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REVERSE MIC EXTRACTION OF ANTIBIOTICS

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Abstract. Many biomol as proteins, enzymes and metal ions could be solubilised effectively into the reverse n anic solvents. Therefore, this opens the possibility of using the existing liquid-liquid extra nology and utilizing reverse micelles for large-scale extraction in downstream processing. ere has been very limited research on the use of reverse micelle for antibiotics extracti n attempt was made to use dioleylphosphoric acid (DOLPA) reverse micelle to extract a It was found that pH, surfactant concentration, as well as antibiotic types and concentration have a significant effect on the solubilizing capacity of antibiotics in the DOLPA reverse micelles system. At optimum conditions, about 63 % of penicillin G could be extracted during forward extraction, and 54 % in backward extraction.

Keywords: Reverse micelles; antibiotics; extraction

Abstrak. Kebanyakan biomolekul seperti protin, enzim dan ion-ion besi boleh dilarutkan secara efektif ke dalam pelarut organik misel balikan. Ini membuka peluang untuk menggunakan teknologi pengekstrakan cecair-cecair yang sedia ada dengan menggunakan misel balikan untuk pengekstrakan berskala besar di dalam pemprosesan hiliran. Setakat ini, kajian yang dijalankan ke atas pengekstrakan antibiotik menggunakan misel balikan adalah terhad. Percubaan untuk mengekstrak antibiotik menggunakan asid dioleylphosphoric (DOLPA) telah dijalankan. Didapati faktor-faktor seperti pH, kepekatan surfaktan, dan kepekatan dan jenis antibiotik memberikan kesan yang penting ke atas kebolehlarutan antibiotik di dalam sistem misel balikan menggunakan DOLPA. Pada keadaan optimum, 63% penicillin G telah diekstrak sepanjang pengekstrakan terus dan 54% sepanjang pengekstrakan berbalik.

Kata kunci: Misel balikan; antibiotik; pengekstrakan

1.0 INTRODUCTION

In today's pharmaceutical industry, one of the most important production technologies is regarding the production of antibiotics [1]. Recently there has been a tremendous increase in the volumetric yield of fermenters and the production of biochemical

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such as antibiotics in large quantities by using cell culture technology. Continuous processes which can be scaled up for the separation of these diluted biological product are still of major concern in biotechnology [2]. The separation of bioproducts from a fermentation broth is troublesome because of their dilute concentrations compared with usual chemical process as a number of impurities in the broth [3]. Improvements in downstream processing are necessary for biotechnological researches to achieve their goals [4]. In biotechnology there is a need for new purification and concentration processes for biologically active compounds such as proteins, enzymes, nucleic acids, antibiotics, or cells that combine a high selectivity and biocompatibility with an easy scale up [5].

A liquid-liquid extraction with a reversed micellar phase might serve these purposes owing to its capacity to solubilize specific biomolecules from dilute aqueous solutions such as fermentation and cell culture media [6]. Liquid-liquid extraction constitutes an attractive alternative for bioseparations since it is well known technique, which is readily scaleable and can be operated in a continuous basis [2]. Therefore, reverse micelles can be applied as the organic solvent system for the solubilization and recovery of hydrophilic biomolecules, showing good selectivity with little denaturation or loss of biological activity [7]. The mechanism of this extraction process involves direct interaction between the reverse micelles generated by the surfactant in the organic phase and the biomolecule in the aqueous phase. In an ordinary liquid-liquid extraction, although it had been difficult to extract large bioactive molecules such as hemoglobin and plasmid, the limitation was overcome by utilizing a nanostructural molecular assembly like reversed micelles [8].

In the past decades, reverse micelles have attracted much attention as novel method for separating and purifying many biological products because reverse micelles provide a special microenvironment in the organic medium [9]. Furthermore, because the separation process using reversed micelles is based on an ordinary liquid-liquid extraction technique, it is easy to scale up and operate continuously. Reverse micelles can be referring to as a nanometer-scale droplet of an aqueous solution, stabilized in an apolar environment by the presence of surfactant interface [10]. The aggregates of surfactant molecules contain an inner core of water molecules and are dispersed in a continuous organic solvent medium. Reverse micelles are used in a tremendous number of applications such as enhanced oil recovery, separation schemes such as liquid-liquid extraction processes, phase transfer catalysis, and various types of chemical reactions [11].

