A new method of extraction of amoxicillin using mixed reverse micelles

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**HIGHLIGHTS**

- Amoxicillin is extracted using novel AOT/TWEEN85 mixed reverse micelle system.
- Final extraction higher than 90\% is obtained under optimised conditions.
- Mixed reverse micelle system reduces total amount of surfactants needed notably.

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**ABSTRACT**

A completely new method of extraction of amoxicillin using solubilisation by mixed reverse micelles was proposed and the optimal conditions for the process were found. Mixed AOT-TWEEN 85 reverse micelles were used for the first time as a new approach for extraction of amoxicillin. The effects of different process variables such as AOT-TWEEN 85 molar fractions, total surfactant concentration, pH of aqueous feed solution and potassium chloride concentration during forward extraction; stripping aqueous phase pH, potassium chloride (KCl) concentration and extraction time during backward extraction were investigated and the optimal conditions were found for all mentioned parameters. With the aid of response surface methodology, the optimum conditions for forward extraction are identified as (i) 5.5:1 molar ratio of AOT/TWEEN 85, (ii) total surfactant concentration 102.57 g/L, (ii) pH 1.90, and (vi) KCl concentration 8.54 g/L. The percentage of amoxicillin solubilised in isooctane was 95.54\% under these optimal conditions. On the other hand, the optimum conditions for backward extraction are identified as (i) stripping aqueous phase pH 6.58, (ii) KCl concentration 11.02 g/L, and (iii) extraction time 15 min. Under these optimal conditions, the percentage of amoxicillin recovered was 90.79\%. AOT-TWEEN 85 mixed surfactant system shows a significant advantage of saving the amount of surfactant for forward extraction. The addition of non-ionic surfactant helps to preserve natural function/activity of antibiotics as compared with a pure AOT surfactant used. The optimum conditions were also found for a backward recovery of amoxicillin.

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1. Introduction

Amoxicillin (a-amino-p-hydroxybenzylpenicillin) is one novel semi-synthetic \(\beta\)-lactam antibiotic and is widely used in medical therapy as a broad-spectrum bactericidal which is to be exposed to a variety of microorganisms \(\beta\)-lactamases produced...
by Gram-positive and many Gram-negative [1]. It is regularly combined with β-lactamase inhibitor Clavulanic Acid for oral antibacterial medicine and better protection against infections of intra-abdominal [2]. In industries, amoxicillin is presently produced through a chemical coupling process by using a β-lactam nucleus and appropriate acyl donors. Chemical coupling of amoxicillin involves the reaction of an amino β-lactam such as 6-aminopenicillanic acid (6-APA) usually having its carboxyl group protected with an activated side-chain derivative, where the protecting group is removed through hydrolysis [3].

There are a number of problems occurred during the purification of semi-synthetic antibiotics from a broth: separation of toxic solvents, waste produced, by-products and other impurities. As a result of separation stages the antibiotic yield and natural function/activity are reduced. Protection and controlled release of bioactive compounds can be done through encapsulation [4]. Reverse micellar extraction (RME) has some clear advantages as compared with other separation process in extracting antibiotics. These advantages are as follows: preservation of native function/activity, higher yield, lower interfacial tension for better phase mixing, ease of scale up, and potential for continuous operation [5,6]. Electrostatic, steric, and hydrophobic interactions between antibiotic and reverse micelles are considered to be the driving forces for the diffusion of antibiotic into the reverse micellar core [7]. RME is usually optimised through adjusting various variables such as solution pH, surfactant concentration, salt concentration, and contact time [8,9]. However, most of the reports on RME deal with ionic surfactants and only a few reports have been published on the application of nonionic surfactants [10]. Extraction of large solutes (such as antibiotics) with nonionic surfactants was reported to be difficult due to a lack of strong electrostatic interaction between antibiotics and surfactant molecules.

Chatterjee et al. [11] found that the alteration of the interfaces between organic and aqueous solutions due to addition of a second surfactant can enhance the solubilisation capacity of mixed surfactant system. The improvement of solubilisation capacity of bio-molecule by reducing the charge density of AOT micellar surface in presence of non-ionic surfactant had been reported earlier [12]. However, studies regarding mixed reverse micellar systems had been restricted on their physic-chemical characterisations. Further, to the authors’ best knowledge, none had been reported for RME of antibiotics using mixed reverse micellar system.

