The Production of Lipopeptides by *Bacillus subtilis* with Desizing Wastewater and Application in Soaping Process

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ABSTRACT: In this article, a low-cost medium for the production of lipopeptides by the *Bacillus subtilis* HSO121 was formulated with desizing wastewater as carbon source, which contains starch and its degradation products, and the lipopeptides were biosynthesized. As the result, the yield of crude lipopeptides was 1.03 g/L, and the critical micelle concentration was ~ 50 mg/L. The chemical oxygen demand of the desizing wastewater after cultivation reduced to half compared with before cultivation. Separation of crude lipopeptides and its application as soaping agents in soaping process were investigated. The optimal soaping process was obtained at pH = 9 and 75°C with 0.05 g/L lipopeptides. Compared with traditional soaping process, lipopeptides soaping showed higher efficiency and higher colorfastness, and it is more energy efficient and more environmentally friendly process. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 1640–1646, 2011

Key words: lipopeptide; desizing wastewater; wastewater medium; starch sizing; soaping

INTRODUCTION

Lipopeptide biosurfactants are surface-active molecules produced by *Bacillus subtilis* HSO121. In the past few decades, lipopeptides have gained attention because they exhibited some advantages such as excellent surface activities, biodegradability, low toxicity, and broad biological activeties.[1,2] Lipopeptides applications have been focusing on bioremediation of pollutant,[3] antiadhesive and antimicrobial agents,[4] enhanced oil recovery,[5] cosmetics,[6] etc.; moreover, these microbial compounds exhibited potential applications in the textile industry,[7] food industry, and environmental fields.

Currently, lipopeptides are not widely utilized because of relatively high production costs coupled with low yields compared with commercially available synthetic surfactants. Using cheaper substrates and simplifying purification procedures are some of the approaches used to cut lipopeptides production costs. Many people have studied to use inexpensive substrates, such as molasses,[8] cassava wastewater,[9] peat hydrolysate,[10] vegetable oils,[11] soybean curd residue,[12] and potato effluents[13] as alternative substrate for biosurfactant production by *B. subtilis*.

Chinese textile industry generates about 300,000 tons of desizing wastewater every year. The disposablity of this residue causes environmental problems because of its high organic load. Soluble starch is the main composition in most of sizing agents, which is a very attractive substrate for the growth of microorganism.[14]

In this article, the effects of additives and byproducts in designing wastewater on the cell culture were investigated, and lipopeptides using desizing wastewater as carbon source by *B. subtilis* HSO121 were produced. Then, the crude lipopeptides were roughly separated from fermentation broth to reduce the separation costs. The soaping process using the crude lipopeptides as soaping agents for dyed fabric was investigated and optimized. The results of lipopeptides soaping process and traditional soaping process were compared. The aims of this work were to decrease the discharge of desizing wastewater, to raise the ratio of the starch size, to reduce the production cost of lipopeptides, and to improve the color fastness of dyed fabrics.

MATERIALS AND METHODS

Microorganisms

The strain used in this work was *B. subtilis* HSO121 supplied by East China University of Science and Technology. It was maintained on nutrient agar slants at 4°C. *B. subtilis* HSO121 secreted lipopeptide biosurfactants.[15]

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Substrate preparation and culture conditions

The composition of starch medium (g/L): soluble starch 20.0, NH_4Cl 2.0, KH_2PO_4 2.7, $Na_2HPO_4{\cdot}12H_2O$ 17, $MgSO_4$ 0.2, $MnSO_4{\cdot}H_2O$ 0.0017, $CaCl_2$ 0.00077, and Na_2EDTA 0.0015, initial pH = 7.2.

The desizing wastewater contains PVA, JFC ($C_{7-9}H_{15-19}O(C_2H_4)_{5-6}OH$), and NaCl except starch sizing agent. The PVA as the synthetic sizing agent was used in starch sizing agent mixture usually; NaCl was the major byproduct in alkali desizing; JFC as the penetrant was added in desizing process. To study the influence of these ingredients on lipopeptides production, effects of content of PVA, JFC, and NaCl in the medium were investigated.

