Review Article

REVERSE MICELLES AS A BIOSEPARATION TOOL FOR ENZYMES

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Abstract: Liquid-liquid extraction is one of the most widely used techniques in various sectors of the biotechnology industries. This technique has found limited application in particular for the separation, concentration and purification of enzymes. The main reason for this is the lack of appropriate solvents with the desired selectivity and the solvents which does not provide the biocompatible environment for the enzymes of interest. However, the organic solvents do not satisfy the selectivity or the preservation of the enzyme activity, structure and its functional properties. Therefore, this necessitates the development of efficient and inexpensive technique, to obtain the high activity, purity and recovery of the enzymes. Reverse micellar technique was found to be safe media in biotechnology industry, because the reverse micellar core avoids the contact between enzymes and solvents. As well as this technique provides compatible surroundings, selectivity, and safeguard the functional properties, structure and activity of the enzymes. In this review we described the concept of reverse micelles, there compositions and the steps involved in extraction of enzymes using reverse micelles method. Also, it highlights the current progress/recent developments in reverse micellar extraction for the improvement of technique by exploiting the reverse micelle phenomenon.

Keywords: Liquid-liquid extraction; reverse micelles; downstream processing; enzymes; liquid emulsion membrane.

Note: Coloured Figures available on Journal Website in "Archives" Section

Introduction

Enzymes are proteins which perform their catalytic function by reducing the energy barrier of the biochemical reactions. Enzymes have unique properties like high turnover number, operates under mild reaction conditions, high biodegradability (Tania *et al.*, 2014; Iyer *et al.*, 2003), high selectivity etc. which makes it attractive for

industrial applications. Enzymes have their own significance inside and outside of the cell, within the cellular spaces like interior of macromolecular chaperones, extracellular spaces and the membranous organelles, as well as enzymes exert their function in many important biochemical reactions (Yeung *et al.*, 2013). Most of the industries carry out the downstream processing of various extracellular enzymes by the use of different conventional techniques such as column chromatography, electrophoresis, salt and solvent precipitation. However, these techniques have not gained industrial recognition due to several reasons like denaturing tendency of the enzymes by the

usage of organic solvents, enzymes poor solubility in the organic solvents and poses substantial problems for scale-up process. In view of the above facts there is need for the development of efficient and economical downstream processing technique, to achieve high purity, activity and recovery of the enzymes.

Reverse micellar extraction (RME) technique is being considered as an alternative for the other available conventional downstream processing techniques (Table 1). The most promising potential of the reverse micelles is separation and/or purification of proteins/enzymes rather achieved in two rapid and simple steps with high degree of activity (Tonova *et al.*, 2008). To achieve the same purpose, the other purification procedure requires more elaborate steps (Liu *et al.*, 2006). The studies have evidenced that, not only enzymes can be extracted by reverse micelles, other biomaterials like organelles (Hochkoeppler *et al.*, 1989), entire cells (Cinelli *et al.*, 2006) and even large biopolymers have been also solubilized into the reverse micelles (Cheng *et al.*, 2014). However, this review highlights only the application of reverse micellar technique for the extraction of enzymes.

Reverse micelles are nanometer sized droplets of aqueous phase stabilized by surfactants in an organic phase. Reverse micellar systems are developed with the use of different organic and aqueous phase and surfactants. Reverse micellar extraction offers unique features such as large interfacial area, less energy requirement, single-stage and continuous mode operation, low cost factors and easy scale up (Krishna *et al.*, 2002). A central challenge of reverse micellar technique is to understand the mechanism involved in the extraction of desired enzymes and the influence of different parameters on enzymes activity, yield and recovery. In this context, an attempt was made to present a review on reverse micelles as a bioseparation tool for enzymes that focuses mainly on the fundamentals of reverse micelle formation, mechanism of RME, the various process parameters that influence the extraction efficiency and some of the current developments in reverse micellar systems.

Surfactants: micelles, reverse micelles and microemulsions

Surfactants are amphiphilic molecules in nature consists of a hydrophilic head and hydrophobic tail.

In the other terms, a surfactant is made up of non-polar group and polar (or ionic) group. According to the nature of the polar and non-polar group, surfactants are categorized into anionic, cationic, zwittterionic and nonionic chemical structures. These surfactants have the ability to form micelles in aqueous solutions and forming reverse micelles in organic solvents (George *et al.*, 2010; Lundberg *et al.*, 2005).

The term "micelle" was coined by J.W. McBain in 1913 to propose the concept of aggregates formation of amphiphilic molecules in aqueous solutions, and this concept was further developed by H.F. Eicke (1982). The structure of micelles (Figure 1a) includes the hydrophilic heads which are oriented towards the dispersing water, while the hydrophobic tails arrange themselves in an interior region forming a micellar core, this micellar core can dissolve substances which can disperse in a polar solvent (Uskokovic *et al.*, 2007). Langevin *et al.* (1992) described that the micelles are formed above the critical micelle concentration (CMC). CMC is the minimum surfactant concentration required for micelle to form and it represents the concentration of free or un-aggregated surfactant molecules in equilibrium with the micelle. The CMC can be obtained by measuring the physicochemical properties such as refractive index, electric conductivity, x-ray diffraction, turbidity, osmotic pressure etc. As CMC determines the characteristic of the micellar system, it is dependent on several factors such as surfactant chemical structure, solvent, pressure & temperature. If the surfactant value is below CMC, then the formation of micelle will get inhibited and the surfactants molecules can only exist as free molecules in the solution.

Formation of reverse micelles requires the dissolution of immiscible organic phase containing surfactants and a polar solvent. The hydrophobic tail surfactant molecules arrange themselves in a polar solvent whereas, hydrophilic head form a water pool/ hydrophilic core, this water pool is protected from direct contact with the organic solvent and can dissolve soluble enzymes/ proteins from the bulk aqueous phase (Figure 1b). Besides enzymes/proteins other biomaterials such as DNA, organelles (ex. Mitochondria) and entire cells have been also solubilized into this water pool. Reverse micelles represent a definite diameter and molecular weight which exhibits relatively ordered structure. Characteristics of reverse micellar

systems comprise of viscosity related to pure organic solvents, spontaneous formation, thermodynamic stability, large surface area and capability to dissolve polar substances (Budker *et al.*, 2002; Ghosh *et al.*, 2004).

The microemulsion/liquid emulsion membranes are made up of three components namely oil/organic solvent, an aqueous phase/water and the surfactant. At times the fourth component co-surfactant is added. There exist 2 types of microemulsions, one is the water in oil (w/o) emulsions, characterized by water droplets dispersed in an oil phase (reverse micelles) (Figure 1c), and the other type is oil in water emulsion (o/w), characterized by oil droplets (reverse micelles) dispersed in a water phase (Fryd *et al.*, 2012). The major distinction involved in the formulation of micelles and microemulsions is the number of components present in each of them, as well as the micelles are represented as a binary system and the microemulsions are characterized as ternary or higher order systems. In several reports, the term microemulsions were referred as reverse micelles (Stamatis *et al.*, 1999). Figure 1 represents the three micellar structures such as micelles, reverse micelles and micoemulsions.