Reverse micelles have already been demonstrated to be capable of hosting large quantities of biomolecules such as protein, hemoglobin and plasmids without causing denaturing by adjusting operational parameters [8]. Thus, reverse micelles are viewed as nanometer-scale microreactors. A substantial amount of work has been aimed at understanding the stabilization of the oil-water interface by the use of surfactant molecules. Indeed, in most studies of extraction by reverse micelles, the conventional

surfactant sodium bis(2-ethylhexyl) sulfosuccinate (AOT) has been used and little attention has been paid to the problem of surfactant optimization [12]. Although a novel reverse micellar system, which is formed by AOT, was reported, a better surfactant system was still required to enhance the extraction efficiency. Consequently, the dioleyl phosphoric acid (DOLPA) reverse micelle system was found to have an excellent ability to extract many biomolecules superior than AOT system [13]. Diolyel phosphoric acid (DOLPA), which has two long alkyl chains in the hydrophobic moiety, and divinylbenzene were employed as a functional host and a cross linking agent, respectively [14]. Goto *et al*. [12] recently developed a novel DOLPA surfactant, which appears to be one of the best surfactants currently available for protein extraction using reversed micelles.

2.0 MATERIALS AND METHODS

2.1 Materials

All salts for aqueous and buffer solutions preparation obtained from E. Merck and used as received. The phosphate and carbonate buffers were used to adjust the aqueous solution pH.

An analytical grade isooctane obtained from Sigma Chemical Co. was used as solvent. Deionized water, obtained from Millipore filtration unit with an electrical conductivity below 0.8 μS/cm was used for aqueous solution preparation. The anionic surfactant, dioleylphosphoric acid (DOLPA) was used as surfactant in this study. The antibiotics used were penicillin G, ampicillin and streptomycin obtained from Sigma Chemical Co., while teicoplanin and rifampicin were donated by Professor P. L. Cen, Institute of Bioengineering, Zhejiang University, Hangzhou, China. Penicillin G was used as the main antibiotic, while the others were used as comparative antibiotics in this study.

2.2 Extraction Procedures

The antibiotic solution was prepared by dissolving antibiotic in 0.1 M salt solutions. The pH of antibiotic solution was adjusted to require pH by adding 20 % 0.1 M either phosphate or carbonate buffer solution. The forward extraction experiment was performed by mixing 5 ml antibiotic solution of the desired pH with an equal volume of reverse micelle solution in a 20 ml bottle, at the speed of 400 rpm for 15 minutes. The dispersion was then centrifuged at 2000 rpm for 15 minutes to obtain a distinct phase boundary and the separated phases were assayed for antibiotic concentration using absorption measurement. The pH before and after mixing with the reverse micelle solution were measured and expressed as initial pH and equilibrium pH, respectively. The antibiotic concentration in the organic phase was determined using mass balance. The percentage of extraction (% E) is used to represent

the transfer efficiency of antibiotics from the aqueous phase to the reverse micelle phase, which is defined as the ratio of the amount of antibiotics extracted into the reverse micelle phase to the total amount of the antibiotics present initially.

The backward extraction process was conducted by contacting the antibiotic loaded reverse micelle solution with an equal volume of aqueous stripping solution having desired pH at 20 minutes mixing time. For stripping study, penicillin G was used as the model antibiotic. The antibiotic concentration was determined using absorption measurement at 257 nm for penicillin G. The antibiotic concentration in the reverse micelles phase was determined also using mass balance. The transfer efficiency (% E) of backward transfer is defined as the ratio of the amount of antibiotics extracted into the buffered aqueous phase to the initial amount of antibiotics loaded in reverse micelle phase from the forward transfer.

Antibiotics concentration were assayed by measuring absorbance at 257 nm (penicillin G), 208 nm (streptomycin), 215 nm (ampicillin), 240 nm (teicoplanin) and 240 nm (rifampicin) using an UV-Spectrophotometer (Shimadzu Co.). The water content of the reverse micelle phase was determined by using Karl Fischer titration using a Metrohm 756 KF Coulometer. All the experiments were carried out at 25.0±1.0 °C.