Desizing wastewater was collected from Printing and Dyeing mill; soluble starch was the main composition in the sizing agent. After filtering the wastewater through cheesecloth, the contents of starch and sugar reduced in wastewater were determined with iodine colorimetry[16] and 3,5-dinitrosalicylic acid colorimetry.[17] Wastewater was evaporated to the concentration of starch, and reducing sugar reached 20 g/L. After cooling, the pH of the concentrated waste solution was adjusted to 7.2 using HCl, and then the other nutrients were added in suitable concentrations according to the composition of starch medium as described above except soluble starch; finally, the desizing wastewater medium for fermentation was prepared after autoclaving (20 min at 121°C).

The culture conditions were as follows: inoculation volume of 5% v/v was used for inoculation of 100-mL medium in 250-mL flask. The culture was incubated at 30°C, 150 rpm in a rotary shaker for 72 h. The experiments were conducted in three independent replicates.

Determination of bacterial growth, surface activity of culture supernatants, and crude of lipopeptides

The crude lipopeptides were obtained by discoloration, acid precipitation, and centrifugation. First, the cell-free broth was discolored by active carbon, and the pH was adjusted to 2.0 using 6*N* HCl and kept at 4°C overnight. Then, the crude lipopeptides were obtained through centrifugation at 5000 × *g* for 20 min, dried, and weighted as a light yellow powder.

Surface tension (ST) was determined with a Du-Nouy Tensiometer (model 322w, Thermo Cahn, USA). All measurements were made on cell-free broth. To ensure that the results were reproducible, an average of three independent measurements was taken. ST was the surface tension of cell-free culture solution, and CMD^{-1} and CMD^{-2} were the ST of 10-times and 100-times diluted cell-free broth, respectively.[18]

To characterize the bacterial growth, the optical density of the 10-times diluted bacterial culture was measured using a U-3310 UV spectrophotometer (Hitachi, Japan) at 600 nm, and the culture biomass was weighed after centrifugation, washed with distilled water, and dried at 105°C for 24 h.[19]

Characterization of lipopeptides

Emulsification activity was performed according to Cooper and Goldenberg[20]; 6 mL of hydrocarbon was added to 4 mL of aqueous solution of lipopeptides, in a screw cap tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h, and the emulsification index (E24) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplied by 100.

The functional groups were determined by infrared (IR) spectra analysis. The IR spectrum of the crude lipopeptides was recorded using KBr pellet in NEXUS-670 FTIR-Raman spectrophotometer (Nicolet, USA). The sample was prepared by dispersing the solid uniformly in a matrix of dry nujol (KBr) mull and compressed to form an almost transparent disc. The spectra showing the functional groups were used to study the composition of the lipopeptides. Absorption spectra were plotted using a built-in plotter. IR spectra were collected from 500 to 4000 wave numbers (cm^{-1}).

The lipopeptide concentrations were determined by diluting the crude lipopeptides up to the critical micelle concentration (CMC), which was determined by plotting the ST as a function of the lipopeptides concentration, and then the ST at that point was designated as CMC.

Textile dyeing

In this study, fabric was bleached poplin (40 * 40; 130 * 70; 57/58). Dyes of reactive red 3BS (150%), reactive yellow 3RS, and reactive blue BRF were purchased from DyStar.

First, the pH of reactive dyes solution (2 g/L) was adjusted to 10–11 using 6*N* NaOH and boiled at 100°C for 2 h to form complete hydrolyzed dyes. Then, the hydrolyzed dyes were mixed with its unhydrolyzed dyes in 50% (w/w) to obtain partially hydrolyzed dyes, which were used in the textile dyeing in this study. Textile dyeing was carried out according to traditional procedure under the following conditions: the concentration of partially hydrolyzed dyes was 2% (on the weight of fabric); dyeing was performed using a liquor ratio of 1 : 50 by Dyeing test machine (Labortex, Taiwan); and dyeing trials were performed in the presence of salt (40 g/L) and sodium carbonate (10 g/L). The previously wetted fabric was immerged into the dye bath at 60°C, and the temperature was raised to 90°C over 30 min and maintained for 30 min. After cooling, the dyed

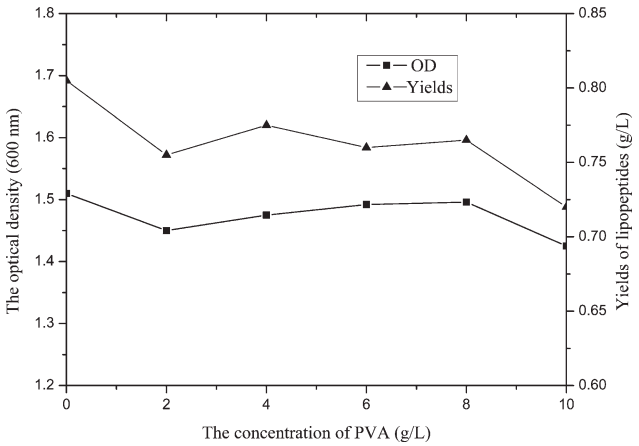

Figure 1 Effects of PVA on the yields of lipopeptides.

