Construction of Surfactin--Polysorbate-80 reversed micelle systems and its application on extraction of cellulasee

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Abstract. This work studied a novel method to construct a mixed reverse micelle system and its application in extracting and purifying enzymes. Mixed reversed micellar extraction formed by biosurfactant surfactin and ionic surfactant polysorbate-80 was applied for extracting cellulase. 40% surfactin and 60% polysorbate-80 were found to be the best system. The activity and protein recovery were obtained with the best conditions: in forward extraction, surfactant concentration was 3.0 mM; pH 7.0; temperature 40 °C; KCl 0.05 M. In backward extraction, pH 7.5; temperature 40 °C; KCl 0.1 M.

1. Introduction
Reversed micelle are water-in-oil microemulsion droplets stabilized by surfactants in apolar solvents, in which the surfactant molecules assemble themselves with the polar head to the inner side and the apolar tail in contact with the organic solvent[1,2]. Due to some limitations of the application of single surfactant reversed micellar extraction, the addition of nonionic surfactant into ionic surfactant modifies the interface and produces considerable decrease in the elastic rigidity of the interface[3]. Thus, the mixed reversed micellar extraction has been paid growing attention recently[4 ~ 7]. The application of mixed reversed micelles is a superior option in terms of total cost, water solubilization capacity, water structure, solubilization and synergistic performance, etc. Getting a better understanding of the mixed surfactants in reversed micellar extraction will be an important matter for improvements in separation and purification of enzymes.

In recent years much attention has been directed to biosurfactants, such as surfactin, because of their broad range of functional properties and the diverse synthetic capabilities of microbes. Moreover, biosurfactants are likely to gain wide market acceptance because they are readily biodegradable and are often significantly less toxic than their chemically synthesized counterparts[8]. Surfactin is a highly surface-active lipopeptide produced by Bacillus subtilis strains which contains a cyclic heptapeptide[9]. The special structure of surfactin makes the electrostatic interaction and hydrophobic force between surfactin and enzymes stronger, which in turn decreases the extraction efficiency. However, the addition of nonionic surfactant polysorbate-80 decreases the interaction between enzymes and head group of surfactant. Moreover, the biggest limitation of the application of surfactin is its low solubility.
Polysorbate-80 possesses high solubility, so the usage of polysorbate-80 to construct mixed reversed micelles can solve the solubility problem\cite{10,11}.

One of the bottlenecks to solve the solid waste problem is the application of lignocellulase\cite{12}. Cellulase can degrade cellulose raw material into sugars. In attempts to maximize efficiency, many studies on extraction and purification have been carried out\cite{13}–\cite{16}. In this study, we employed surfactin and polysorbate-80 at different ratios to form mixed reversed micelles. The aim of this work is to find a new way to separate and purify enzymes and unveil the possibility of mixed reversed micellar extraction. This work studied the effects of different parameters during the extraction process on the extraction efficiency.

2. Materials and methods

2.1. Chemicals and methods

Cellulase was purchased from Shanghai Beta Biological Products Co., LTD. In the forward extraction: first, the surfactants were dissolved in isooctane by adding hexanol at the ratio of 1:1. Next, 10 ml of aqueous cellulase solution was prepared. The mixture was mixed thoroughly for 1 h using magnetic stirrer and separated by a centrifuge at 10000 rpm for 10 min. For the backward extraction, the organic phase obtained from the forward extraction was mixed with an equal volume of stripping phase which was the new aqueous phase for backward extraction. Each experiment was conducted in triplicate and the standard deviations of all analyses were less than 5%.

2.2. Estimation of protein content and cellulase activity

The activity and protein content of cellulase was measured by the 3, 5-dinitrosalicylic acid and Bradford methods\cite{14}. Each sample was analyzed three times while the standard deviations of all analyses were less than 5%.

2.3. Definition

\[
AR = \frac{A_2}{A_1} \times 100\% 
\]

(1)

\[
PR = \frac{P_2}{P_1} \times 100\% 
\]

(2)

where, AR refers to activity recovery of cellulase; A1 is activity of cellulase before extraction; A2 is activity of cellulase after extraction; PR refers to protein recovery of cellulase; P1 is protein content of cellulase before extraction; P2 is protein content of cellulase after extraction.

3. Results and discussion

3.1. Forward extraction parameters

3.1.1 Effect of surfactant concentration. The reversed micelle systems tested were surfactin/polysorbate-80 at molar ratios of 0%-100%, 20%-80%, 40%-60%, 60%-40%, 80%-20%, and 100%-0%. From the results in Figure 1, the ratio of surfactin to polysorbate-80 had great effect on extraction efficiency. Among all the systems, mixed system 40% surfactin and 60% polysorbate-80 showed better activity and protein recovery.