3.0 RESULTS AND DISCUSSION

3.1 Forward Extraction

3.1.1 Effect of pH

Figure 1 shows the effect of pH on penicillin G extraction from penicillin solution made of various salts. The degree of extraction for NaCl is significantly higher than the other two due to the fact that the NaCl equilibrium constant for the formation of the complex is higher than that of the other two salts [15], which allows a better interaction between antibiotic and micelles head group. The highest percentage of penicillin G extraction was achieved at pH around 7 in general. However, it was found that at low pH, there is no extraction of penicillin G into the organic phase. This was confirmed by the findings from water content study, which showed that there was no formation of reverse micelles at pH lower than 5. However, the degree of extraction also depends on the types of the antibiotics used. For penicillin G, the extraction increases at higher pH, which might be due to the extraction of penicillin G by DOLPA reverse micelles.

It is postulated that at lower pH, DOLPA exits as a monomer of undissociated form. It does not adsorb at interface and hence there is no lowering of interfacial tension to form reverse micelles due to high concentration of hydrogen ions in the aqueous solution. At medium pH range, some DOLPA molecules dissociate to

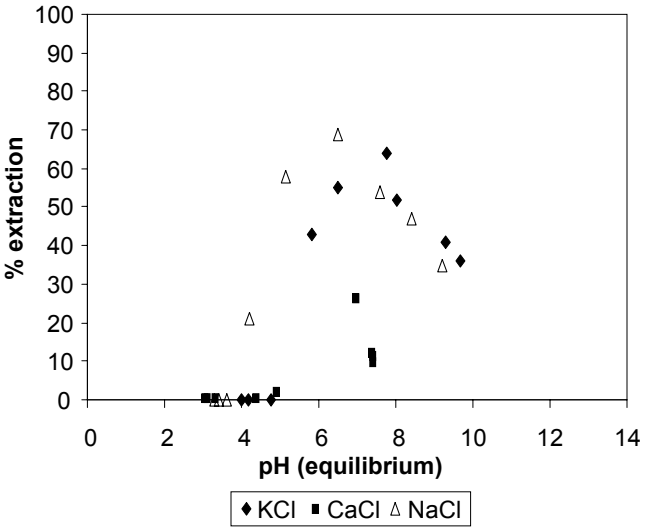

Figure 1 Effect of pH on antibiotic extraction

form anions, which adsorb at the interface and reduce the interfacial tension. However, the interfacial tension is not low enough to form clear and thermodynamically stable reverse micelles solution due to the lack of DOLPA ions adsorb at the interface. At higher pH, the water solubilisation reaches saturation indicating that all molecules dissociate (exist as anions) and form reverse micelles.

The extraction at higher pH can be postulated as a result of hydrophobic interaction between non-polar group surfactant and antibiotics through the formation of the hydrogen bonding. In conventional penicillin G extraction, penicillin G is found to be soluble and extracted by polar solvent like butyl acetate at lower pH in undissociated form. The solubility of penicillin G decreases with increasing pH owing to the dissociation of penicillin G. The formation of hydrophobic complex of the antibiotic and DOLPA molecules makes the antibiotics possible to be solubilised into the organic phase since the solubility of the antibiotics in pure isooctane is zero even at low pH. While the results point to the ionic interactions as being the main controlling factor in the extraction of penicillin G in the reverse micelles, it is also interesting to note that there are other phenomena which also come to play. Among the questions that have been raised is how precisely the penicillin G molecule is partitioned within the reverse micelles. It is also uncertain that the antibiotic molecule is contained within the polar core of reversed micelles. According to Hatton [18], it is possible that extraction occurs via simple ion pairing by the positively charged antibiotic molecule and the charged surfactant head groups. At higher pH, the decrease in penicillin G extraction might be due to denaturation of penicillin G.