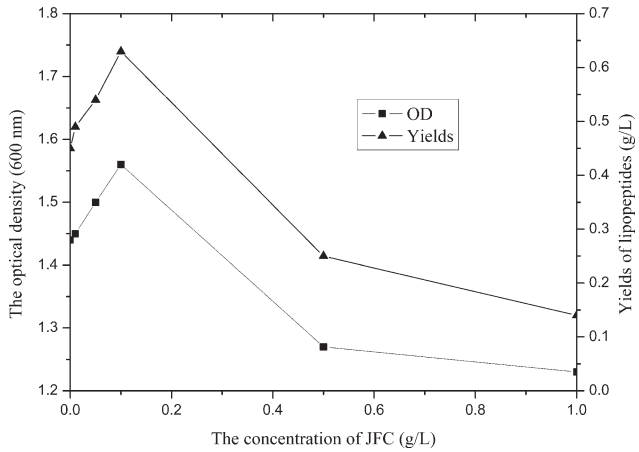

Figure 3 Effects of JFC on the yields of lipopeptides.

fabric was removed, rinsed, and dried at room temperature. The dyed fabric was used as the test sample in preceding soaping experiment.

Soaping tests

After dyeing, the samples were soaped using soaping agents at 95°C for 10 min to remove the loosely fixed dye on the surface, rinsed, and then dried at the room temperature.

All the residual soaping liquids were mixed and diluted to a constant volume, and its absorbance (Abs) at the wavelength of maximum absorption (λ_{max} = 541 nm) of the dye was measured using a UV spectrophotometer. The greater the values of absorbance (Abs) were, the stronger the effects on soaping were.

RESULTS AND DISCUSSION

Lipopeptides production and cell growth

The effect of PVA as the additives in medium on lipopeptides production is shown in Figure 1. The influence of PVA on culture was weak. When the concentration of PVA was increased to 10 g/L, a sharp decrease appeared in the growth of bacteria and the yields of lipopeptides. As shown in Figure 2, the presence of NaCl caused an enhancement of bacterial growth. When the salt concentration increased more than 10 g/L, too much salt will cause osmotic pressure of medium loss balance. However, the salt concentration in desizing wastewater was less than 10 g/L by calculated, so the NaCl existing in the desizing wastewater was not affected the bacterial growth. From Figure 3, the effect of JFC on lipopeptides production was very notable; the growth of bacteria and the yields of lipopeptides increased evidently with the concentration of JFC increased to 0.2 g/L. The bacterial growth was inhibited after the concentration of JFC was increased more than 0.2 g/L. Therefore, the concentration of JFC was restricted below 0.2 g/L in later.

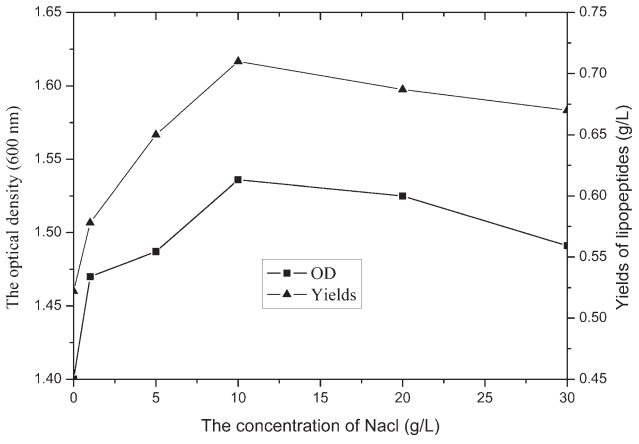

Figure 2 Effects of NaCl on the yields of lipopeptides.