Surfactant concentration in the organic phase was varied between 1.0 mM to 4.0 mM. As the concentration of surfactant increased from 1.0 to 3.0 mM, both the activity recovery and protein recovery of cellulase increased. The increase in surfactant concentration resulted in an increased number of reversed micelles, which enhanced the contact chances for reversed micelles and cellulase, so the activity and protein recovery increased. When the concentration of surfactants increased further, the activity recovery and protein recovery both decreased. When the number of surfactant molecules increased further, the reversed micelles aggregated, which made it harder for cellulase to transfer from crude into reversed micelles\cite{17}–\cite{19}.
3.1.2. Effect of aqueous phase pH. The aqueous phase pH of forward extraction was varied from 3.0 to 9.0. The effects of aqueous pH on activity and protein recovery are presented in Figure 2. The activity and protein recovery were found to increase with an increase in aqueous phase pH from 3.0 to 7.0. Again the system of 40% surfactin and 60% polysorbate-80 possessed best activity compared with other systems.

Former researchers assumed the optimal pH for forward extraction should be lower than the pI value of biomolecules\cite{20}. The pI of cellulase used in the experiment is around 5.0\cite{21}. However, from the results showed in Figure 2, best pH was 7.0. This indicates that hydrophobic interaction is also a significant for mixed reversed micellar extraction.

3.1.3. Effect of temperature. The effects of temperature on the activity and protein recovery are demonstrated in Figure 3. As illustrated in Figure 3, the system of 40% surfactin and 60% polysorbate-80 was the best system for extracting cellulase. It was found that the variations of activity and protein recovery were both bell-shaped. The optimum temperature was 40oC. With the temperature increasing, the molecular movement became more frequently. However, when the temperature grew higher, both the activity and protein recovery decreased, mainly because the temperature was then out of the proper range of enzyme stability and inactivation in terms of temperature\cite{22}.

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Figure 1. Effect of surfactant concentration on activity and protein recovery

Figure 2. Effect of pH in forward extraction on activity and protein recovery

Figure 3. Effect of temperature in forward extraction on activity and protein recovery
3.1.4 Effect of salt (KCl) concentration. The ionic strength of the aqueous phase is closely related to the enzyme purification process. Hence, in the present study, KCl concentration was varied from 0.01 to 0.07 M (as shown in Figure 4). The concentration of salt in aqueous phase has been reported to enhance the stability of the reversed micelles. The reversed micelles formed at lower salt concentration are unstable due to the absence of adequate electrostatic forces between the head group of surfactant and the target biomolecule[23]. The best activity and protein recovery were obtained at a KCl concentration of 0.05 M with the system of 40% surfactin and 60% polysorbate-80 employed. The existence of larger amounts of salt can reduce the electrostatic repulsion between the surfactant head groups, decreasing the size of the reversed micelles[22].

Figure 4. Effect of KCl concentration in forward extraction on activity and protein recovery

3.2. Backward extraction parameters

3.2.1 Effect of aqueous phase pH. The effects of pH of in backward extraction of cellulase on the activity and protein recovery are presented in Table 1. In the paper, pH was varied from 5.5 to 9.5. The purpose of changing pH in backward extraction was to generate minimum electrostatic interaction for releasing the biomolecule into fresh aqueous solution. In the present study, the highest activity and protein recovery were obtained at pH 7.5 by the system of 40% surfactin and 60% polysorbate-80. Meanwhile, for other systems, 7.5 was also the best pH value. For higher pH values, the alkaline environment led to the decrease of cellulase activity, which was responsible for the decrease of activity and protein recovery[21].

3.2.2 Effect of temperature. Table 1 depicts the effect of temperature on the backward extraction process. The best system was 40% surfactin and 60% polysorbate-80. The optimum temperature for activity and protein recovery during backward extraction was 40oC. The activity and protein recovery increased with rising temperature from 25oC to 40oC. This was because with the temperature increasing, the energy level of reversed micelles became higher, which decreased the relaxation time for enzyme transfer through oil-water interface. Meanwhile, the migration rate of cellulase passing through the oil-water interface toward the water phase was higher[22]. However, the activity and protein recovery decreased when the temperature increased further, which can result from the activity loss of cellulase in higher temperature environment.