Below AOT/TWEEN 85 mixed anionic and non-ionic surfactant system was used as a new approach in RME and an alternative for conventional liquid–liquid extraction with a chemical solvent. TWEEN 85 is ethoxylated derivatives of sorbitan esters in which the substitution of the hydroxyl groups on the sorbitan ring with polyoxyethylene groups makes the surfactant hydrophilic heads bigger and able to occupy larger area on the interface. The focus of this research is extraction of the antibiotic amoxicillin using the reverse micelles mentioned above to enhance the extraction processes from aqueous solution of amoxicillin. The optimum conditions for extraction process to give a highest yield are determined.

2. Methodology

2.1. Chemicals

The bio-molecule used is amoxicillin trihydrate obtained from bio-WORLD, USA. The aqueous phase was prepared from fresh deionised water using Purite Select AN HP40 (Purite Ltd, England) with resistivity 15–16 MΩcm. Reagent grade sodium di-2-ethylhexylsulfosuccinate (AOT) was used as anionic surfactant and non-ionic TWEEN 85 surfactant was used as the co-surfactant (Sigma Adrich Co). Various concentrations of KCl in aqueous phase were used to compose the aqueous solution containing biomolecule of amoxicillin. Isooctane was used as an organic phase for the reverse micelles formation. HCl or NaOH solutions were used to adjust pH of the aqueous phase during the experiment. All the chemicals were purchased from Sigma Adrich Co. (M). All reagents used in the experiment were of analytical grade.

2.2. Selection of surfactants for mixture and optimal surfactant molar ratio

AOT is capable of solubilising a huge quantity of water without any addition of co-surfactant [5]. However, denaturing of biomolecule may occur due to the strong electrostatic interaction between the polar head of reverse micelles and bio-molecules. This results in a deactivation of bio-molecule and in a low yield of extraction. Most researchers are more focused on the extraction capability of anionic surfactants such as AOT. The potential of non-ionic surfactants or combination of ionic and non-ionic surfactants in reverse micelles for bio-molecules separation, especially antibiotic molecules is still to be investigated. Only a few studies have been reported on the application of non-ionic surfactant and these studies are limited to protein extraction [13].

There are several publications available where TWEEN85 reverse micelles were used for protein extraction and retention of enzyme activity [14]. TWEEN 85 was used in this research because it is able to solubilise a larger volume of water and protein than the classic anionic surfactants such as AOT [15]. However, single non-ionic surfactant systems were reported to be not effective in forward extraction of protein [16] because the electrostatic interactions between protein and surfactant are weak. Therefore, it should be used together with ionic surfactant to provide sufficient electrostatic interactions for forward extraction.

It was found earlier that TWEEN85 does not have detrimental effect on the structure, function, and stability of cytochrome-c subtilisin [17]. Pfammatter et al. [18] also had demonstrated the solubilisation and growth of whole cells in reverse micelles, composed of TWEEN 85 and Span 80. TWEEN 85 is biodegradable and has been successfully tested for use as an additive in fertilizer. TWEEN 85 also has a hydrophilic/lipophilic balance (HLB) of 11, which indicates that it is soluble in organic solvents [19]. It is the reason why TWEEN 85 was selected as a co-surfactant for formation of mixed reverse micelles.

In the course of the RME it is important to have a clear phase and avoid emulsion formation during the extraction process. The latter can be achieved by selecting a proper ratio of surfactant in the mixture. The optimum molar ratio of AOT/TWEEN 85 was determined beforehand. Various amount of TWEEN 85 was added into isooctane while keeping AOT concentration at 150 mM and forward extraction was conducted. Experimental results showed that only molar ratio of AOT/TWEEN 85 above 1.38:1.00 produced a clear transparent phase. Below this ratio (region to the right in Fig. 1, line not shown) precipitation of surfactants was observed during forward extraction. Furthermore, when the molar ratio is less than 1.00:1.00, a small precipitation was observed at the interface and emulsion was easy to occur. However, emulsion formation substantially reduced the amount of amoxicillin solubilised in the reverse micelle phase. The cloudiness observed also indicates that amoxicillin was denatured. The following ratio 5.50:1.00 was found to be the best ratio in the parameter range being investigated as shown in Fig. 1. All subsequent experiments were carried out with AOT/TWEEN 85 ratio of 5.50:1.00.