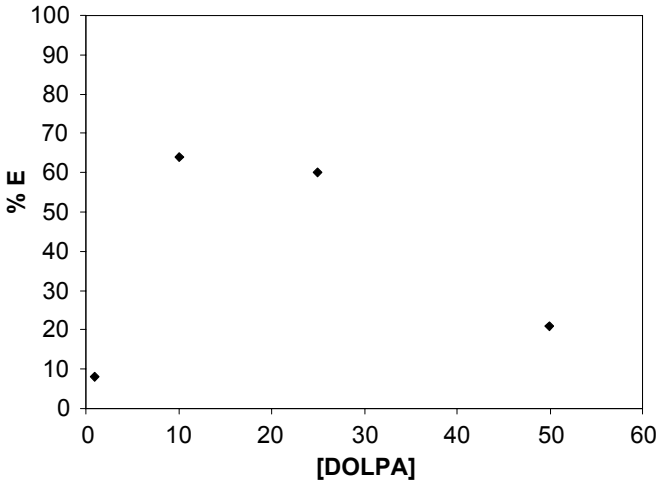

Figure 2 Effect of surfactant concentration on antibiotic extraction

3.2 Effect of Surfactant Concentration

Increase in the surfactant concentration caused several interesting phenomena to occur as shown in Figure 2. An increase in the surfactant concentration increases the number of reverse micelles available for the extraction of penicillin G. This increases the capacity of the reverse micelle to solubilize more solute molecules. This effect was noticed for the extraction of protein [16, 17] and antibodies [18] in the reverse micelle phase. However, as for penicillin G extraction using the DOLPA reverse micelle system, a different result was noticed. The experimental investigations show that there is an optimal level of surfactant concentration where the maximal extraction could be obtained. The maximal extraction of penicillin G occurs when the surfactant concentration is 10 mM. Increasing the surfactant concentration above 10 mM decreases the maximal extraction of penicillin G into the reverse micelle phase. When surfactant concentration above 10 mM was adopted, a large amount of precipitate was observed at the interface and this suggests that the DOLPA might form a complex with the penicillin G molecules at the interface. Therefore, it leads to a decrease in the extraction of penicillin G into the reverse micelle phase. One of the possible reasons for this effect could be that by using DOLPA concentration of above 10 mM, the reverse micelle system becomes saturated with DOLPA. This leads to the formation of a complex with penicillin G molecules when the extraction is carried out.

3.3 Effect of Antibiotic Types and Concentration

Figure 3 shows the percentage of extraction for five antibiotics from different antibiotic groups and molecule structures. Rifampicin exhibited a very high degree of extraction

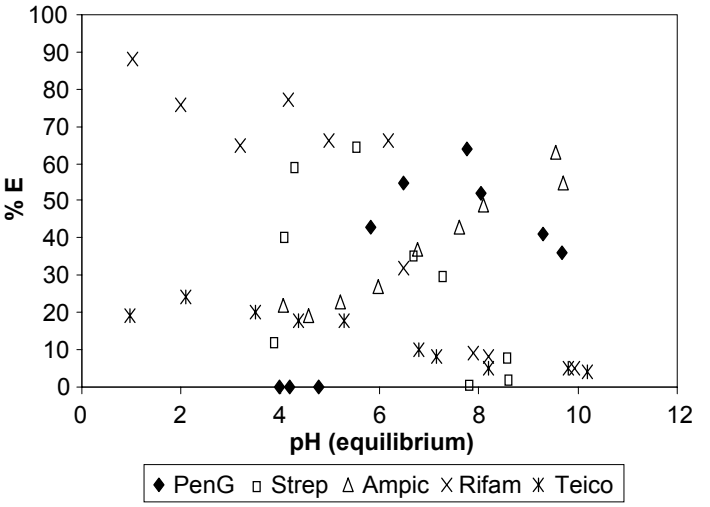

Figure 3 Effect of antibiotic types on antibiotics extraction micelles

using DOLPA at low pH, while penicillin G, streptomycin and ampicillin were found moderately extractable by using the same reverse micelle system. All the antibiotics employed in this study have high solubility in most organic solvents except teicoplanin. Teicoplanin is relatively insoluble in non-polar/organic solvents [19]. Figure 3 also shows that there is no penicillin G extraction at pH<5. Besides the factor that DOLPA exists as a monomer of undissociated form and does not adsorb at the interface at lower pH, it is important to consider the effect of antibiotic types in reverse micelle extraction. Penicillin G is a weak acid. It is extremely unstable and tends to decompose in low pH range and ambient temperature [20]. This answers the question why certain antibiotics could not be extracted at low pH and high temperature.