The growth studies of *B. subtilis* HSO121 using starch medium and wastewater medium after 72 h of cultivation and the performance of cell-free culture solution are shown in Table I. The normal starch as carbon source was used in starch medium (A), and the starch of the desizing wastewater as carbon source was used in wastewater medium (B). After cultivation using the starch medium, the lowest ST of cell-free broth was dropped to 26.8 mN/m, the dry cell weight was 4.82 g/L, and the yield of lipopeptides was 1.24 g/L. Moreover, the results of cultivation using the desizing wastewater as substrates had the lowest ST of cell-free broth of 27.4 mN/m, the yield of lipopeptides was 1.03 g/L, and the dry cell weight reached 6.33 g/L. Compared with using the starch medium, there was no significant difference between the lowest ST. However, the CMD^{-1} and CMD^{-2} showed a slight increase. Additionally, the lipopeptides yields of using the wastewater medium were lower than using the starch medium. This indicated that the concentrations of

TABLE I
Results from the Growth Studies of *Bacillus subtilis* HSO121

Medium	OD (600 nm)	Biomass (g/L)	ST (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	Yield (g/L)
A	1.678	4.82	26.82	30.17	43.68	1.24
B	2.081	6.33	27.41	32.68	47.57	1.03

A: The starch medium; B: the wastewater medium; OD: the optical density of the 10-times diluted culture solution after cultivation at 600 nm; biomass: dry cell weight; ST: the surface tension of cell-free broth; CMD^{-1}: the surface tension of 10-times diluted cell-free culture solution; CMD^{-2}: the surface tension of 100-times diluted cell-free culture solution; and yield: the weight of crude lipopeptides.

lipopeptides in the cell-free culture solution were relatively lower compared with using the starch medium, but the differences were not very obvious. Whether from the dry cell weight or the optical density of the 10-times diluted culture solution, it seemed the wastewater medium obtains the better results. Because desizing wastewater contained some impurities that were not been filtered could add the dry cell weight, and desizing wastewater with deep color could affect the optical density.

The results demonstrated that desizing wastewater proved to be a suitable substrate for lipopeptides biosynthesis and could get stable productivity. The chemical oxygen demand of the desizing wastewater after cultivation reduced to half compared with before cultivation. This not only had an effect of cutting the production cost of lipopeptides but also achieved some effect of treating the effluent. Although the crude lipopeptides were too dark, it could have application in dyeing and finishing process by simple decolorization treatment.

Emulsification characterization

Experimental results on the emulsification activity of lipopeptides obtained from *B. subtilis* on wastewater medium are presented in Figure 4. The product was capable of forming stable water-in-oil emulsions with all hydrocarbons tested.

The emulsification activity of lipopeptides revealed that it could be used as an emulsion-forming agent for hydrocarbons and oils, and the emulsions were stable. Biosurfactants obtained from *Pseudomonas fluorescens*[21] and *B. subtilis* LB5a strain[9] had lower emulsifying index than the lipopeptides. The ability to form emulsions with hydrocarbons suggests that the lipopeptides have other potential application, such as a cleaning and emulsifying agent in textile industry.

CMC value of lipopeptides

The crude lipopeptides were dissolved in distilled water, and the ST of the water solution was measured with various concentrations of lipopeptides. The lipopeptides produced exhibited excellent ST reducing activity. From Figure 5, the ST of water solution decreased from 72.0 to 28.5 mN/m with increasing the concentration of the water solution up to $\sim$ 50 mg/L. There was almost no change in the ST of water solution after the concentrations were increased more than 50 mg/L. It indicated that the CMC was reached at this concentration. The crude lipopeptides from wastewater medium showed a

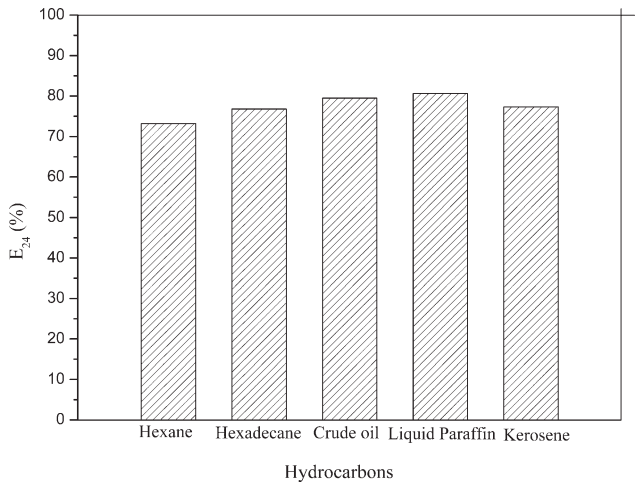

Figure 4 Emulsification activity (E_{24}) of lipopeptides against hydrocarbons.