Reverse micelle composition

The formation of reverse micelles occurs by dissolving the surfactants in the organic solvents. The majority of the existing surfactants have a partial/less solubility in organic solvents, when such surfactants are used the addition of a co surfactant helps the surfactants to dissolve in organic solvents and to form appropriate reverse micelles (Costa *et al.*, 2000). Co-surfactants which are in the group of oil-soluble components having the ability to form the hydrogen bonding over and above having great attraction for surfactant is chosen (Zemb *et al.*, 2016). Such co-surfactants are chloroform, amides, long chain amines, ethoxylates, fatty acids etc. The co-surfactant reduces the repulsive ion–ion interaction between the surfactant head group in the organic phase, thus allows the close packing of surfactant head groups to form a stable hydrophilic inner core of reverse micelles (Goncalves *et al.*, 2000). The major benefit of using co-surfactants is that, the micelle's shape and diameter can be changed either to larger or to smaller values (Chen *et al.*, 2015; Liu *et al.*, 2009).

The co-surfactants is chosen for its several importance as described above likewise it has its own limitations which have been reported in some of the papers, few of them are discussed here. Co-surfactants generally interpose between the surfactant chains, which increase the interface flexibility and the interdroplet interaction (Aboofazeli *et al.*, 2000). The complexity of the phase diagrams increases with the use of co-surfactants, which makes the reversed micellar system hard to build up its physical picture and their hosted solutes (Goto *et al.*, 1997).
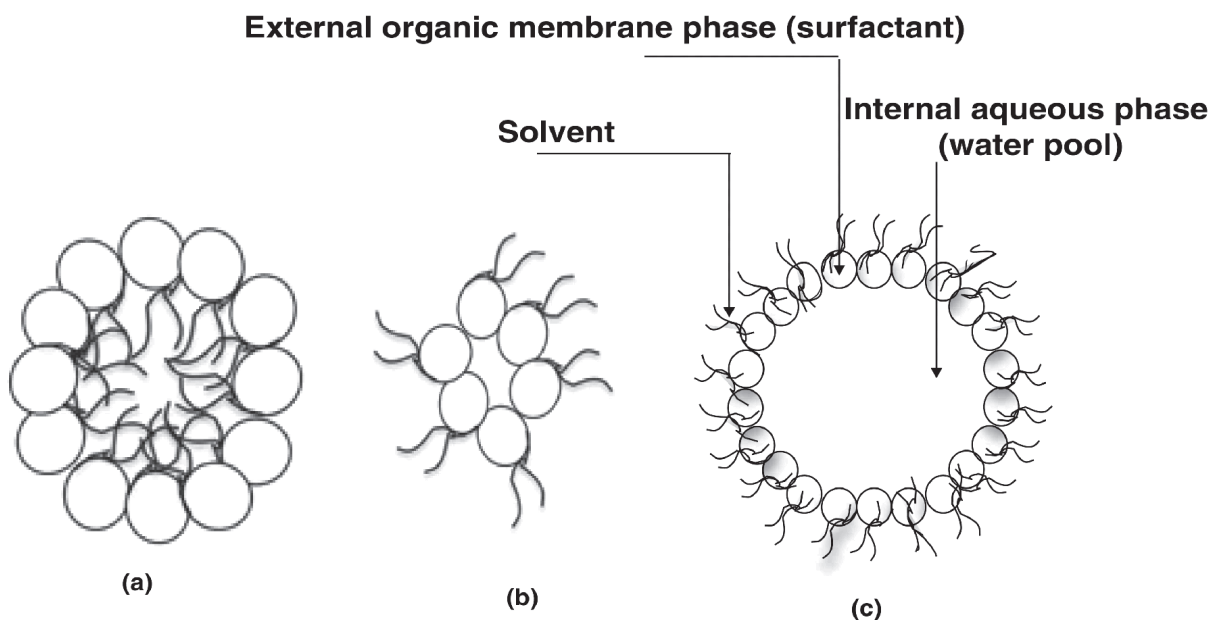
External organic membrane phase (surfactant)

Solvent

Internal aqueous phase (water pool)

(a)   (b)   (c)

Figure 1: **A schematic representation of (a) Micelles (b) Reverse Micelles (c) Water in oil Emulsion**

Several studies indicate that, not only co-surfactant are used to improve the solubility of surfactants in solvents, co-solvents are also used during the formulation of reverse micelles in order to facilitate the surfactants to dissolve in the organic solvent. The properties which determine the suitability of co-solvents are dielectric constant, molecular weight and water solubility are some of the major factors (Krishna *et al.*, 2002). Co-solvents normally used in reverse micelle formation should have low solubility in water. Some of the co-solvents which are in use are hexane, long-chain alcohols, *n*-octane, acetates and isooctane etc. Stamatis *et al.* (1999) have used *n*-octane and isooctane as co-solvents for the formation of cationic reverse micelles, the results indicates that these co-solvents do not effect on the reversed micelle size and structure (Stamatis *et al.*, 1993).

Extraction of enzymes by reverse micellar system

Reverse micelles should have two characteristic features in order to selectively extract and purify the target biomolecules. Primarily, they should be able to selectively solubilize the target biomolecules and next it should be capable to release the extracted biomolecules into an aqueous stripping phase thus it is possible to achieve the recovery of the purified biomolecules.

The reverse micelle extraction cycle is essentially composed of two processes: forward and backward extraction (Marcozzi *et al.*, 1991). The diagrammatic representation of each step in the reverse micellar extraction of enzymes is shown in Figure 2. The forward extraction involves 3 steps: reverse micelle formulation, incorporation of enzymes into reverse micelles and phase separation. The back-extraction process is carried out in rather only one step, which includes the breakage of reverse micelles to release the microencapsulated enzymes into a fresh aqueous strip solution (Nandini *et al.*, 2010).

The first step of the forward extraction is reverse micelles formulation. In order to formulate the reverse micelles, the different components like desired surfactants and solvents should be brought in contact with each other. To achieve the reverse micelle formulation, generally 2 techniques are followed, they are: (i) contact method (ii) titration method.

The second step of the forward extraction involves the incorporation of enzymes into formulated reverse micelles. There are different methods available to facilitate the transfer of enzymes into reverse micelles, they are: (i) phase transfer between bulk aqueous and surfactant-containing organic phases (ii) Injection of a concentrated aqueous solution (iii) addition of dry lyophilized protein to a reverse-micellar solution (Matzke *et al.*, 1992). Any of these three methods are used to incorporate the enzymes into reverse micelles. Whereas the mechanism by which the selective entry of enzymes into reverse micelles is governed by the various reverse micellar systems. The details of these reverse micellar systems are given in the next section. The reverse micelles composition is decided based on the selection of the reverse micellar systems.