2.3. Critical micelle concentration (CMC)

Since reversed micelles formed in organic phase, the CMC refers to the concentration of surfactants in isooctane. The variation of
interfacial tension between the isooctane containing AOT/TWEEN 85 at different concentrations and aqueous phase is shown in Fig. 2. Fig. 2 allows determining CMC as shown. The CMC value of AOT/TWEEN 85 at 5.50:1.00 ratio was determined as 0.1064 g/L. This CMC value is lower as compared with CMC of single AOT in isooctane which is ∼23 g/L but higher than CMC of single TWEEN 85 which is 0.04 g/L. High CMC of single AOT can be explained by a strong repulsion between charged AOT heads inside reversed micelles. The presence of TWEEN 85 neutral molecules in reverse micelles decrease the repulsion between charged AOT heads and as a result the CMC of the mixture is lower than that of single AOT molecules. Our data show that addition of TWEEN 85 substantially reduce the CMC of AOT/TWEEN 85 mixture and the resulting CMC of the mixture is in between of those of single AOT and single TWEEN 85 surfactant molecules.

2.4. Forward extraction

The antibiotic aqueous solution was obtained by dissolving of amoxicillin powder (4 g/L) with KCl in de-ionised water. The pH of the solution was adjusted using HCl or NaOH solutions. Then 5 mL of the obtained aqueous solution was added into 5 mL of isooctane with AOT/TWEEN 85 pre-dissolved at a fixed concentration. A slow stirring using a magnetic stirrer at 350 rpm (IKA RET Basic safety control) was used for gentle mixing. The stirring time and rotational speed were controlled carefully to avoid denaturation of antibiotic molecules and formation of a stable emulsion. The rotational speed of stirring started from zero rpm and gradually increased until 350 rpm to avoid a vigorous mixing. The mixture was stirred for 15 min until a clear phase was obtained. The temperature for extraction process of amoxicillin was kept constant at 20 ± 1°C throughout the experiment. Then, the mixture was stored for 24 h for settling. The organic phase loaded with amoxicillin was extracted from the mixture after the settling using a syringe for further backward processing and analysis. The reverse micelle solutions were analysed using water determination balance (Karl Fisher Titration Method) and surface tension meter (Wilhelmy Ring Method) to measure surface tension. The steps of forward extraction process are shown in Fig. 3.

2.5. Backward extraction

The antibiotic-loaded organic phase from previous forward extraction was used for backward extraction. The organic solution was added into 5 mL of fresh aqueous solution containing fixed concentration of KCl. The mixture was then stirred for 15 min using a magnetic stirrer type IKA RET Basic safety control at 350 rpm for backward extraction. The temperature for extraction process of amoxicillin was maintained at 20 ± 1°C throughout the experiment. Then the mixture was left for 24 h to settle. After settling process, the two phases was separated using a syringe. The organic phase was extracted from the mixture after the settling processes using a syringe. The concentration of amoxicillin in the remaining aqueous phase after extraction was analysed using UV–vis Spectrophotometer.

3. Result and discussion

3.1. Forward extraction

3.1.1. Effects of total surfactant concentration

The solubilisation of antibiotic depends on the conditions in both the aqueous phase and the organic phase. However, the nature of surfactant and its concentration are the major factors affecting solubilisation of antibiotic. It is the reason why the effects of surfactant concentration on reverse micelles formation and extraction efficiency in mixed AOT/TWEEN 85 system were examined firstly. The surfactants molar ratio was kept constant at 5.50:1.00 while total surfactant concentration was varied. Water content and micellar size were determined before forward extraction was conducted and after forward extraction was conducted. The results are given in Fig. 4 and Table 1.

Water content in reverse micellar phase is defined as the molar ratio between solubilised water and surfactants. Fig. 4 shows that the water content in isooctane after forward extraction increased when total surfactant concentration was increased to 80 g/L. Increasing the total surfactant concentration increases
Fig. 4. Effect of mixed AOT/TWEEN 85 concentration on water content in reverse micellar phase, $A_{w}$, 4 g/L; amount of KCl, 10 g/L; stirring speed, 350 rpm; stirring time, 15 min; temperature, 20 °C ± 1; pH, 3.5 ± 0.1.

Table 1

| AOT/TWEEN 85 (g/L) | Reverse micellar size, $R_m$ (nm) |
|-------------------|----------------------------------|
|                   | Before extraction | After extraction |
| 20.0              | 0.32              | 0.66              |
| 40.0              | 0.65              | 1.55              |
| 60.0              | 0.90              | 3.03              |
| 80.0              | 1.17              | 4.67              |
| 100.0             | 1.49              | 1.32              |

the number of reverse micelles [20] and this caused more water to be taken by the reverse micelles. “Before Extraction” refers to the water content before forward extraction was conducted. The increase in water content and micellar size after forward extraction was conducted show that water and consequently amoxicillin had been solubilised into the reverse micelles.

The data presented in Table 1 also suggests that higher total surfactant concentration may increase the size of reverse micelles. However, at total surfactant concentration higher than 80 g/L, there was a reduction in the amount of water in organic phase. This may be due to the collapse of reverse micellar structure, which is generally observed at high surfactant concentration. This lead to lower amoxicillin transferred to the reverse micelles.

