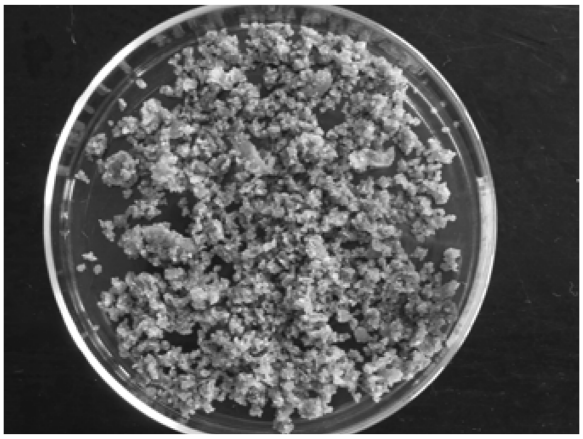

Fig. 1 Purified biosurfactant product.

Through the FT-IR spectrum of the pure biosurfactant (Fig.2), the presence of a rhamnolipid structure, which is composed of rhamnose rings and long hydrocarbon chains, is clearly indicated by the absorbance bands at the wave numbers of 2,920 cm^{-1}, 1,720 cm^{-1}, and 1,300–1,100 cm^{-1}. The strong adsorption peak at 2,920 cm^{-1} is expected to be the C–H stretching vibrations of the hydrocarbon chain positions. The strong absorbance at 1,720 cm^{-1}, which is considered to be the characteristic peak of biosurfactants by many researchers (Li *et al.*, 2002), must be assigned to the C–H stretching vibrations of the hydrocarbon chain positions. However, the strong absorbance in the range of 1,300–1,100 cm^{-1} indicates the presence of bands formed between carbon atoms and hydroxyl groups in the chemical structures of rhamnose rings (Wu and Ju, 1998; Pornsunthorntawee *et al*, 2007).

The chemical shifts of purified biosurfactants, observed from ^{1}H NMR and ^{13}C NMR analysis, are shown in Table 1, which indicates that the sample has the molecular structure of *L*-rham-nosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1) and *L*-rhamnosyl-*L*-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL2), which are two major types of rhamnolipids produced by the *Pseudomonas aeruginosa* species (Wei *et al*., 2005; Wu *et al*., 2008; Monteiro *et al*., 2007). Hence, both ^{1}H NMR and ^{13}C NMR spectrometry results indicate that the purified biosurfactant is a mixture of RL1 and RL2.

Table 1 Chemical shifts of purified biosurfactant in ^{1}H NMR and ^{13}C NMR spectra

^{1}H chemical shift (ppm)	Assignment	^{13}C chemical shift (ppm)	Assignment
0.871	$-CH_3$	95.366	RL1
1.284	$-(CH_2)_6-$	103.254	RL2
2.509	$-CH_2-COO-$		
4.077	–O–CH–		
5.368	–COO–CH–		

RL1: *L*-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate; RL2: *L*-rhamnosyl-*L*-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate.

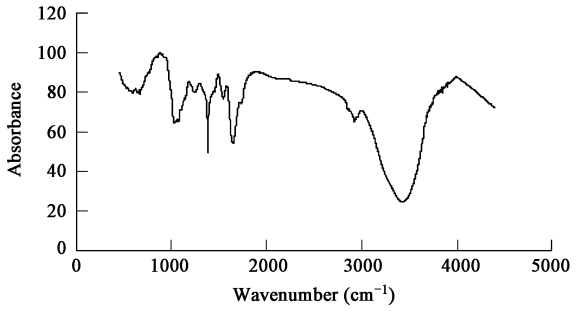

Fig. 2 FT-IR spectrum of purified biosurfactant.

2.2 Stimulatory effects of pure biosurfactant on rice straw decomposing

Different concentrations of purified rhamnolipid were added to the rice straw decomposing course, as shown in Fig.3. It was observed that as more rhamnolipid was added, more reducing sugars were produced by *Trichoderma reesei* ZM4-F3. When the concentration of rhamnolipid was below 0.3 g/L, there was only a slight increase in the reducing sugars. When 0.3 g/L rhamnolipid was added, the production of reducing sugar was only 8.16% higher than that of the control. However, when the concentration of rhamnolipid was increased to 0.5 g/L, the production of reducing sugar was 22.32% higher than that of the control, which clarified that rhamnolipid had a remarkable stimulatory effect on rice straw decomposing. When the concentration of rhamnolipid was above 0.5 g/L, the increase of reducing sugar started to tardiness. Thus, 0.5 g/L was chosen as the optimum adding concentration of rhamnolipid.