In the third step, the mixture should result into separation of two phases by allowing the mixture to settle in a separation funnel. The upper phase consists of the enzyme entrapped in the reverse micelles whereas the denser crude extract settles down in the lower phase (Marcozzi *et al.*, 1991).

During the fourth step of back extraction, the reverse micelle enriched with enzyme in the upper phase is collected and destabilized by the addition of stripping solution to release the enzyme. Then the mixture is centrifuged, which result into upper and lower phases, upper phase is referred as the organic phase and consist of reverse micellar components like surfactants and solvents, which can be filtered and recycled. Whereas the lower phase consists of recovered enzyme, for which the activity of enzyme/protein content can be estimated. The overall experimental procedure of reverse micellar extraction is represented as flow diagram in Figure 3.

Reverse micellar systems / Mechanism of reverse micelle extraction

The protein/enzyme extraction by reverse micelles is governed by the various mechanisms/ reverses micellar systems like electrostatic interactions, electrostatic and hydrophobic interactions, hydrogen bonding interactions, affinity interactions between proteins and their affinity ligands, hydrophobic and hydrogen bonding interactions. Below is the description of these different mechanism by which enzymes are solubilised into reverse micelles. The overview of the different micellar systems is given in Table 2.
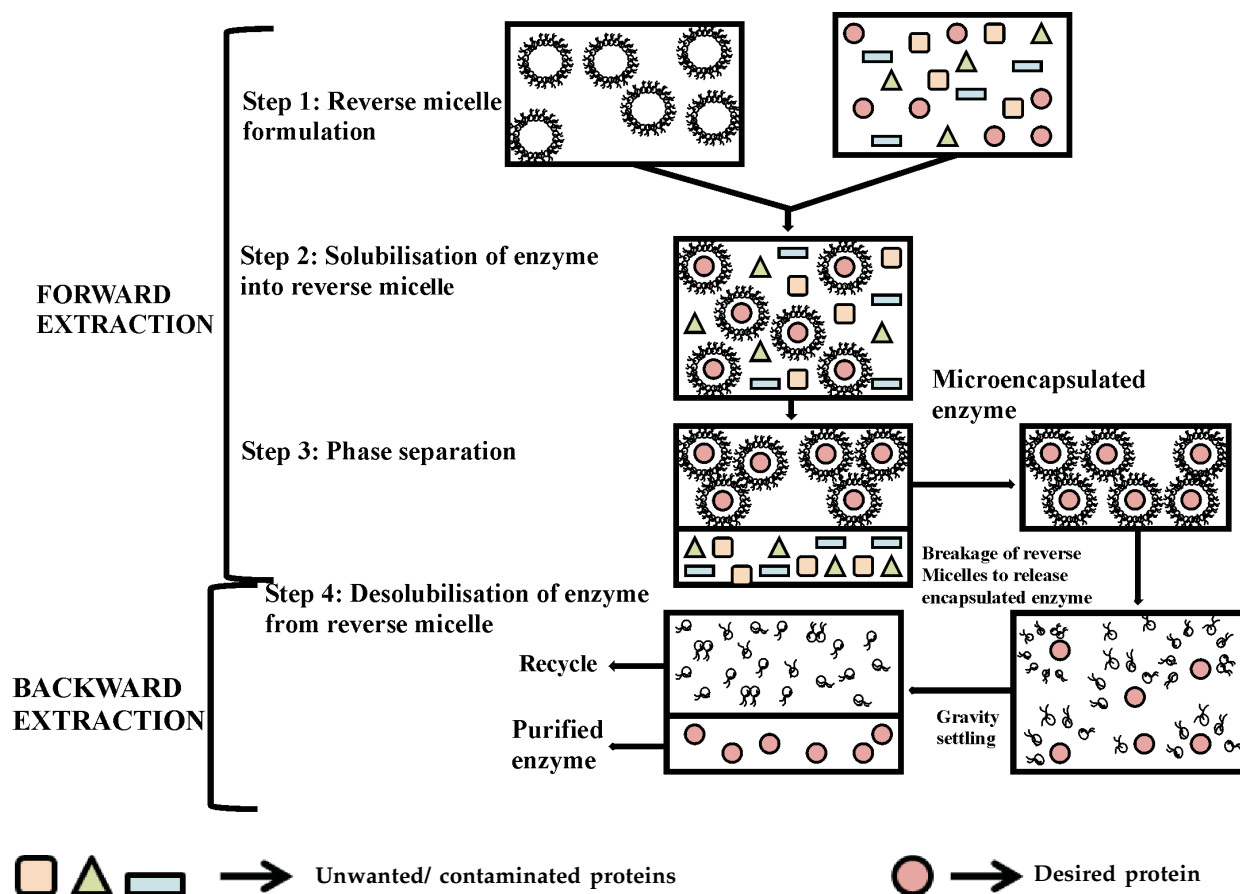
Figure 2: **A diagrammatic representation for the preparation of reverse micelles and extraction of desired enzyme from aqueous feed**

Table 1
Comparison of conventional methods and reverse micellar method for the purification of enzymes