Fig. 5 shows the forward extraction efficiency of single AOT, single TWEEN 85, and mixed AOT/TWEEN 85 surfactant systems. The total surfactant concentrations were varied from 0 to 200 g/L at constant aqueous and isooctane volume of 5 mL. The pH and NaCl concentration were maintained at 3.5 and 10 g/L, respectively, in aqueous phase.

The final amount of amoxicillin solubilised increased as the surfactant concentration was increased from 5 to 50 g/L in mixed AOT/TWEEN 85 reverse micellar system. High amoxicillin extraction was achieved at 50 g/L and reaches around 88%. The amount of amoxicillin extraction decreased at higher total AOT/TWEEN 85 concentration. At concentrations of 100 g/L and at higher concentrations the originally transparent reverse micellar solution became turbid, which in undesirable as mentioned earlier because it caused the separation of phases more difficult. At high surfactant concentration, more reverse micelles are present in isooctane.

In Fig. 5 comparison was presented between forward extraction efficiency of AOT/TWEEN 85 mixed reverse micelle with the forward extraction efficiency of single surfactant AOT and TWEEN 85. The comparison was undertaken to evaluate the effectiveness of each process. There is no extraction of amoxicillin by up to 23 g/L, which is equal to CMC of AOT in isooctane. The latter is in agreement with that reported by Mohd-Setapar et al. [21]. Further increasing the AOT concentration above CMC to 200 g/L increased the amount of amoxicillin solubilisation. However, the AOT concentration for maximum extraction would be at least 200 g/L or higher as shown in Fig. 5. Hence, AOT/TWEEN 85 mixed surfactant system shows a significant advantage of saving the amount of surfactant for forward extraction. On the other hand, single TWEEN 85 surfactant system shows extremely low forward extraction efficiency indicating that it alone is not suitable for amoxicillin extraction. Important to emphasise, that the addition of non-ionic surfactant makes the arrangement of surfactant molecules at the interface more compact and rigid, and the latter decreases the electrostatic repulsion between the head groups of AOT and protects the bio-molecule from unfavourable action of surface charge of AOT and, hence, helps to preserve natural function/activity.

Experimental result clearly shows that there exists maximum of efficiency of forward extraction, which is around 50 g/L of total surfactant concentration. In order to determine more precisely the maximum point, forward extraction was further conducted for AOT/TWEEN 85 total concentration between 50 g/L and 100 g/L. The result is included in Fig. 5. The highest extraction efficiency was obtained around 92% at 80 g/L of total surfactant concentration. It was observed that when the AOT/TWEEN 85 concentration was 90 g/L or higher, the turbidity of the solution increased, making the amount of solubilised amoxicillin decreased from 75% to 70%.

3.1.2. Effects of feed pH

Earlier, Hemavathi et al. [10] found that the initial aqueous feed pH determines the net charge of proteins affecting the electrostatic interaction between surfactant and protein. Hence, we expect that in the case under consideration, the pH of aqueous feed solution should influence the partitioning of amoxicillin molecules in mixed reverse micellar phase. In this section, the effect of pH on amoxicillin extraction using mixed AOT/TWEEN 85 reverse micellar system was examined using pH range from 1.0 to 6.0 and the results are presented in Fig. 6. The mixed reverse micellar system consisted of mixed AOT/TWEEN 85 surfactants at already determined optimal concentration of 80 g/L.

The isoelectric point (pI) of amoxicillin is 4.7 [22] and at pH below this value amoxicillin charge is positive and it becomes negative at pH above this value [21]. Fig. 7 shows a well pronounced maximum extraction of amoxicillin in the region close to pH = 1.9. This region of pH corresponds to the positively charged amoxicillin molecules. When the pH was increased to near pI (4.7), forward
extraction percentage dropped due to weakening of positive charge at the surface of amoxicillin thus lower electrostatic attraction with negatively charged AOT molecules. When pH was further increased to above pH, the net opposite surface charge increased which resulted in drastic drop in forward extraction percentage.

3.1.3. Effects of KCl concentration

Ionic strength is known as one of the factors which can affect the reverse micelle extraction efficiency. It was found earlier that reverse micelles extraction of proteins substantially depends on the salt concentration in the feed solutions [9]. The organic phase was observed to become cloudy at high salt concentrations because the surfactant molecules started to migrate from the isoctane into the aqueous phase [21]. KCl is found favourable for RME of proteins [23].