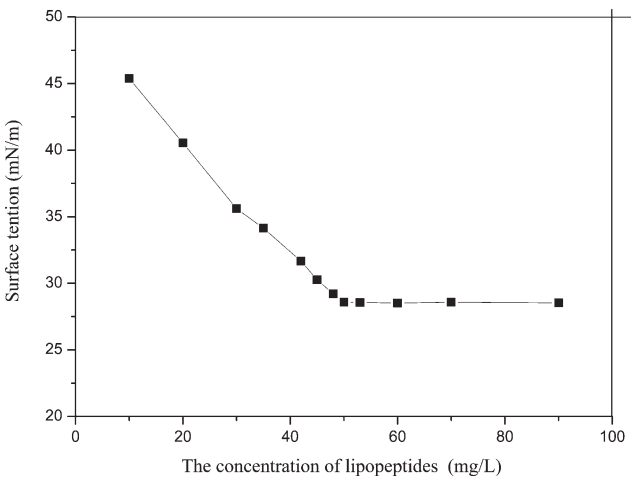

Figure 5 Surface tension of lipopeptides at different concentrations.

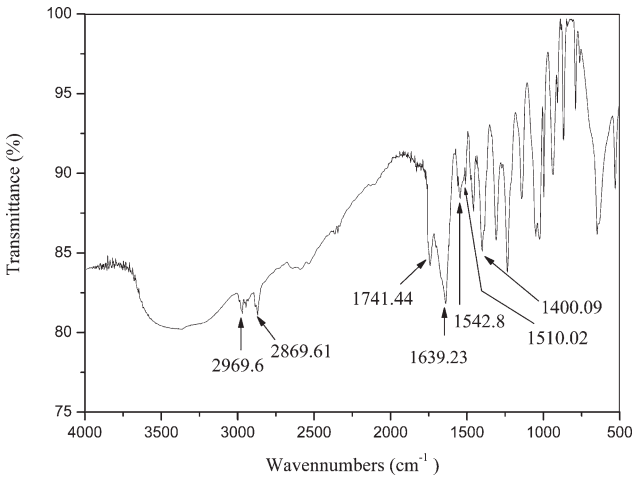

Figure 6 Infrared spectrum of lipopeptides.

lower minimum ST than that of the purified lipopeptides from *Klebsiella* sp.Y6-1 (32 mN/m).[22] Although the CMC value was obtained from a crude preparation and impurities in the medium can increase the CMC, the CMC value of crude lipopeptides was similar with purified lipopeptides from *Klebsiella* sp.Y6-1 (40 mg/L),[22] the surfactant from *B. subtilis* LB5a strain (33 mg/L).[9]

IR spectra of lipopeptides

IR spectra revealed that lipopeptides from the *B. subtilis* strains showed strong absorption bands. Figure 6 shows the presence of a peptide component at 1639.23 and 1542.8 cm^{-1} resulting from amide bands I and II; the presence of an aliphatic chain was indicated by the C—H stretching modes at 2969.9–2869.61 cm^{-1} and 1510.02–1400.09 cm^{-1}. The band at 1741.44 cm^{-1} was due to lactone carbonyl absorption. These results indicated that this compound is a lipopeptide biosurfactant. This spectrum showed similarity with the reported lipopeptide biosurfactant.[23,24]

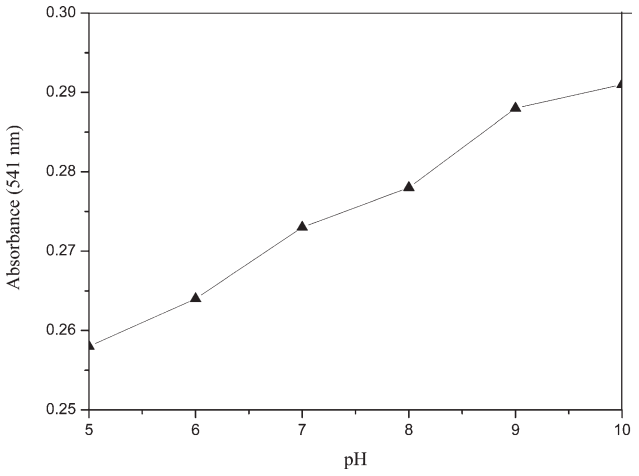

Figure 7 Effect of different pH values on soaping process using only crude lipopeptides as soaping agents.