| Enzyme	Methods	Activity recovery (%)	Purification factor (fold)	References
Lipase	Acetone precipitation (50–70% saturation)	56.89 ±0.54	2.31 ±0.07	Nandini *et al.*, 2009
	Ammonium sulphate precipitation	62.71 ± 0.88	1.62 ±0.05	
	Reverse micellar extraction	82.72 ± 0.71	4.09 ±0.04	
Bromelain	Acetone precipitation (40–80% saturation)	85.97 ± 0.53	4.90	Chaurasiya *et al.*, 2013
	Ammonium sulphate precipitation	72.0 ± 1.47	1.82	
	Reverse micellar extraction	78.90 ± 0.82	3.96	
Peroxidase	Acetone precipitation followed by chromatography	2.7%	4.6	Duarte-Vazquez *et al.*, 2007.
	Reverse micellar extraction	60%	7	
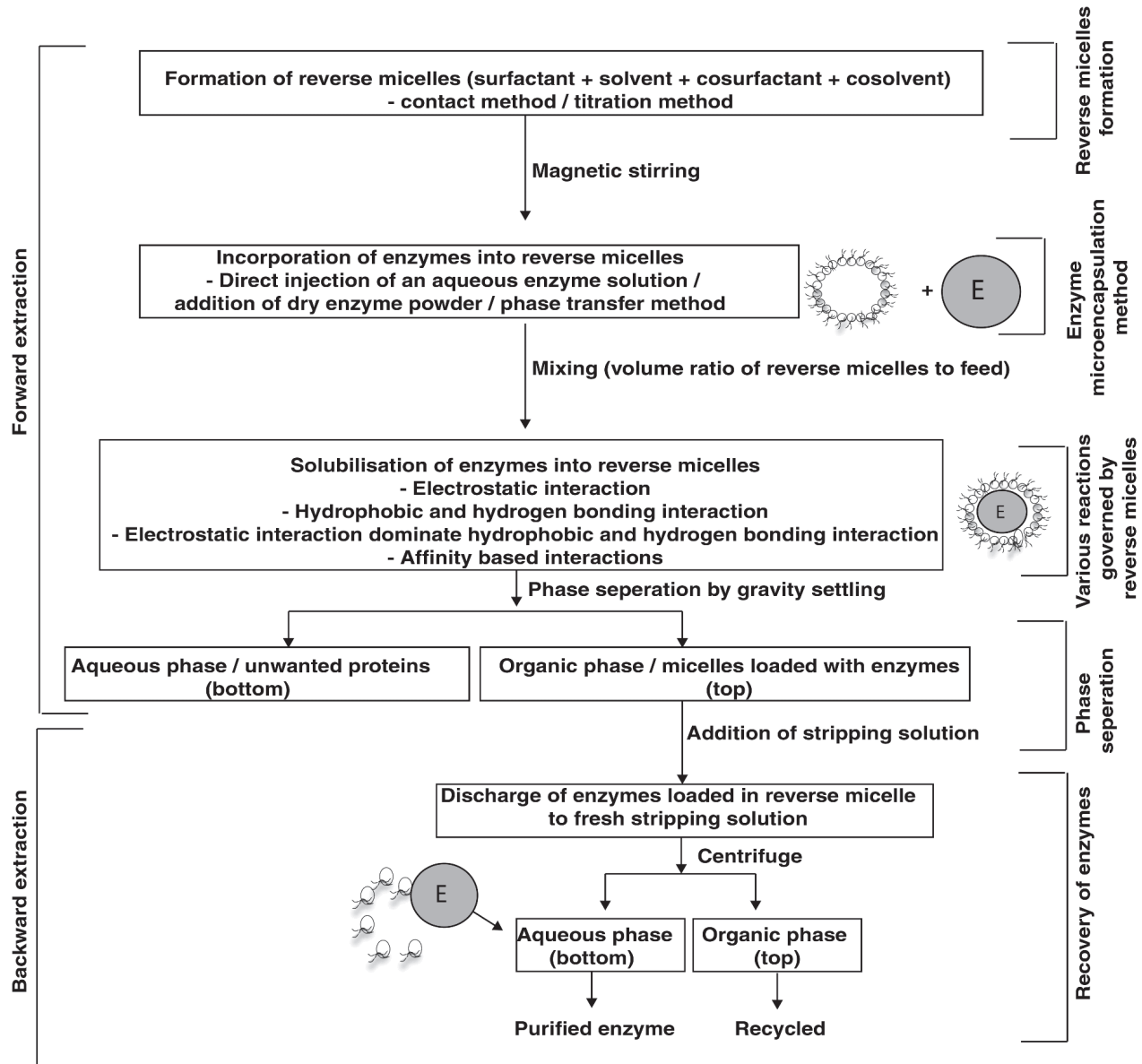
Figure 3: **A flow-sheet representing the overall experimental procedure involved in the reverse micelle extraction**

Electrostatic interaction (Ionic surfactant based reverse micelles)

Protein solubilizes in reverse micelles due to the domination of electrostatic interactions between the ionic surfactant head groups in the inner layer and the charged enzymes. The main driving force behind the electrostatic interaction is the opposite charge between the ionic surfactant head groups and the charged enzyme. Opposite charge is favored at pH values above the isoelectric point (pI) of the protein for the cationic surfactant where as in case of anionic surfactant it is favored at pH values below the isoelectric point (pI) of the protein (Vardanega *et al.*, 2014).

In the study investigated by Liu *et al.*, 2006, AOT (Sodium bis-2-ethyl-hexyl-sulfosuccinate)/ isooctane reverse micelles are applied to extract and purify nattokinase enzyme from fermentation broth. The result indicates the 80% activity recovery and 2.7 purification factor of nattokinase. Here 6.5 is the pH maintained for nattokinase enzyme extract, which is below the pI (8.6-8.7) value of nattokinase enzyme. The nattokinase enzyme carries the positive charge, since the pH value of nattokinase enzyme is maintained below its pI value. As the positive charge carrying enzyme and the anionic surfactant has the opposite charges, it creates the electrostatic interaction between the enzyme and the surfactant (Liu *et al.*, 2006). This electrostatic interaction played

Table 2
Reverse micellar systems

Reverse micellar system	Type of surfactant molecule	Major interaction forces	Example of surfactant molecules	Proteins/ enzymes purified	References
Ionic surfactant based reverse micellar system	Ionic surfactant molecules	Electrostatic interaction	Sodium bis-2-ethyl-hexyl-sulfosuccinate (AOT)	Nattokinase	Liu *et al.*, 2006
			Sodium bis-2-ethyl-hexyl-sulfosuccinate (AOT)	Cytochromec and lysozyme	Shen *et al.*, 2007
Nonionic surfactant based reverse micellar system	Nonionic surfactant molecules	Hydrophobic and hydrogen bonding interactions	Polyoxyethylene sorbitan trioleate (Tween-85) and polyoxyethylene *tert*-octylphenyl ether (TX-100)	α-chymotrypsin and subtilisin	Sawada *et al.*, 2004
Mixed reverse micellar system	Ionic and nonionic surfactant molecules	Electrostatic and hydrophobic, hydrogen bonding interactions	Cetyltrimethyl ammonium bromide (CTAB) & Polyoxyethylene (20) sorbitan monooleate (Tween 80)	Lipase	Bhowal *et al.*, 2014
			anionic surfactant Sodium di-2-ethyl hexylsulfosuccinate (AOT) and non-ionic surfactant TWEEN 85	Amoxicillin	Chuo *et al.*, 2014
Affinity based reverse micelles	Ionic/ Nonionic surfactants + affinity ligands	Affinity interactions	Ionic surfactant Sodium bis-2-ethyl-hexyl-sulfosuccinate (AOT) and affinity ligand Concanavalin A	Peroxidase	Paradkar *et al.*, 1993
			Two nonionic surfactants (Triton X-45 and Span 80)and Di (2-ethylhexyl)phosphoric acid used as affinity ligand.	Green fluorescent protein (EGFP) expressed in Escherichia coli	Dong *et al.*, 2010

an important role during the forward extraction, so this has contributed towards obtaining high activity and purification factor of nattokinase enzyme after the extraction.