As previously discussed on pH effect, the degree of extraction depends strongly on the types of antibiotics and pH. For penicillin G, streptomycin and ampicillin, a sharp increase in the extent of extraction at higher pH is most probably due to the solubilisation of antibiotics by DOLPA reverse micelles. However, for penicillin G and streptomycin, there is an optimum pH that can give the highest extraction efficiency. For example, the optimum pH for penicillin G extraction is pH 7. At pH greater than 7, the extraction decreases (Figure 4). It was found that the maximal extraction of antibiotic was obtained at initial antibiotic concentration of 5 mM for penicillin G, streptomycin and ampicillin. An increase of antibiotic concentration above 5 mM reduced the extent of extraction. One of the possible explanations for this phenomenon is that the reverse micelle system becomes saturated when the antibiotic concentration is increased to above 5 mM. Thus, further increase in the antibiotic concentration would only reduce the extraction of antibiotics into the reverse micelle phase.

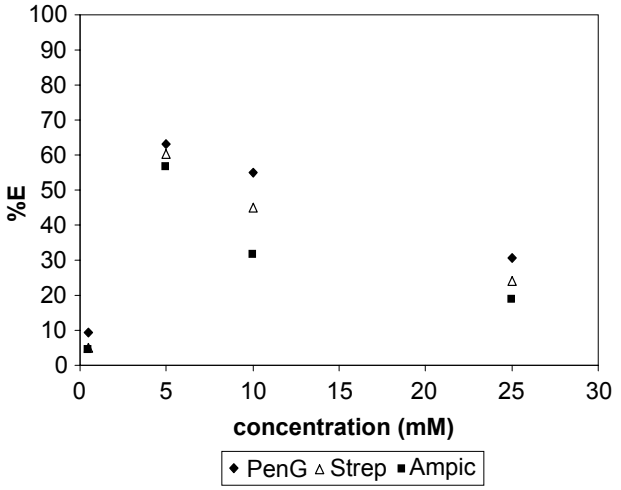

Figure 4 Effect of antibiotic concentration on antibiotics extraction

3.4 Backward Extraction

3.4.1 Effect of pH

Figure 5 shows the extent of penicillin G backward extraction from the loaded penicillin G reverse micelle system at various pHs using 10 M KCl. It could be observed that the optimum backward extraction was found at pH 3. The degree of backward extraction decreased sharply as the backward extraction pH was increased further. At lower pH region, the DOLPA reverse micelles start to beak-up and exist as monomer. Consequently the antibiotics solubilised in DOLPA reverse micelles

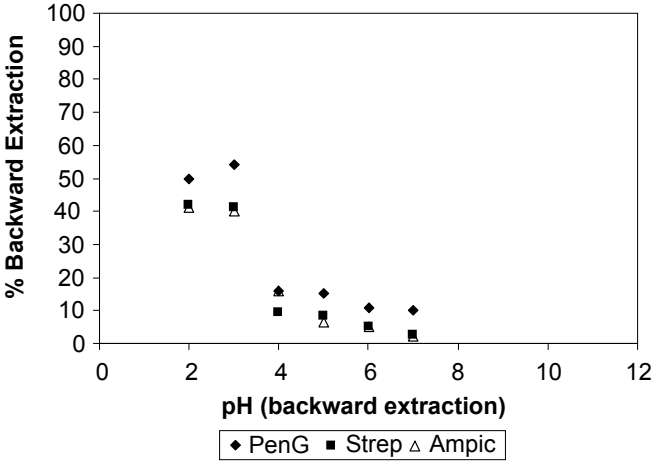

Figure 5 Effect of initial backward aqueous phase pH on backward extraction of antibiotics

can be released into 1.0 M KCl buffered solution. Decrease in backward extraction efficiency at higher pH might be due to the high solubility of antibiotics in reverse micelle phase.

