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REVERSE MICELLAR SEPARATION OF LIPASES : A CRITICAL REVIEW

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ABSTRACT

Production of lipases in large scale through conventional methods is quiet expensive; and the activity/stability so obtained is low. This review discusses about the potential of improving the downstream processing of lipases by using reverse micellar solvent separation and also highlights the fundamental aspects of its application in the biotechnology industry. This paper aims at bringing out the features of reverse micelle extraction of lipase using surfactants, through experimental data. The activity recovery, extraction efficiency and purification factors were improved many-folds. It is one of the best technology for safe liquid-liquid extraction of enzymes.

Key words: Lipases, Reverse micellar separation.

INTRODUCTION

Enzymes have been used since the dawn of mankind. Most of the reactions in living organisms are catalyzed by protein molecules called enzymes. Enzymes are also termed as the catalytic machinery of living systems. They are responsible for the biocatalytic fermentation of sugar to ethanol by yeasts, a reaction that forms the basis of beer and wine manufacturing. Enzymes oxidize ethanol to acetic acid, a reaction that has been used in vinegar production for thousands of years.

Industrial enzyme business is reaching new heights due to improved production technologies, engineered enzyme properties and new application fields. Usually the production organism and the individual enzyme have been genetically engineered for maximal productivity and optimized enzyme properties. At least 75% of the industrial enzyme market was occupied by hydrolytic enzymes such as protease, lipase and amylase. Lipases (E.C. 3.1.1.3) not only possess the natural function of hydrolyzing triacylglycerol (TAG) to glycerol and free fatty acids, but also catalyze esterification, transesterification and ammonolysis in non-aqueous media. They are one of the most promising hydrolases of industrial potential. And as a matter of fact, wide usage of lipases is found in food, detergent, chemical and pharmaceutical industries.

The industrial demand for lipase is growing significantly, in spite of its low proportion in current enzyme market. One of the main obstacles is the relatively high cost which comes

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from the feedstock cost as well as the time-consuming and complex downstream-processing.

Lipases are produced from microbial, plant and animal species. Large scale level of production is from microbial sources. Generally Regarded As Safe (GRAS) organisms are used for this purpose which includes *Aspergillus* species and *Penicillia* species. Additional advantage of these species is that they are stable over a wide range of pH, temperature and organic solvents. Lipases can be of intracellular and extracellular nature, of which the latter is easy to extract, hence most widely used.

Till date, there are two types of fermentations: Solid Substrate Fermentation (SSF) and the Submerged Fermentation (SmF). SSF is defined as the fermentation on moist solid substrate in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted, whereas, in the latter case the water content plays a major role in the fermentation. SSF was found to be more attractive from the economical viewpoint, as the unitary lipase cost was 47% lower than the selling price in SSF, while 68% higher than that in SmF. More importantly, SSF offers a useful tool for processing agro-industrial residues[1].

Most purification schemes for lipases involve multi-step strategies. No conclusions can be drawn regarding the optimal sequence of chromatographic methods that maximizes the recovery yields and purification fold. Based on the nature of the lipase produced by the organism, purity, quantity and its use, one has to design the protocol for purification. Often the extraction and separation processes that include chemical/biological methods leads to loss of quantity and activity of enzyme, because of its sensitivity towards pH, temperature, substrate conditions, etc. With the upcoming technologies and exploiting the very basic laws of chemistry, reverse micelle extraction is now in the trend of enzyme technology.

Reverse micelles are thermodynamically stable, nanometer-sized water droplets dispersed in an organic phase by means of surfactant. The size and shape of reverse micelles vary significantly with the nature and the concentration of the surfactant and the solvent used. Size and shape depend also on the temperature, pressure, and the ionic strength. Reverse micelles have an inner core of water, often called as water pool, can solubilize hydrophilic biological substances such as DNA, proteins and amino acids. Reverse micelles are used as a reaction system for enzymatic catalysis, liquid–liquid extraction of proteins, and protein refolding in the field of biotechnology[2].

Surfactants are amphiphilic molecules that possess both hydrophilic and hydrophobic parts. Surfactants can be classified according to their ionic character as: anionic, cationic, ampholytic or zwitterionic and non-ionic surfactant. The former group includes AOT as well as calcium dodecyl sulfate (CDS) and sodium dodecyl sulphate (SDS). Among the cationic surfactants there are CTAB, didodecyl dimethyl ammonium bromide (DDAB), tetradecyl trimethyl ammonium bromide (TTAB) and trioctyl methyl ammonium chloride (TOMAC). Surfactants are mostly used in solvents like isooctane, hexane, heptane, benzene and n-hydrocarbons.

The overall liquid–liquid extraction process by reverse micelles consists of two fundamental steps: a forward extraction in which a protein is transferred from an aqueous solution into a reverse micellar organic phase and a back extraction in which the protein is released from the reverse micelles and transferred into an aqueous phase to be recovered. The enzyme molecules are entrapped in the water pools, avoiding direct contact with the organic solvent that is potentially denaturing to enzyme.[3]

Increasing the interfacial area is of great technological interest because this results in an increase in the number of substrate molecules available to react. By incorporating this surfactant based separation in the downstream processing one can obtain better yields, increased purification factor and most importantly the specific activity is increased many folds.