Shen, *et al.*, (Shen *et al.*, 2007) worked on the separation of protein mixture consisting of myoglobin, cytochrome *c*, and lysozymes using the AOT (Sodium bis-2-ethyl-hexyl-sulfosuccinate)/ n-hexane reverse micelles. The pI values for Myoglobin, cytochrome *c* and lysozyme is reported as 7.0, 9.6 and 11 respectively in the paper. Throughout their work, separations were manipulated mainly by pH gradients, which controlled the electrostatic interactions between the protein molecules and reverse micelles. Though authors have attempted to separate the three enzymes simultaneously, due to non-specific protein/micelle interactions, only the cytochrome *c* and lysozymes complete separation was achieved but not the myoglobin. The activity recovery of cytochrome *c* and lysozyme using the reverse micelles simultaneous extraction was found to be 90% and 82% respectively. The possibility of simultaneous separation and enrichment of different enzymes is demonstrated in this work.

Hydrophobic and hydrogen bonding interactions (nonionic surfactant based reverse micelles)

In this type, reverse micellar system consists of nonionic surfactant and organic solvents. In such case the solubilization of enzymes in reverse micelles is governed by hydrophobic and hydrogen bonding interactions. By the results obtained through isothermal titration calorimetry (ITC) study, Arnulphi *et al.* (2007) has reported that the nonionic surfactant (TX-100) molecules bound to the protein surface only by the hydrophobic and polar interactions (Arnulphi *et al.*, 2007).

Reverse micelles is of great importance and have attracted considerable interest, due to the solubilizing property of reverse micelles inner core/water pool. This inner core resides the polarized biomolecules, such as proteins, enzymes, DNA, and amino acids that one wishes to extract from the bulk aqueous phase. Since, the product of interest preserve in this inner core, the polarity of the inner core plays a major role in retaining the function and activity of the product. As the inner core has a great significance in the reverse micellar extraction of enzymes, Sawada *et al.*, 2004 has evaluated the polarity of water in the inner core as well as the activity of enzymes in the non ionic reverse micelle. In their study, the enzymes like the proteases *á*-chymotrypsin and subtilisin are purified by two different non-ionic surfactants reverse micellar systems like, polyoxyethylene sorbitan trioleate (Tween-85) and polyoxyethylene *tert*-octylphenyl ether (TX-100). For the polarity study small amounts of water incorporated on the inner core of both Tween-85 and TX-100 reverse micelles, the incorporated water is found to have low polarity compared with bulk water. These differences in the polarity maybe explained in terms of the hydration state of the surfactant molecule and the penetration of the outer solvent. The activity study reveals that Tween-85 reverse micelles and TX-100 reverse micelles have successfully extracted the enzymes by hydrophobic and hydrogen bonding interactions. Further investigations confirmed that the Tween-85 reverse micellar system preserved their activities and followed Michaelis–Menten kinetics. Whereas, the TX-100 reverse micellar system did not show much activity. This is attributed to the lack of sufficient micellar size to solubilize the enzyme (Sawada *et al.*, 2004). This study shows that non ionic surfactant reverse micelles extract the enzymes due to hydrophobic and hydrogen bonding interactions. As well as it concludes that difference in polarity of water inside the core effects the solubilized enzyme inside the reverse micelle.

Electrostatic and hydrophobic, hydrogen bonding interactions (mixed reverse micelles)

The efforts have been made to bring in new concept of mixed reverse micelles in order to address the protein deactivation problem in ionic reverse micelles, size concerns in ionic/non ionic reverse micelle and selectivity of protein issue in nonionic reverse micelles. The composition of mixed reverse micelles is similar to reverse micelle composition, only difference is that, mixed reverse micelles have both the ionic and nonionic surfactant molecules (sometimes co-surfactant is also added to overcome the reverse micelle size issue), whereas reverse micelles will have either the ionic or nonionic surfactant molecules. The papers studied on the extraction of lipase and amoxicillin enzyme using the mixed reverse micelle is reported below.

Recently Bhowal *et al.* (2014) used the mixed reverse micelles for the purification of lipase enzyme obtained from *Aspergillus niger* source by solid-state method from fermented rice bran. The authors have attempted to extract and purify the lipase enzyme using mixed reverse micelles consisting of cationic surfactant Cetyltrimethyl ammonium bromide (CTAB) and non-ionic surfactant Triton X-100 (Bhowal *et al.*, 2014). In their previous study Bhavya *et al.*, 2012, extracted the lipase enzyme from the same *Aspergillus niger* source by using only the cationic surfactant Cetyltrimethyl ammonium bromide (CTAB). The results show the activity recovery (78.6%) and purification (3.14-fold). While in this mixed reverse micelle extraction, maximum lipase recovery (100%) and purification fold (17.0-fold) were achieved. Comparision of reverse micelles and mixed reverse micelles work indicates that, incorporation of the Tween 80 nonionic surfactant resulted in significant progress in the purification fold (3.1–17.0) of the lipase. This drastic increase in the recovery and purification fold of lipase in mixed reverse micelle is due to the increase in the diameter of the reverse micelles after the addition of nonionic surfactant and this was confirmed by the increase in water content (Wo) of the micelles (Bhavya *et al.*, 2012). The study concludes that the reverse micelles containing a mixture of nonionic and cationic

surfactants can be effectively used for the improvement in the activity recovery and purification fold during downstream processing of enzymes.

Chuoa *et al.*, 2014 carried out work on extraction of amoxicillin using the mixed reverse micelles. In this study sodium di-2-ethylhexylsulfosuccinate (AOT) as anionic surfactant and TWEEN 85 as nonionic surfactant is used for the formulation of mixed reverse micelles. And the authors have procured the enzyme amoxicillin trihydrate from bio-WORLD, USA. The study includes the optimization of various process parameters and its effect on the extraction of amoxicillin. The process parameters like potassium chloride (KCl) concentration, pH of aqueous feed solution, AOT-TWEEN 85 molar fractions, surfactant concentration and extraction time were examined. At the optimal conditions for all mentioned parameters, 90.79% of amoxicillin is recovered. As the interactions between two or more factors are not explored in real time experiments, further response surface methodology (RSM) is employed in this study to know the precise optimum point of experiments. Central Composite Design with STATISTICA 8.0 software is used to implement the data. Analysis of variance (ANOVA) statistical method is used to check the significance level. The Experimental and RSM results confirm that the experimental values found are in agreement with the predicted values. The authors conclude through this study that, recovery of enzymes by mixed reverse micellar system is higher as compared to only AOT reverse micelles (Chuoa *et al.*, 2014).