3.4.2 Effect of Surfactant Concentration

From the literature, it is well known that the backward extraction process using conventional surfactant like AOT is relatively difficult although at high ionic strength [9]. Figure 6 shows the effect of DOLPA concentration on the backward extraction of penicillin G. The results indicate that the highest degree of backward extraction was obtained at DOLPA concentration between 10 to 25 mM. At the concentration

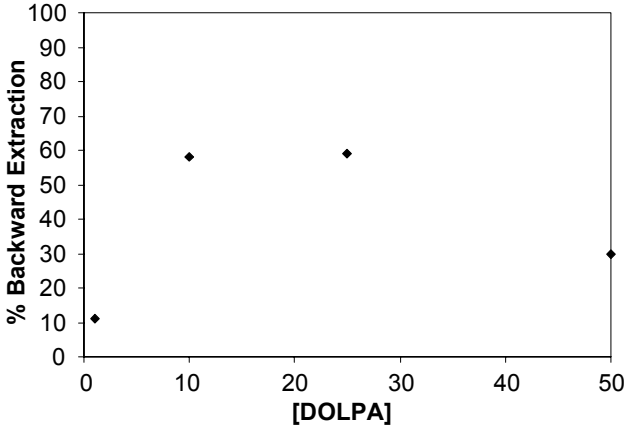

Figure 6 Effect of surfactant concentration on backward extraction of antibiotics

above 25 mM, the backward extraction of antibiotics decreases dramatically. As discussed in the forward extraction, decreased backward extraction might largely due to the saturated reverse micelle system when surfactant concentration is very high. This is followed by the formation of a complex with penicillin G molecules, which reduces the backward extraction efficiency.

3.4.3 Effect of Antibiotic Types and Concentration

The effect of antibiotic concentration on the backward extraction of penicillin G and streptomycin is shown in Figure 7. It was found that the degree of backward extraction of penicillin G is higher than streptomycin. It was also found that the effect of antibiotic concentration on the backward extraction process depends on the initial antibiotic concentration that could be extracted into the reverse micelle phase by the forward extraction process. An increase in the initial antibiotic concentration reduced the backward extraction efficiency as a result of reduced capacity of the backward aqueous

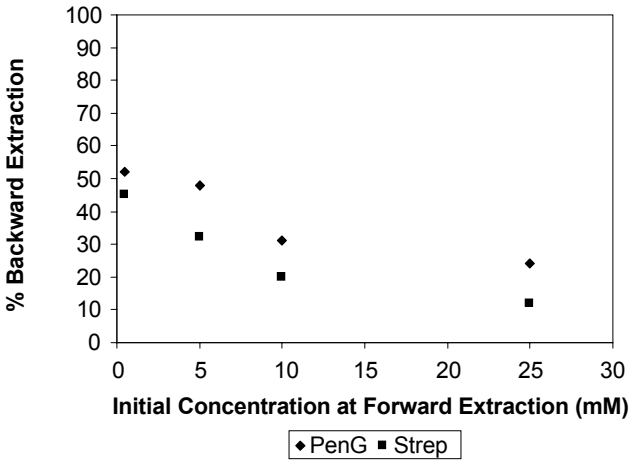

Figure 7 Effect of antibiotic concentration on the extent of backward extraction

phase to recover the increasing amount of antibiotics solubilised in reverse micelles. As the forward extraction, the types of the antibiotic also affect the extent of backward extraction of antibiotics.

4.0 CONCLUSION

The equilibrium studies on reverse micelle extraction of antibiotics were carried out for forward extraction and backward extraction. Results show that the highest forward and backward extractions that could be achieved are 63% and 54% respectively. It was found that there is an optimal DOLPA concentration for maximal forward extraction. Rifampicin exhibited a very high degree of extraction using DOLPA as surfactant at low pH, while penicillin G, streptomycin and ampicillin were found moderately extractable by using the same reverse micelle system at higher pH. It may suggest that the attractive electrostatic and hydrophobic interactions could be responsible for the reverse micelle extraction of antibiotics.

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