To further prove the stimulatory effect of rhamnolipid on rice straw hydrolysis, the influence of fermentation time on the production of reducing sugar, by *Trichoderma reesei* ZM4-F3, was compared with rhamnolipid-free samples and rhamnolipid-added (0.5 g/L) samples, as illustrated in Fig.4. In the rhamnolipid-free samples, 2.231 g/L reducing sugar could be produced by *Trichoderma reesei* ZM4-F3 in 96 h, which was considered as the optimal fermentation time. However, in the rhamnolipid-added samples, the production of reducing sugar could achieve 2.730 g/L in 84 h. Therefore, the authors discovered that the addition of rhamnolipid to the rice straw decomposing course could not only increase the production of reducing sugars, but could also cut down the fermentation time distinctly, thus reducing the production cost effectively.

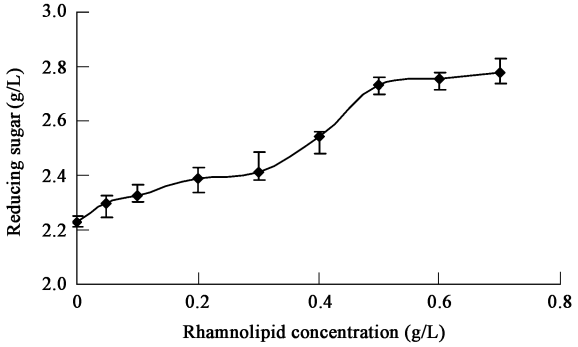

Fig. 3 Influence of rhamnolipid concentration on rice straw decomposing.

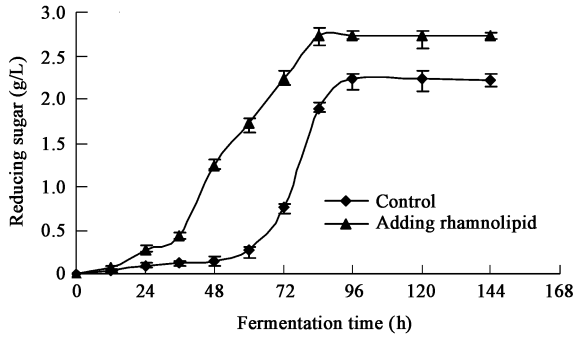

Fig. 4 Stimulatory effect of adding 0.5 g/L rhamnolipid on rice straw decomposing.

2.3 On-site production of biosurfactant and its stimulatory effects on rice straw decomposing

To omit the complex purification process and reduce the cost of rice straw decomposing, on-site production of the rhamnolipid bioprocess was realized. As the growth periods for *Trichoderma reesei* ZM4-F3 and *Pseudomonas aeruginosa* BSZ-07 are 96 and 48 h, respectively, *Trichoderma reesei* ZM4-F3 is first cultivated, for rice straw decomposing, for 48 h at its suitable temperature 30°C. For the next 48 h, the exponential inoculum of *Pseudomonas aeruginosa* BSZ-07 is inoculated into it to keep these two strains working together. However, this on-site rhamnolipid production bioprocess requires compatible fermentation conditions for both microorganisms. For example, a similar pH, temperature, and optimum substrate concentration are all needed. As temperature is one of the most important factors, *Trichoderma reesei* ZM4-F3 is considered as the only microorganism in the first 48 h fermentation.

As shown in Fig.5, the production of reducing sugar increased with an increase in temperature. But when the temperature was above 34°C, the production of the reducing sugar decreased rapidly, which indicated that 34°C was the optimum temperature in the final 48 h. This might be because of the stronger temperature tolerance ability of *Trichoderma reesei* ZM4-F3 than that of *Pseudomonas aeruginosa* BSZ-07. At the optimum temperature for this two-stage fermentation, namely, 30°C for the first 48 h and 34°C for the next 48 h, the production of reducing sugar could increase to 2.504 g/L, which was 12.20% higher than that of the rhamnolipid-free sample.

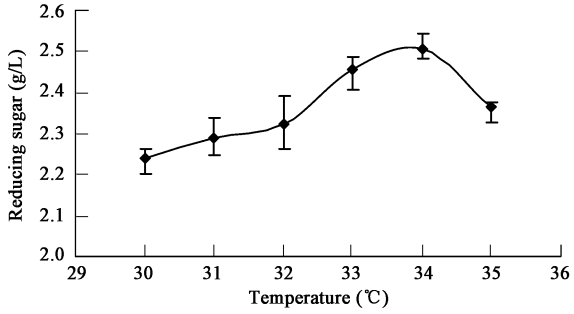

Fig. 5 Influence of temperature on rice straw decomposing in the on-site biosurfactant production system.