Affinity interactions (affinity based reverse micelles)

The approach is called affinity-based reverse micellar extraction and separation (ARMES). This technique involves the affinity interaction between affinity ligands and their proteins which are introduced into reverse micelles, which is the main driving interaction in extraction process. ARMES technique can be carried out either by using ionic/ non ionic surfactants. Several difficulties were encountered with ionic surfactants compared to non ionic surfactants for the extraction of enzymes by ARMES technique. The difficulty reported in using the ionic surfactants is the, electrostatic interactions between the proteins and ionic surfactants, which impede the affinity effect under usual extractive conditions. That is, the enhancement of selectivity by affinity is hindered by the strong electrostatic interactions under normal extractive conditions. Hence, it was proposed to incorporate the affinity ligands into reverse micelles of nonionic surfactants (Matveeva *et al.*, 1996). Since there is less ability to extract proteins by nonionic surfactants, addition of affinity ligands to the system can increase the selective solubilisation of the protein merely by the affinity interaction. There are several papers available, which represents the usage of ionic/ non-ionic surfactants for the extraction of enzymes by ARMES technique.

Paradkar *et al.*, 1993 has used ARMES technique to purify the peroxidase enzyme from soybean hulls. In this research work lectin concanavalin **A** (con A) is used **as** a sugar-binding affinity ligand and the ionic surfactant Bis (2- ethylhexyl) sulfosuccinate (AOT)/ solvent isooctane is used to prepare ARMES. The purified peroxidase enzyme has attained the purification factor of 30; the same has been confirmed through HPLC and SDS-PAGE analysis with the regeneration of the con A ligand. The authors propose that ARMES technique is more applicable to purify the complex biomolecules. As well as for the purification of glycoform protein variants, affinity ligand lectins can be used and for the purification of therapeutic significant protein, affinity ligand antibodies are preferable (Paradkar *et al.*, 1993).

Dong *et al.*, 2010 has demonstrated the ARMES technique for the purification of recombinant hexahistidine-tagged Enhanced Green fluorescent protein (EGFP) expressed in Escherichia coli. In this study, Di (2-ethylhexyl) phosphoric acid is used as affinity ligand and two nonionic surfactants (Triton X-45 and Span 80) are used to formulate the reverse micelles. This research focuses on the optimization of extraction operating conditions. At the optimized conditions, 87% activity recovery of EGFP is reported. In addition Fluorescence spectrum analysis result indicates the preservation of protein structure after the separation process, which confirms that ARMES method is promising for the purification of proteins (Dong *et al.*, 2010).

The other groups (Leser *et al.*, 1993; Matzke *et al.*, 1992) also worked on the selective separation of chymotrypsinogen using anti chymotrypsinogen-antibodies as affinity ligands, which was immobilized by covalently combining cholesteryl groups in reverse micellar system composed of tetra-oxy ethylene monodecyl ether. The results

indicated that, extraction through affinity interactions is highly selective which can be used for the other protein purification, if its antibody is available.

Factors affecting forward extraction/ solubilisation: intrinsic parameters for the enzyme extraction

It has been proved by several studies that, the extraction of enzymes into reverse micelles occurs only during the forward extraction step (Chaurasiya *et al.*, 2015). The mechanism by which the enzyme enter into the reverse micelles during the forward extraction is governed by the various interaction forces like electrostatic interactions, electrostatic and hydrophobic interactions, hydrogen bonding interactions, affinity interactions between proteins and their affinity ligands, hydrophobic and hydrogen bonding interactions.

These various interaction forces are influenced by several parameters like the size of the reverse micelle, the nature of the surfactant used, the kinetic effect on microencapsulated enzymes, pH and ionic strength of the aqueous phase. Below is the description about few of these parameters influence on the forward extraction of enzymes (Matzke *et al.*, 1992).

The pH controls the solubilization of enzymes into reverse micelles to a large extent. Hence, the control on solubilization of enzymes into reverse micelles depends on the enzymes aqueous phase pH. As well as, after the solubilization of enzymes into reverse micelles, it is equally important to examine the pH inside the core, which will have a direct effect on enzymes activity. Hence, it makes a point to study the influence pH in aqueous phase and pH inside the micellar core, during the forward extraction of enzymes by reverse micelles.

Considering the pH in aqueous phase: The solubilization of enzymes into ionic surfactant based reverse micelles is controlled to a great extent by pH. In general, the enzymes would be transferred selectively into reversed micellar phase, only when the enzymes net charge is opposite to that of the surfactant head groups (Castro *et al.*, 1988). The pH in the aqueous phase will determine the net charge of the enzymes. To maintain the opposite charges between enzyme and surfactant head groups, it is required to formulate the enzymes to carry the opposite charge as per the pre-determined surfactant head group charge. The net

charge of enzymes opposite to surfactants is governed by the iso-electric point (pI) of the enzymes. The negative charge of enzyme is favored at pH values above the isoelectric point (pI) of the enzyme in the case of cationic surfactants, whereas the positive charge is favored at pH values below the isoelectric point (pI) of the enzymes for anionic surfactants. As a result, the state of enzyme ionization depends on the enzyme aqueous source pH.

Considering the pH inside the micellar core: It is reported by Castro *et al.*, 1988 that, a significant shift in pH profile is observed when the enzymes are encapsulated inside the reverse micelle, this occurs due to the heterogeneous distribution of protons within the micellar core. Therefore, research work needs to be done on this parameter in order to enhance the preservation of enzyme activity inside the micellar core after the solubilisation.

Adalberto Pessoa *et al.* (1998) worked on the reverse micellar extraction of inulinase from *Candida kefyr* using cationic surfactant BDBAC [N-benzyl-N-dodecyl-N-bis(2-hydroxyethyl)ammonium chloride]. The various factors affecting the efficiency of forward extraction are studied in this paper. The factors such as pH, surfactant concentration, temperature and buffer concentration are investigated. By the results obtained from the influence of pH parameter, authors conclude that, the solubilization of inulinase in cationic surfactant BDBAC is achieved by increasing the pH above the pI=4.5 value of inulinase enzyme. Since, the inulinase aqueous phase pH is maintained above 4.5, this gives the negative charges on inulinase enzyme, which is opposite to cationic surfactant BDBAC. Thus, the opposite charge on enzyme and surfactant creates the electrostatic interaction forces. The maximum solubilization (~86%) of the enzyme was observed optimally at pH 6.5 (Adalberto Pessoa *et al.* 1998). Similarly, several other studies also indicated the dependence of protein solubility on its pI value.