3.2.3 Effect of salt (KCl) concentration. Ionic strength in backward extraction is an important factor for mixed reversed micellar extraction. The effects of KCl concentration (0.02-0.18 M) on activity and protein recovery are presented in Table 1. In the present case, the activity and protein recovery of cellulase were found to increase with an increase in salt concentration up to 0.1 M. The maximum activity recovery was 85.68% and the highest protein recovery reached 57.83%, by the system of 40% surfactin and 60% polysorbate-80. However, further increase in KCl concentration led to decrease in both activity and protein recovery. The addition of KCl during back extraction destabilized the reversed micelles, which make it easier for cellulase to transfer from reversed micelles into water phase[22,23].
Table 1(A). Effects of factors in backward extraction on activity recovery (%)

| Surfactants P-80 | 20% Surfactin + Surfactin | 40% Surfactin + Surfactin | 60% Surfactin + Surfactin | 80% Surfactin + Surfactin |
|------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| pH               |                           |                           |                           |                           |
| 5.5              | 68.72                     | 69.21                     | 70.55                     | 68.82                     | 69.15                     | 70.11                     |
| 6.5              | 74.93                     | 73.85                     | 76.93                     | 75.23                     | 75.43                     | 76.57                     |
| 7.5              | 82.34                     | 82.77                     | **84.71**                 | 83.28                     | 82.88                     | 84.63                     |
| 8.5              | 81.01                     | 80.73                     | 81.69                     | 80.29                     | 80.65                     | 79.86                     |
| 9.5              | 75.43                     | 76.52                     | 78.19                     | 74.23                     | 76.98                     | 77.43                     |
| 25               | 71.32                     | 72.84                     | 73.88                     | 72.49                     | 74.25                     | 74.68                     |
| 30               | 74.92                     | 75.28                     | 76.93                     | 77.36                     | 79.41                     | 79.55                     |
| 35               | 78.99                     | 79.85                     | 80.61                     | 79.94                     | 80.01                     | 81.98                     |
| Temperature (°C) |                           |                           |                           |                           |                           |                           |
| 40               | 82.45                     | 83.25                     | **85.27**                 | 83.86                     | 83.23                     | 84.68                     |
| 45               | 81.09                     | 80.52                     | 82.84                     | 80.31                     | 79.74                     | 80.41                     |
| 0.02             | 74.39                     | 73.77                     | 74.21                     | 73.67                     | 74.23                     | 75.23                     |
| 0.06             | 77.38                     | 79.25                     | 79.73                     | 78.42                     | 79.26                     | 80.76                     |
| KCl concentration (mM) |                           |                           |                           |                           |                           |                           |
| 0.1              | 82.53                     | 81.37                     | **85.68**                 | 82.55                     | 83.15                     | 85.19                     |
| 0.14             | 81.12                     | 81.01                     | 83.23                     | 82.11                     | 81.77                     | 82.13                     |
| 0.18             | 80.13                     | 79.94                     | 81.27                     | 79.89                     | 80.16                     | 80.01                     |

Table 1(B). Effects of factors in backward extraction on protein recovery (%)

| Surfactants P-80 | 20% Surfactin + Surfactin | 40% Surfactin + Surfactin | 60% Surfactin + Surfactin | 80% Surfactin + Surfactin |
|------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| pH               |                           |                           |                           |                           |
| 5.5              | 43.26                     | 44.85                     | 47.65                     | 45.36                     | 46.29                     | 47.27                     |
| 6.5              | 49.81                     | 48.76                     | 49.53                     | 49.17                     | 48.77                     | 49.58                     |
| 7.5              | 56.27                     | 56.72                     | **58.69**                 | 57.66                     | 56.98                     | 57.88                     |
| 8.5              | 50.43                     | 51.23                     | 52.37                     | 51.22                     | 51.93                     | 52.03                     |
| 9.5              | 49.78                     | 50.07                     | 51.78                     | 49.78                     | 50.12                     | 51.04                     |
| 25               | 50.18                     | 51.25                     | 51.09                     | 51.23                     | 51.37                     | 51.84                     |
| 30               | 52.64                     | 52.16                     | 52.99                     | 52.08                     | 52.13                     | 52.19                     |
| Temperature (°C) |                           |                           |                           |                           |                           |                           |
| 35               | 55.76                     | 54.68                     | 56.46                     | 54.56                     | 54.63                     | 54.78                     |
| 40               | 56.94                     | 56.73                     | **57.18**                 | 55.98                     | 55.96                     | 56.85                     |
| 45               | 55.43                     | 53.26                     | 54.53                     | 51.37                     | 50.31                     | 52.31                     |
| 0.02             | 48.69                     | 47.33                     | 48.62                     | 47.65                     | 48.16                     | 48.83                     |
| KCl concentration (mM) |                           |                           |                           |                           |                           |                           |
| 0.06             | 50.72                     | 50.86                     | 51.38                     | 50.87                     | 50.31                     | 50.69                     |
| 0.1              | 55.88                     | 55.73                     | **57.83**                 | 55.81                     | 54.57                     | 56.99                     |
| 0.14             | 54.67                     | 54.25                     | 55.48                     | 54.97                     | 51.29                     | 53.15                     |
| 0.18             | 50.12                     | 50.22                     | 52.17                     | 51.79                     | 50.77                     | 50.44                     |