It is the reason why investigated below the effects of KCl salt for AOT/TWEEN 85 mixed reverse micellar system by varying salt concentration between 0 g/L and 16 g/L (0 M–0.21 M). Fig. 8 shows the forward extraction efficiency at different concentrations of KCl. When KCl does not present in the system, there is no amoxicillin solubilisation detected. This indicates that AOT/TWEEN 85 reverse micelles are not formed.

Data presented in Fig. 8 show that the forward extraction efficiency increased when KCl concentration was increased up to 10.0 g/L (0.13 M in Fig. 7), with maximum forward extraction of 92%. However, above KCl concentration of 10 g/L (0.13 M), the amount of amoxicillin solubilised decreased with further increase of salt concentration. Adding low amount of KCl helps to stabilise the reverse micelles. At the same time, KCl reduces the electrostatic interaction between AOT and amoxicillin. When KCl concentration becomes too high, less amoxicillin is captured by the reverse micelles due to lower electrostatic interaction. In addition, KCl also reduces electrostatic interactions between AOT head groups and leads to smaller reverse micellar size. Thus forward extraction efficiency decreases at high KCl concentration because the reverse micelles become too small for solubilisation of amoxicillin.

3.2. Backward extraction

3.2.1. Effects of aqueous phase pH

The protein extracted into the reverse micellar phase can be controlled during backward extraction through electrostatic repulsion by altering the pH of the fresh aqueous solution. Since the aqueous phase pH affects the net surface charge of the protein, therefore in backward extraction the pH must be such that the bio-molecules have the same charge as the surfactants in order to create repulsion forces between the bio-molecules and the reverse micelles so that the bio-molecules can be recovered into fresh aqueous phase [21]. Therefore, during backward extraction using an anionic AOT surfactant-based reversed micelle, the aqueous phase pH should be elevated above the pI to recover the amoxicillin in backward extraction process. It is the reason why we investigated below the influence of pH on the backward extraction of amoxicillin using the mixed micellar system under consideration.

The effect of fresh aqueous solution pH during backward extraction on the backward extraction efficiency is presented in Fig. 8 for mixed AOT/TWEEN 85. For comparison purpose, we show in the same Fig. 8 efficiency for single AOT reverse micellar system. The presence of maximum recovery for both reverse micellar systems (Fig. 8) shows that backward extraction is very sensitive to aqueous phase pH.

Fig. 8 clearly shows that in the pH region below pI of amoxicillin (4.7) no amoxicillin was extracted. This is because the amoxicillin and surfactants have opposite charges at pH below pI. Thus, the presence of strong electrostatic interactions between the
amoxicillin and surfactants hindered back transfer of amoxicillin into the aqueous phase. When aqueous phase pH was increased above pH = 4.7, the amoxicillin molecules were recovered successfully for both reverse micellar systems (Fig. 8). When the pH of the aqueous solution is above pl value of amoxicillin, the net charge of amoxicillin will be negative charge, hence repelled by the negatively charged head groups of the AOT surfactant in the reversed micelles. Therefore, the amoxicillin was able to transfer into the aqueous phase.

The percentage of recovered amoxicillin in aqueous phase was found to be highest at pH 6.0 for mixed AOT/TWEEN 85 system (93%) (Fig. 8). This value is higher than in pure AOT system at pH 5.0 (84%) (Fig. 8).

Fig. 8 shows that at higher pH the amount of amoxicillin recovered for mixed AOT/TWEEN 85 reverse micelles decreased at higher pH from 90% to 64% as the aqueous phase pH was increased from 6.5 to 8.0. This is because the amoxicillin molecular structure was destroyed under strong alkali condition.

Fig. 8 also shows that amoxicillin was recovered with higher efficiency when using mixed reverse micelles formed by using AOT/TWEEN 85 compared to using AOT only. The efficiency of backward extraction was above 80% for aqueous phase pH range between 5.8 and 7. For AOT only system the recovery efficiency was slightly above 80% only in narrow range around pH 5.0. Biotechnological process is considered viable when the final recovery of target molecule is higher than 80% and if the process does not meet this requirement the recovery step is considered unviable with overall loss too high [24]. Thus, mixed AOT/TWEEN 85 reverse micelle system provides better recovery as compared with AOT only system.

3.2.2. Effects of alcohol

The addition of alcohol was reported to improve the backward extraction of bio-molecule from reverse micellar solutions [21]. According to Hemavathi et al. [10] addition of alcohol is able to reduce the interactions between reverse micelles and bio-molecule by reducing the micellar interfaces resistance, hence, improving the back transfer.