Along with pH, ionic strength is also the essential parameter to be considered in the purification of enzymes by reverse micelles. The influence of aqueous phase ionic strength on enzyme solubilization depends on the presence of ionic salts and by the Debye screening effect. Kinugasa *et al.* (1994) studied the effects of ions on Reverse micellar extraction of enzymes and classified the ions as water structure forming (WSF)

and Water structure breaking (WSB) salts. According to the authors observation by research, the addition of water structure forming salts (WSF) like NaCl increases the reverse micelles stability, which results in higher solubilization of enzymes. Li^+, Na^+, Ca^{2+}, Sr^{2+}, and Ba^{2+} are regarded as the WSF ions (Kinugasa *et al.* 1994). The second ionic strength effect Debye screening effect was explained by Debye as, the presence of salt content in the aqueous phase changes the size of the reverse micelles. Decrease in the size of the reverse micelles is caused by higher ionic strength by means of Debye screening. This reduction in size of reverse micelles decreases the repulsive forces between the two surfactant heads, leading to the formation of smaller reversed-micelles for the solubilisation of enzymes and also it improves the interaction between the enzyme and surfactant head (Krishna *et al.*, 2002). Thus, selective transfer of enzymes based on size exclusion effects is possible to achieve. At very less concentration of salt (ionic strength) in the aqueous phase, solubilisation/transfer of enzymes into reverse micelles does not occur. It is required to have a minimum of ionic strength in the aqueous phase for the transfer of enzyme into reverse micelle. Krishna *et al.*, 2002 found a minimal concentration of the salt NaCl around 0.75M to achieve 100% of solubilization for Bovine Serum Albumin (BSA) with an AOT/isooctane system.

After the forward extraction, the solubilized enzymes in the reverse micelle (microencapsulated enzymes) can be used directly as reaction media for the bioconversion process. Hence, the backward extraction of solubilized enzymes from reverse micelles can be omitted and microencapsulated enzymes can be directly used as a catalyst to carry out the enzymatic reaction (Sharma *et al.*, 2014). Fletcher *et al.*, 1984 carried out the kinetic studies of reactions catalysed by microencapsulated á-chymotrypsin enzyme in AOT/heptane reverse micelles. The main intend of the kinetic studies is to determine *K*m- the Michaelis constant and *k*cat-the turnover number of the enzyme for á-chymotrypsin enzyme catalysed reactions in reverse micelles. The conclusion drawn from this study report that, kinetic constants for microencapsulated enzymes considerably vary from the ones observed with the free enzyme in aqueous solution. The microencapsulated enzymes obey the Michaelis-Menten model for the chemical reaction catalysis. The most significant observation of α-chymotrypsin kinetics in reversed micelles is the increment of *K*m

by 100 to 1000-fold and a simultaneous decrease in *k*cat by a factor of 2–5 times. The á-chymotrypsin enzyme preserved its activity inside the reverse micelles, with much more stability than in the aqueous medium (Fletcher *et al.*, 1984). This shows that reverse micelles can be utilized as reaction media for the enzymatic catalysis of water-insoluble substrates.

Factors affecting backward extraction/ desolubilization: extrinsic parameters for the enzyme extraction

The efficacy of reverse micellar extraction for enzymes depends on the easy recovery of enzymes encapsulated/solubilized in the reverse micelles and also the extent to which enzymatic activity is preserved by the recovered product. Recovery of solubilized enzymes from the reverse micelles is aided by de-assembling the reverse micelles in aqueous media. Most of the study indicates that, the addition of Water Structure Breaking (WSB) ionic solution or alcohols to reverse micelle disturbs the reverse micelles to release the encapsulated enzyme.

The role of ionic solution in the recovery of enzymes from reverse micelles is explained by 2 phenomenons. The first phenomena state that, increase in the ionic strength decreases the electrostatic interaction between the surfactant head group of the reverse micelles and the protein molecule. Second phenomena explain that, increasing the ionic strength reduces the electrostatic repulsion between the reverse micelles surfactant head group, this decreases the size of reverse micelles, resulting in the expulsion of enzymes through size exclusion effect/ squeezing-out effect. Along with ionic strength, the type of the ions also plays an important role in recovery of proteins from reverse micelles. In general, WSB ions are used for the back extraction process, the K+, Rb+ and Cs+ are regarded as WSB ions.

Nandini and Rastogi, 2010, have studied on the extraction of lactoperoxidase enzyme from whey using reverse micellar extraction. The various parameters affecting the forward and backward extraction are studied in this work. The effect of KCl concentration on the backward extraction of lactoperoxidase was studied, the activity recovery and purification factor was reported to be increasing with increase in the salt concentration from 0.5 M to 1.0 M. Further increase in salt concentration

resulted in the aggregation and precipitation of enzyme. Therefore, it is reported that 1M KCl is the optimum salt concentration to be used for the backward extraction of lactoperoxidase enzyme. At this optimized condition the activity recovery and purification factor of enzyme resulted as 86.60% and 3.25 fold respectively (Nandini and Rastogi, 2010).

As referred earlier in this review Bhavya *et al.*, 2012 demonstrated on the extraction of lipase from *Aspergillus niger* using reverse micelles. Authors in this work used KCl ionic solution in the back extraction process for the recovery of lipase from reverse micelles. The results indicate that increase in KCl concentration from 0.5 to 1.0 M has increased the activity recovery of lipase from 40 to 78.6% and purification fold from 1.8 to 3.14. For further increase in KCl concentration there was no significant change in the activity recovery and purification fold. The reason for this was given as; at lower concentration of salt all the enzymes in the reverse micelles has not transferred to the freshly contacted aqueous phase which lead in lower extraction. And at higher concentration, due to reduction in the repulsive interaction between surfactant head groups, resulted in decrease in micelle size. Thus, due to squeezing out effect there was an increase in the recovery of lipase from reverse micelles with an increase in the concentration of KCl (Bhavya *et al.*, 2012).

Along with KCl, the reverse micelle needs an intervention of an alcohol to release the encapsulated enzyme. Alcohol is considered to be good means for the enzyme release from reverse micelles, as it reduces the hydrophobic protein–surfactant/solvent interactions, as well as it reduces the micelle–micelle interactions (attractions between solubilized enzymes and micelles). Aires-Barros *et al.*, 1991 has carried out selective separation and purification of lipase from *Chromobacterium viscosum*. This study describes the influence of different parameters like pH, ionic strength and water content on the extraction of lipase by reverse micelles. The study on the effect of ionic strength on the back extraction reveals that, 50% recovery of lipase was achieved at the optimum concentration of 50mM KCl. The effect of added alcohol content is studied at the same optimized condition. The addition of alcohol improves the degree of back extraction of lipase from reverse micellar phase. The increase in the addition of ethanol concentration from 0.5% to 2.5% has increased the recovery of lipase from 50% to 85%. These results suggest that the alcohol is the best promoter to improve the efficiency of back extraction of enzymes from the reverse micelles. Further the study also suggests that, alcohol chain length significantly affects the degree of back transfer. The effect of alcohol chain length from C2 to C10 was studied on the recovery of lipase (Aires-Barros *et al.*, 1991). The results indicate that, recovery of enzyme decreased with the increase in the alcohol chain length. In a review published by Mathew and Juang (2007) has summarized that, the addition of short-chain alcohols such as ethanol, *iso*-propyl alcohol, *iso*-butanol gave the best results for AOT reverse micelles as compared to long-chain alcohols (*n*-octanol, *n*-hexanol) (Mathew and Juang 2007).