4. Conclusion
This research verified that mixed reversed micellar extraction can be used as an efficient method to extract cellulase. Surfactin, a biosurfactant, was mixed with ionic surfactant polysorbate-80, with the most effective and efficient mixed system of 40% surfactin and 60% polysorbate-80. The activity and protein recovery were greatly enhanced by optimizing the conditions. During forward extraction, best surfactant concentration was 3.0 mM. The optimal pH, temperature and KCl were 7.0, 40°C, and 0.05
M. In backward extraction, best pH, temperature and KCl were 7.5, 40°C, and 0.1 M. Mixed reversed micellar extraction is an efficient and economical method, which has great potential in extraction and purification work.

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References
[1] Peng X, Yuan X-z, Zeng G-m, Huang H-j, Zhong H, Liu Z-f, et al. 2012 J. Process Biochemistry 47 (5) 742-8.
[2] Hong D-P, Lee S-S, Kuboi R 2000 J. Journal of Chromatography B: Biomedical Sciences and Applications 743 (1) 203-13.
[3] Ma Y-j, Yuan X-z, Xin P, Hou W, Huang H-j, Shan B, et al. 2015 J. Journal of Molecular Liquids 203 181-6.
[4] Liu D, Ma J, Cheng H, Zhao Z 1998 J. Colloids and Surfaces A: Physicochemical and Engineering Aspects 143 (1) 59-68.
[5] Mitra RK, Paul BK 2005 J. Colloids and Surfaces A: Physicochemical and Engineering Aspects 252 (2) 243-59.
[6] Kundu K, Paul BK 2013 J. Colloids and Surfaces A: Physicochemical and Engineering Aspects 433 154-65.
[7] Paul BK, Mitra RK 2005 J. Journal of Colloid and Interface Science 288 (1) 261-79.
[8] Maity JP, Lin T-J, Cheng HP-H, Chen C-Y, Reddy AS, Atla SB, et al. 2011 J. International Journal of Molecular Sciences 12 (6) 3821.
[9] Shen H-H, Lin T-W, Thomas RK, Taylor DJF, Penfold J 2011 J. The Journal of Physical Chemistry B 115 (15) 4427-35.
[10] Spernath A, Aserin A 2006 J. Advances in Colloid and Interface Science 128-130 47-64.
[11] Sun X-H, Zhu K-X, Zhou H-M 2008 J. Journal of Cereal Science 48 (3) 829-35.
[12] Cheng Shu I, Stuckey David C 2011 J. Biotechnology Progress 27 (6) 1614-22.
[13] Shin YO, Weber Martin E, Vera Juan H 2008 J. Biotechnology Progress 19 (3) 928-35.
[14] Yuan X-Z, Peng X, Huang H-J, Wang H, Ma Y-J, Bao S, et al. 2014 J. Separation Science and Technology 49 (14) 2249-54.
[15] Komesvarakul N, Do LD, Nguyen TT, Scamehorn JF 2005 J. Separation Science and Technology 40 (12) 2463-78.
[16] Ruso JM, González-Pérez A, Prieto G, Sarmiento F 2003 J. International Journal of Biological Macromolecules 33 (1) 67-73.
[17] Noritomi H, Ito S, Kojima N, Kato S, Nagahama K 2006 J. Colloid and Polymer Science 284 (6) 604-10.
[18] Kai LI, Cheng-Fu LI, Jia-You LI, Qian L, Jiao QC 2008 J. Fine Chemicals.
[19] Wang J, Cao X 2007 J. China Biotechnology 27 (3) 93-9.
[20] Zhang YX, Zhao JH, Du ZY, Fang J, An XQ, Shen WG 2007 J. Acta Physico-Chimica Sinica 23 (9) 1483-6.
[21] Lei M 2002 J. Journal of Cellulose Science & Technology.
[22] Krishna SH, Srinivas ND, Raghavarao KSMS, Karanth NG. Reverse Micellar Extraction for Downstream Processing of Proteins/Enzymes. In: Dutta NN, Hammar F, Haralampidis K, Karanth NG, König A, Krishna SH, et al., editors. History and Trends in Bioprocessing and Biotransformation. Berlin, Heidelberg: Springer Berlin Heidelberg; 2002. p. 119-83.
[23] Biasutti MA, Abuin EB, Silber JJ, Correa NM, Lissi EA 2008 J. Advances in Colloid and Interface Science 136 (1) 1-24.