Below we investigated influence of added alcohol on backward extraction of amoxicillin using mixed AOT/TWEEN 85 system. 5% v/v hexanol was added into aqueous solution with 10 g/L KCl at different pH. The obtained recovery is presented in Fig. 9. Fig. 9 shows that the recovery of amoxicillin was enhanced at pH 3.5–5.5 although the pH of aqueous stripping was lower than the pl. It appeared that the pl value became less significant in the presence of alcohol.

However, a complex precipitate was observed between aqueous and organic phases throughout the experiment when the extraction was carried out at pH lower than pl. Although up to 51% of amoxicillin was recovered at pH below pl, the activity of amoxicillin was lost to a considerable extent. The presence of white emulsion formed during reverse micelle extraction indicated the loss of bio-molecule’s activity due to structural change of the bio-molecules. On the other hand, addition of hexanol enhanced the backward extraction efficiency at pH above pl. However, it may also favour the formation of emulsion and thus was omitted in following experiments.

3.2.3. Effects of KCl concentration

Various concentrations of KCl between 2 g/L and 16 g/L (0.03 M–0.21 M) were used during backward extraction. KCl was chosen because the larger K+ ions are capable to cause higher solubilisation as compared to ions with smaller sizes such as Na+ [22]. K+ cations are chaotropes which can break water structure [9].

Fig. 10 shows the effects of KCl concentration on the backward extraction of amoxicillin. The results show that recovery of amoxicillin increased when the concentration of KCl was increased from 2 g/L to 12 g/L (0.03 M–0.16 M). At this range, the backward efficiency was increasing from 7% to 94%. The latter finding differs from the forward extraction (see Fig. 7). The amoxicillin was successfully recovered in backward extraction at high concentration of KCl. Addition of salt is known to reduce the size of reverse micelles thus “squeezing out” the solutes contained in the reverse micelles. This size exclusion effect enhanced the backward extraction efficiency. Furthermore, addition of higher amount of salt also destabilises the reverse micelles, causing more amoxicillin to be released into the aqueous phase.

However, a further increase in KCl concentration above 12 g/L (0.16 M) from 14 g/L to 16 g/L (0.19 M–0.21 M) resulted in a decrease of amoxicillin recovery from 67% to 52%. White precipitates were observed in this range, making the recovery process less efficient. These white precipitates indicate that some amoxicillin molecules were denatured. When the salt concentration became too high, the amoxicillin molecular structures changed due to the squeezing out of the amoxicillin from small reverse micelles. Hence,
Table 2
Predicted and experimental value at optimum conditions for forward extraction.

| Model | Optimum conditions | Predicted value (g/L) | Experimental value (g/L) |
|-------|--------------------|-----------------------|--------------------------|
|       | AOT/TWEEN 85 concentration (g/L) | pH of aqueous fresh solution X_{F} | KCl salt concentration (g/L) X_{S} | Y_{pre} | Y_{exp} |
| Y_{1} (g/L) | 102.57 | 1.90 | 8.54 | 4.06 | 3.82 (95.54%) |

Table 3
Predicted and experimental value at optimum conditions for backward extraction.

| Model | Optimum conditions | Predicted value (g/L) | Experimental value (g/L) |
|-------|--------------------|-----------------------|--------------------------|
|       | pH of stripping solution X_{B} | KCl salt concentration (g/L) X_{S} | Backward extraction time (min) X_{T} | Y_{pre} | Y_{exp} |
| Y_{1} (g/L) | 6.58 | 11.02 | 19.8 | 17.42 | 17.25 (90.79%) |

Fig. 11. Effects of the backward extraction time on the stripping of amoxicillin from mixed AOT/TWEEN 85 reverse micelle. Experimental conditions: Initial mass of amoxicillin in organic phase, A_{org}, 19 mg; pH of stripping solution, 6.0; amount of KCl, 12 g/L; stirring speed, 350 rpm; temperature, 20°C ± 1.

the aggregation and precipitation of amoxicillin become dominant in the back transfer.

3.2.4. Effects of backward extraction time
The influence of mixing time on recovery of biomolecules is substantial because it directly affects the contact between the two phases. Fig. 11 shows the effects of mixing time on the recovery of amoxicillin. The mixing time was varied from 5 min to 40 min. When the mixing time was increased from 5 to 15 min, the recovery of amoxicillin increased from 19% to 93%. The optimum mixing time is around 15 min (see Fig. 11).