After the first time recovery of enzymes from reverse micelles, the remaining organic phase in the upper layer can be recycled for the second time extraction. The same has been represented as recycle in the figure 2 and figure 3. Hemavathi *et al.*, 2010 has worked on the extraction and purification of β-glucosidase enzyme using sodium bis (2-ethylhexyl) sulfosuccinate (AOT) and nonionic surfactants (Tween 20, Tween 80, Tween 85 and Triton X-100) in iso-octane. To maximize the efficiency of extraction the influence of various process parameters on forward and backward extraction were studied in this work. At the optimized conditions the results obtained were reported as 95.18% of activity recovery and 4.8 fold of purification. Further to confirm the efficiency of remaining organic phase after the extraction, the recovered organic phase was reused for the second time extraction along with fresh feed. The recycled organic phase extraction has resulted in 90.2% of activity recovery and purification of 4.5-fold (Hemavathi *et al.*, 2010). This study shows the possibility of recycling the reverse micellar organic phase for subsequent extractions. Very few reports are available on the studies with recovered phase. Still work needs to be done on the recovered organic phase to evaluate the number of effective recycles.

Future trends / current developments

There are several directions of the developments in reverse micelles which have been very actively studied; some of those current interested topics in the field of reverse micelles are discussed in this section. To look upon one such topic, the ordinary

organic solvents used in the formulation of reverse micelles has the high volatile organic compound (VOC) emissions, which causes the poor stability of enzyme, reduces the recoverability of enzymes and recyclability/reusability of organic solvents. Ionic Liquids (ILs) have been extensively employed in liquid-liquid extractions especially in reverse micellar extractions, in place of organic solvents which are volatile in nature and have several toxic effects. Ionic liquids possess many unique physicochemical properties, like no vapor pressure, fire resistant, having high thermal stability, non-flammable and wide liquid temperature ranges, high conversion rates, high selectivity, better enzyme stability etc. Muhammad Moniruzzaman *et al.*, 2008 have successfully solubilized the lipase in sodium bis (2-ethyl-1-hexyl) sulfosuccinate (AOT) reverse micelles formulated by using ionic liquids as solvent. This study also shows that solubilized lipase has the catalytic activity and has catalyzed the hydrolysis of pnitrophenyl butyrate (p-PNB) to p-nitrophenol (p-NP). This study has reported the possibility of carrying out enzymatic reactions in ILs. Hence, this approach unlocks the new opportunity for studying enzymes in ILs (Muhammad Moniruzzaman *et al.*, 2008).

There has been a significant interest in studying reverse micelles as a reaction media, since, reverse micelles serve as media for reactions between species that are soluble in different reverse micelle phases. It has been proved that the biomolecules enclosed in surfactant assemblies would retain their structure and function when they are compared with the biomolecules in bulk aqueous solution (Griffiths *et al.*, 2000). There is a considerable amount of evidence that the reverse micelles have been used as a biological membrane to solubilize DNA, proteins and enzymes (Luisi *et al.*, 1988).

Though few studies indicate the preservation of structure and function of enzymes inside micellar core, there are several other studies which report the possibility of enzyme denaturation inside the reverse micelles. To scale-up the reverse micellar technology for industrial purposes, it is essential for enzymes to retain the structure and function inside the reverse micelles. Hence, the techniques like protein engineering and site-direct mutagenesis have been in use to avoid the denaturation of enzymes. The first reports by Melo *et al.*, 1994 revealed that the microencapsulated cutinase in AOT reversed micelles is unstable and showed reduction in their activity. Since then it was prioritized to study on the stabilization and activity retention strategies of microencapsulated enzymes (Melo *et al.*, 1994). Carvalho *et al.*, 1999 applied factorial design methodology to optimize both the activity and stability of cutinase in the reversed micelles. The study discloses that it is possible to preserve the cutinase activity by the addition of hexanol to microencapsulated cutinase which greatly avoids deactivation. The hexanol protects microencapsulated cutinase by binding to a specific site on microencapsulated cutinase surface which safeguard the cutinase against deactivation. Further, study applied medium engineering and thermostability experiment, which has increased the stability of cutinase to 509-fold when compared to results achieved in the absence of hexanol. A considerable progress was observed by improving cutinase stabilization in AOT reversed micelle, which makes the system competent for continuous operation (Carvalho *et al.*, 1999).

Few researches employ spectroscopic studies, in order to get an insight about the conformational changes of encapsulated enzymes, to understand the folding and kinetic behavior of the enzymes. So far, spectroscopic studies including photon correlation spectroscopy, NMR spectroscopy and IR spectroscopy have indicated the different results for the various enzymes. These studies were found to be helpful for the optimization of the reverse micelles model (Yeung *et al.*, 2013).

Conclusion

The review has pointed out several features of Reverse Micellar Extraction system, different extraction mechanisms governed by various Reverse Micellar systems and the effect of various parameters on the forward and backward extraction of enzymes by reverse micelles. As well as a detailed discussion is provided on the recent developments of reverse micellar extraction process. This includes, ionic liquids as a substitute for ordinary organic solvents, reverse micelles as a reaction media and the determination of protein structure using NMR and X-ray.

Reverse micelles are useful in the recovery of both the extracellular enzymes and the other biomolecules, which are from the source of fermentation broths and tissue cultures. As well as reverse micelles finds its application in the separation of intracellular products, obtained after the cell lysis. Although this review focused only on

the proteins/enzymes, they are not the only biomolecules which can be separated by reverse micelles. We can say that reverse micelles application is not only limited to proteins/enzymes, it can also be employed for the extraction of other biomolecules. Reverse micellar extraction for enzymes is well established in the laboratory scale, but to the best of our knowledge no reports are available on the industry/large scale extraction of enzymes using this technique. Therefore there is a need to investigate the applicability of reverse micellar extraction on real systems using industrial equipment. There should be more in-depth study and research in scale-up, mathematical modeling and engineering aspects to provide statistical interpretation of data and to evaluate the effect of multiple factors on the extraction of enzymes. It has also been shown that organic solvents and surfactants are easily recovered and reused, which would reduce the cost of the process and makes it economically feasible. There is a need to use the green solvents in the formulation of reverse micelles, instead of using pollutant hydrocarbons. Keeping this in view, it is more significant to continue the current trend work on the usage of benign, environmental friendly and non-pollutant green solvents as an alternative to common organic solvents. Through this review, it has been attempted to give an insight about the reverse micelle that would shortly develop into a novel bioseparation tool for biotechnology.

Acknowledgement

Abbreviations

AOT: Sodium di (2-ethylhexyl) sulfosuccinate; CMC: Critical micelle concentration; ILs: Ionic liquids; ITC: Isothermal titration calorimetry; pI: Isoelectric point; RME: Reverse micellar extraction; VOC: Volatile organic compound; WSB: Water Structure Breaking; WSF: water structure forming salts

Conflict of interest

The authors declare that they have no conflict of interest.

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