2.4 Comparison of the stimulatory effect

The effects of chemically synthesized surfactants and two different adding methods of biosurfactants on rice straw decomposing were studied as follows. Five different surfactants, including Triton X-100, SDS, Tween 20, Tween 80, and pure rhamnolipid, were evaluated for their ability to enhance the enzymatic hydrolysis of rice straw. Moreover, a new method for adding rhamnolipid, on-site production, was also compared, as described in Fig.6. For each surfactant, the adding dosage was 0.5 g/L. But for the on-site production of rhamnolipid, the adding inoculation concentration ratio of *Pseudomonas aeruginosa* BSZ-07 to *Trichoderma reesei* ZM4-F3 was 4%. As seen in Fig.6, 2.231 g/L reducing sugar was obtained in enzymatic hydrolysis of rice straw without surfactant. Fig.6 also revealed that all nonionic surfactants, including Triton X-100, Tween 20, and Tween 80, improved the hydrolysis slightly. Among them, Tween 80 showed the best ability to improve rice straw decomposing, which could increase the production of reducing sugar to 2.423 g/L, 8.6% higher than that of the control. However, the negatively charged surfactant SDS reduced the production of reducing sugars. Moreover, it could obviously be seen that these two adding methods of rhamnolipid greatly increased the production of reducing sugars, both of which were better than any other kind of chemically synthesized surfactant discussed here. The pure rhamnolipid adding method and on-site production of rhamnolipid could increase the production of reducing sugar to 2.730 and 2.504 g/L, 22.30% and 12.20% higher than that of the control, respectively, which provided a bright future for the biosurfactant in rice straw decomposing, especially for the on-site production method.

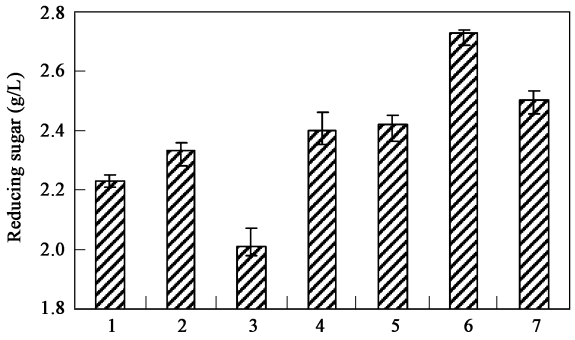

Fig. 6 Comparison of the stimulatory effects of various types of surfactants (0.5 g/L) on rice straw decomposing. (1) control; (2) Triton X-100; (3) SDS; (4) Tween 20; (5) Tween 80; (6) pure rhamnolipid; (7) on-site production of rhamnolipid.

3 Conclusions

The stimulatory effect of the biosurfactant produced by *Pseudomonas aeruginosa* BSZ-07 on rice straw decomposing was verified in this article. The biosurfactant was first purified and then analyzed by FT-IR and NMR. It

was considered to be a mixture of two different types of rhamnolipids, RL1 and RL2. Following the usual adding method, the purified rhamnolipid sample was added to the rice straw decomposing system directly. The result showed that 0.5 g/L was the optimum concentration for rhamnolipid, and it could increase the production of reducing sugar to 2.730 g/L, 22.30% higher than that of the control. However, another new adding method of rhamnolipid, on-site production, was tried, to enhance the hydrolysis of rice straw. As the optimum temperature and growth periods for *Trichoderma reesei* ZM4-F3 and *Pseudomonas aeruginosa* BSZ-07 were different, *Trichoderma reesei* ZM4-F3 was first cultivated for rice straw decomposing for 48 h at 30°C. For the next 48 h, the exponential inoculum of *Pseudomonas aeruginosa* BSZ-07 was inoculated into it to keep these two microorganisms working together. The result showed that 34°C was the optimum temperature for the next 48 h. At the optimum temperature for this two-stage fermentation, the production of reducing sugar could be increased to 2.504 g/L, which was 12.20% higher than that of the rhamnolipid-free sample. Finally, four other chemically-synthesized surfactants, including Triton X-100, SDS, Tween 20, and Tween 80, were compared with rhamnolipid for the stimulatory effect on rice straw decomposing. It obviously showed that both the adding methods of rhamnolipid were more effective than those four. As the on-site production of rhamnolipid could leave out the purification process, thus reducing the production cost effectively, it seemed to be a prospective adding method of the biosurfactant, for enhancing the hydrolysis of rice straw.

The common interpretation for the stimulatory effect of biosurfactants was that surfactants improved the permeability of the cell membrane and led to more enzymes being excreted outside the cell (Reese and Manguire, 1969). However, Helle *et al*. (1993), revealed that the surfactants might improve the cellulase stability and prevent the denaturation of enzymes during hydrolysis by desorbing it from the cellulose substrate (Shi *et al*., 2006). Therefore, the mechanism of the stimulatory effect of rhamnolipid on the rice straw decomposing should be observed in depth in the future study. Moreover, as *Trichoderma reesei* ZM4-F3 and *Pseudomonas aeruginosa* BSZ-07 were not compatible with fermentation conditions, such as pH and substrate concentration, the optimum fermentation conditions for these two strains should be studied in the future as well.

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