3.3. Response surface modelling

Interactions between two or more factors are known to mask the true optimum point of experiments. Thus, response surface methodology was employed to optimise the extraction condition of amoxicillin recovery using mixed AOT/TWEEN 85 reverse micellar system. All data were implemented using Central Composite Design with STATISTICA 8.0 software. The results were fitted into following equations:

For amount of amoxicillin extracted during forward extraction, Y_{1} : (1)

\[ Y_{1} = -2.495 + 0.045X_{1F} + 0.898X_{2F} + 0.794X_{3F} - 0.236X_{2F} + 0.46X_{2F}^{2} \]

where \( X_{1F} \) is the total concentration of AOT/TWEEN 85 in isooctane, \( X_{2F} \) is pH of the fresh aqueous phase and \( X_{3F} \) is salt concentration in aqueous solution.

For amount of amoxicillin recovered during backward extraction, \( Y_{2} : (2) \)

\[ Y_{2} = -121.15 + 28.039X_{1B} - 2.128X_{2B}^{2} + 4.283X_{2B} - 0.194X_{2B} + 2.289X_{3B} - 0.058X_{3B}^{2} \]

where \( X_{1B} \) is pH of stripping solution, \( X_{2B} \) is KCl concentration and \( X_{3B} \) is backward extraction time.

Analysis of variance (ANOVA) shows that both models are significant with 1% significance level. This suggests that both models are able to represent the RME process in the required experimental region. Using these models, the optimum conditions were determined and compared with the experimental results. Table 2 and Table 3 show the predicted and experimental values for forward extraction and backward extraction. The results show that the experimental values found are in agreement with the predicted values. The extraction method using mixed reverse micelle can be considered as a good alternative since the recovery of amoxicillin is able to exceed 90%.

4. Conclusion
A completely new method of extraction of amoxicillin using solubilisation by mixed reverse micelles was proposed and the optimal conditions for the process were found. For the first time AOT/TWEEN 85 mixed reverse micellar system was successfully used to solubilise amoxicillin. Experimental results showed that mixed surfactant system gives better forward extraction efficiency compared to single AOT or TWEEN 85 systems. Addition of TWEEN 85 into AOT reverse micelles was found to reduce the total surfactant concentration required up to 77%. This shows that mixed reverse micelle system can be an economic alternative for extracting of bio-molecules. The forward extraction was found to depend on several process parameters. Analysis showed that the dominant factors affecting forward extraction are molar ratio of AOT to TWEEN 85 molecules in isooctane, total surfactant concentration, aqueous phase pH, and KCl concentration. The optimal AOT/TWEEN 85 molar ratio was determined as 5.5:1.0. This system is able to give high extraction at pH lower than pl. A minimum amount of KCl is found needed for best extraction. After optimisation, the forward extraction efficiency can go up to 95.54%.

The effects of aqueous phase pH, alcohol addition, KCl concentration, and extraction time on backward extraction of amoxicillin using AOT/TWEEN 85 reverse micelle were also investigated. High recovery can be obtained at pH higher than pl of amoxicillin
whereas no recovery was observed at pH lower than pl. Addition of hexanol enhances the backward extraction efficiency even at pH less than pl. However, white precipitates formed at pH less than pl indicating loss of amoxicillin. Increasing KCl concentration is able to enhance the recovery up to 93.68%, then reducing the recovery with further increase of KCl concentration. Increasing mixing time enhanced back transfer due to longer contact time between two phases but excessive contact time may causes the recovery to drop. Extraction of amoxicillin from AOT/TWEEN 85 reverse micelle was shown to be viable. Mixed reverse micellar system is able to give higher recovery compared to AOT only.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfa.2014.03.107.