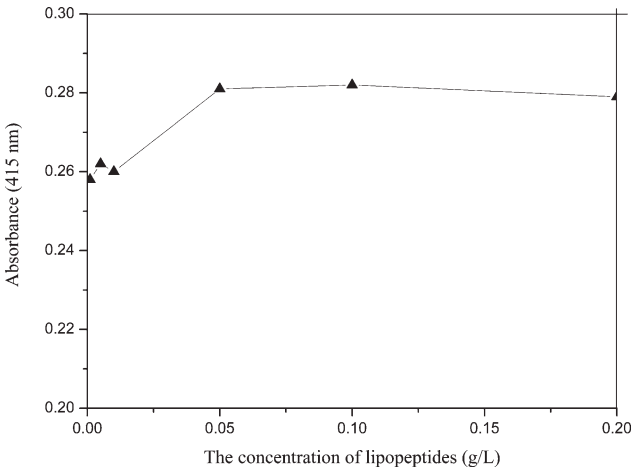

Figure 8 Effect of different lipopeptides concentrations on soaping process using only crude lipopeptides as soaping agents.

Influence of pH value on lipopeptides soaping process

Lipopeptides are amphiphilic molecules and anionic surfactants. In condition of lipopeptides concentrations 0.1 g/L and soaping temperature 95°C, the test results of soaping process using only crude lipopeptides as soaping agents at different pH values are shown in Figure 7. The test sample was poplin dyed with partially hydrolyzed reactive red 3BS dye.

It can be observed that different pH values were able to affect the result of soaping process using only crude lipopeptides as soaping agents. The absorbance (Abs) of residual soaping liquid was better with increasing pH. When the pH value was increased over 9, the Abs increased slightly. Therefore, pH = 9 was selected as the optimal pH value.

Influence of lipopeptides concentrations on lipopeptides soaping process

In the condition of pH = 9 and soaping temperature of 95°C, the test results of soaping process using only crude lipopeptides as soaping agents at different lipopeptides concentrations are shown in Figure 8. The concentration of lipopeptides was 0.2, 0.1, 0.05, 0.01, 0.005, and 0.001 g/L, respectively. The test sample was poplin dyed with partially hydrolyzed reactive red 3BS dye.

As shown in Figure 8, when the concentrations of lipopeptides were less than 0.05 g/L, the Abs was about 0.26, and it increased to 0.28 when the concentrations of lipopeptides were more than 0.05 g/L. It indicated that when the concentrations of lipopeptides were more than 0.05 g/L, better results can be

obtained. It may be because those surfactants begun to form micelles at the CMC. Lipopeptides could remove the loosely fixed dye easily when its concentrations were above CMC. No significant differences were observed when its concentrations were increased more than 0.05 g/L. From the perspective of economy, the concentrations of lipopeptides in the latter soaping experiment were 0.05 g/L.

Influence of soaping temperature on lipopeptides soaping process

The results of the soaping process using only crude lipopeptides as soaping agents at different soaping temperatures at pH = 9 and lipopeptides concentrations of 0.05 g/L are shown in Figure 9. Poplin dyed with partially hydrolyzed reactive red 3BS dye was used as the test sample.

From Figure 9, the data of Abs increased with the soaping temperature increased from 55 to 75°C. No appreciable change was observed on the absorbance (Abs) of residual soaping liquid when soaping temperature was higher than 75°C. It may be because the loosely fixed dye molecules have relatively low kinetic energy at low temperatures and were not easily removed from the surface of dyed fibers. As the temperature increased, molecules moved intensely, and the loosely fixed dye fell off easily. For saving energy, we selected 75°C as best soaping temperature in the latter experiments.

The comparison of soaping results between lipopeptides soaping and traditional soaping

As stated above, the optimum conditions of lipopeptides soaping were studied. In this section, we compared the lipopeptides soaping in the optimum conditions with traditional soaping process (Fig. 10).

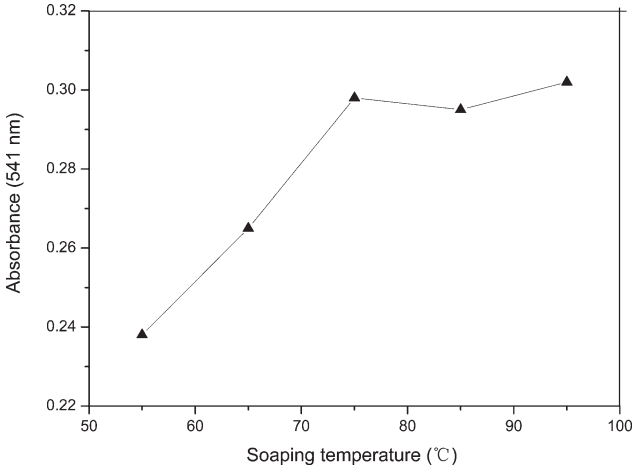

Figure 9 Effect of different soaping temperature on soaping process using only crude lipopeptides as soaping agents.

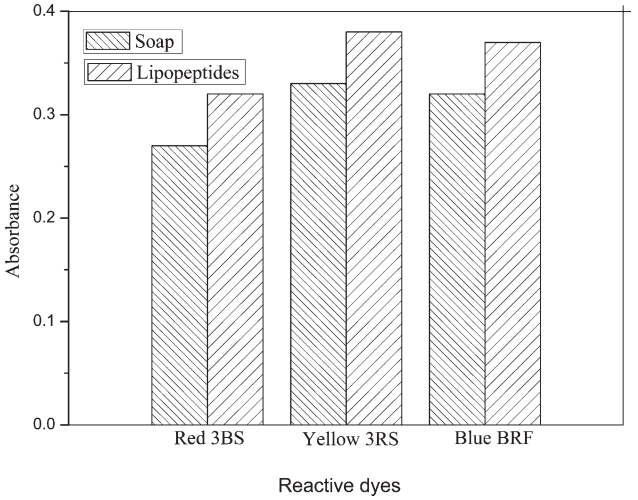

Figure 10 Comparing the Abs of soaping bath using lipopeptides with traditional soaping process.

The test sample was poplin dyed with partially hydrolyzed reactive red 3BS, reactive yellow 3RS, and reactive blue BRF.

Regardless of dyeing by partially hydrolyzed reactive red 3BS, reactive yellow 3RS, or reactive blue BRF, the results of soaping with lipopeptides were better than traditional soaping process. As can be seen from results of the test sample dyed with partially hydrolyzed reactive yellow 3RS, the Abs of traditional soaping was 0.33 and lipopeptides soaping was 0.38.

The wet rubbing fastness rating of the fabric after soaping with lipopeptides was 5, and the fabric after soaping with soap was 4. The lipopeptides soaping improves wet rubbing fastness compared with traditional soaping process. It was demonstrated that lipopeptides as soaping agents were capable of removing floating color and were better than traditional soaping agents.

CONCLUSIONS

The results showed that *B. subtilis* could produce lipopeptides from substrates prepared by desizing wastewater. Traditional carbon sources for lipopeptides production could be replaced by desizing wastewater. The ST was decreased to 28.5 mN/m when *B. subtilis* was grown on desizing wastewater medium, and the yield of crude lipopeptides was 1.03 g/L. The chemical oxygen demand of the desizing wastewater after cultivation reduced to half compared with before cultivation. Therefore, utilization of desizing wastewater for lipopeptides production could alleviate waste management problem in dyeing and finishing industries while addressing the economic issues related to lipopeptides production costs.

Compared with traditional soaping process, lipopeptides soaping showed higher efficiency and higher colorfastness, and it is more energy efficient and more environmentally friendly process. With the improvement of living standards, we began to pursue green textiles. Biosurfactant will be a new direction of development of dyeing and finishing auxiliaries. Because of their good biodegradability and low toxicity, they have promising future in dyeing and finishing technologies. However, further research including decreasing production costs of lipopeptides, application on fabric softeners, antibacterial agent, and others will be required.

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