References

[1] Q. Pei, Y. Guo-Ping, L. Zuo-Jun, P. Xiang-Dong, F. Jing-Hui, L. Zhao-Qian, Simultaneous analysis of amoxicillin and sulbactam in human plasma by HPLC-DAD for assessment of bioequivalence, J. Chromatogr. B 879 (2011) 2000–2004.
[2] F. Mosiman, P. Cornu, Z. N’Ziya, Amoxicillin/clavulanic acid prophylaxis in elective colorectal surgery: a prospective randomized trial, J. Hosp. Infect. 37 (1997) 55–64.
[3] I. Alemzadeh, G. Borghesi, L. Vafi, R. Roostazad, Enzymatic synthesis of amoxicillin with immobilized Penicillin G Acylase, Chem. Chem. Eng. 17 (1) (2010) 106–113.
[4] P.N. Ezhilarasi, P. Karthik, N. Chhanwal, C. Anandharamakrishnan, Nanocapsulation techniques for food bioactive components: a review, Food Bioprocess Technol. 6 (3) (2012) 628–647.
[5] S.A. Moore, R.M. Palepu, Fluorometric investigations on the transition from reverse micelles to microemulsions in non-aqueous microemulsions, J. Mol. Liq. 135 (2007) 123–127.
[6] S.R. Patil, N. Buchavzov, E. Careya, C. Stubenrauch, Binary mixtures of b--dodecylmaltoside (B-C12E2) with cationic and non-ionic surfactants: micelle and surface compositions, Soft Matter 4 (2008) 840–848.
[7] K. Tonova, Z. Lazarova, Inversed micelle solvents as tools of enzyme purification and enzyme-catalyzed conversion, Biotechnol. Adv. 26 (2008) 516–532.
[8] S.H. Mohd-Setapar, S.N. Mohamad-Aziz, Backward extraction of Penicillin G using AOT reverse micelles, Adv. Sci. Lett. 19 (12) (2012) 3688–3694.
[9] R.P. Gaikwari, S.A. Wagh, B.D. Kulkarni, Efficient lipase purification using reverse micellar extraction, Bioresour. Technol. 108 (2012) 224–230.
[10] A.B. Hemavath, H.U. Hebbar, K.S.M.S. Raghavarao, Mixed reverse micellar systems for extraction and purification of β-glucosidase, Sep. Purif. Technol. 71 (2) (2010) 263–268.
[11] S. Chatterjee, R.K. Mitra, B.K. Paul, S.C. Bhattacharya, Interface of AOT/Brij mixed reverse micellar systems: conductometric and spectrophotometric investigations, J. Colloid Interface Sci. 298 (2006) 935–941.
[12] R.K. Mitra, B.K. Paul, Effect of NaCl and temperature on the water solubilization behavior of AOT/nonionics mixed reverse micellar systems stabilized in IPM oil, Colloids Surf., A: Physicochem. Eng. Aspects 255 (2005) 165–180.
[13] K. Naoe, M. Murata, C. Ono, M. Kawagoe, M. Imai, Efficacy of guanidium salts in protein recovery from reverse micellar organic media, Biochem. Eng. J. 10 (2002) 137–142.
[14] M. Vassudevan, J.M. Wienecek, Mechanism of the extraction of protein into Tween 85 nonionic microemulsions, Ind. Eng. Chem. Res. 35 (1996) 1085–1089.
[15] M.J. Hossain, T. Takeyama, Y. Hayashi, T. Kawanishi, N. Shimizu, R. Nakanura, Enzymatic activity of chromobacterium viscosum lipase in an AOT/tween 85 mixed reverse micellar system, J. Chem. Technol. Biotechnol. 74 (1999) 423–428.
[16] R.P. Gaikwari, S.A. Wagh, B.D. Kulkarni, Extraction and purification of tannase by reverse micella system, Sep. Purif. Technol. 89 (2012) 288–296.
[17] C.A. Ayala, B.J. Kamath, A.J. Bechman, A.J. Russel, Protein extraction and activity in reverse micelles of a nonionic detergent, Biotechnol. Bioeng. 39 (1992) 806–814.
[18] N. Pfammatter, A. Hochkoeppler, P.L. Luisi, Solubilization and growth of candida pseudotropicalis in water-in-oil microemulsions, Biotechnol. Bioeng. 40 (1992) 67–172.
[19] Y. Moroi, Micelles: Theoretical and Applied Aspects, Plenum, New York, 1992.
[20] H.U. Hebbar, A.B. Hemavath, B. Sumana, K.S.M.S. Raghavarao, Reverse micellar extraction of bromelain from pineapple (Ananascomosus L. Merryl) waste: scale-up, reverse micelles characterization and mass transfer studies, Sep. Sci. Technol. 46 (2011) 1656–1664.
[21] S.H. Mohd-Setapar, R.J. Wakeman, E.S. Tarleton, Penicillin G solubilisation into AOT reverse micelles. Chem. Eng. Res. Des. 87 (2009) 842–883.
[22] S. Feng, N. Shan, K.J. Carpenter, Crystallization of amoxicillin trihydrate in the presence of degradation products, Org. Proc. Res. Dev. 10 (2006) 1212–1218.
[23] X. Zhao, Y. Li, X. He, N. Zhong, Z. Xu, L. Yang, Study of the factors affecting the extraction of soybean protein by reverse micelles, Mol. Biol. Rep. 37 (2010) 669–675.
[24] F.A. Hasman, D.V. Cortez, D.B. Gurpilhares, V.C. Santos, L.C. Roberto, A. Pessoa-Júnior, Response surface methodology for the evaluation of glucose-6-phosphate dehydrogenase enrichment process by soybean lecithin reversed micelles, J. Chromatogr. B 847 (2007) 262–266.