The presence of lipases has been observed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which represent today's best studied lipase producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively.[4]

Lipases (EC 3.1.1.3) are a class of hydrolases that are primarily responsible for the hydrolysis of acylglycerides. They are ubiquitous and indispensable for the bioconversion of lipids in nature. In addition to their biological significance, lipases hold tremendous potential for exploitation in biotechnology. They possess the unique feature of acting at the aqueous and non – aqueous interface which distinguishes them from other enzymes.[5,6]

Recent studies on the structure of several lipases have provided some clues for understanding their hydrolytic activity, interfacial activation and stereoselectivity of lipases.[7] Lipases have other additional advantages of biocatalysts, such as high catalytic activity, mild reaction conditions, environmental friendliness, exquisite chemical, enantio- and regioselectivity, very broad substrate range and excellent stability. Lipases differ greatly with regard to both their origins (which can be bacterial, fungal, mammalian, etc.) and their properties. They all show highly specific activity towards glyceridic substrates.[4]

Although most of commercial enzyme preparations need not be purified to homogeneity, a certain extent of purity allows successful usage in the industries.[1]

Production strategies

Among the microorganisms, fungi are widely recognized as the best lipase sources, and are used preferably for industrial applications, especially in the food industry.[8] SSF process opened new opportunities for the lipase production since agro-industrial residues with low cost could be used as the feedstock.[1]

Mahadik *et al*.[9] reported the highest yields of enzyme using *Aspergillus niger*. SSF was done using wheat bran in combination with olive oil as lipid substrate. Maximum lipase activity (630 IU/g dss) was recovered when fermented wheat bran was extracted with NaCl supplemented with Triton X-100. The enzyme also exhibited high activity (75%) at extremely

acidic pH 1.5. This organism, being GRAS cleared, can be used for large-scale production of enzyme for commercial purpose.[9]

Conventional methods for optimization of medium and fermentation conditions involve varying one parameter at a time and keeping the others constant. It is time consuming and expensive, when a large number of variables are to be evaluated. To overcome this difficulty and to evaluate and understand the interactions between different physiological and nutritional parameters, response surface methodology (RSM) has been widely used. This methodology brings about the effect of interaction of various parameters, generally resulting in higher production yields and simultaneously limits the number of experiments. It is currently used for optimization studies in several biotechnological and industrial processes.[10]

Wolski *et al*.[11] studied the partial characterization of the crude enzymatic extract of *Penicillium sp*. obtained by SSF and SmF systems showed optimum activity at pH 5.5 and 47°C, and pH 7.0 and 37°C, respectively (15.17 U/mL and 11.28 U/mL). These results confirmed the interesting potential of SSF, because, besides the higher activities obtained in this system, the half-life time at 25°C was higher than that observed for the lipase extract obtained in the SmF system.[11]

A study related to this advanced study was done on *Rhizopus delemar*.[8] The production process of a lipase from *R. delemar* was optimized by RSM and a Box–Behnken experimental design to study the interactive effects of fermentation medium components on lipase activity and microorganism growth. The optimum fermentation medium composition for lipase production by *R. delemar* were sucrose concentration 4.19 g/L, molasses 1.32 g/L, yeast extract 0.53 g/L, sunflower oil 1.11% (v/v), and tween-80 1.80% (v/v). In these conditions, the biomass concentration of 4.52 g/L with a lipolytic activity of 1585 µmol/L min was reached.

In choosing the production strain several aspects are considered. Firstly, the enzyme is secreted from the cell. This makes the recovery and purification process much simpler compared to the production of intracellular enzymes. Secondly, the production host should have a GRAS-status. This is especially important when the enzyme produced by the organism is used in food processes. Thirdly, the organism should be able to produce high amount (50 g/L) of the desired enzyme in a reasonable time frame.

Most of the industrial enzymes are produced by a relatively few microbial hosts like *Aspergillus sp, Trichoderma sp, Streptomyces imperfecti* and *Bacillus* bacteria. Yeasts are not good producers of extracellular enzymes and are rarely used for this purpose. In nature, the *Penicillia* are versatile and opportunistic fungi. They are good producers of extracellular enzymes such as lipase, protease, cellulase and xylanase.[12]

Extraction and purification

Most commercial applications such as enzyme preparation for detergents do not require pure lipases, but a certain degree of purity simplifies there successful usage as biocatalyst because it reduces side product formation and simplifies product downstream processing

complexity. Extensive lipase purification is considered when structural studies are performed or when it will be used as a biocatalyst in the synthetic reactions in a pharmaceutical industry.

Most of the microbial lipases are extracellular and the fermentation processes are followed by the removal of culture cells from the broth either by centrifugation or by filtration. Pre-purification steps involve the concentration of cell free culture broth by ammonium sulfate precipitation, ultrafiltration, or extraction using an organic solvent and are often followed by chromatographic separation techniques for getting the required level of purity. Based on the nature of the lipase produced by the organism, one has to design the protocol for purification involving precipitation and chromatographic steps. However, it will also be guided by the purity of the enzyme required for its usage, which is important from the economic viewpoint.[13]

Applications of reversed micelle systems are also limited since the surfactant in high concentration severely complicates the separation of products and hinders the enzyme recovery. A method was developed by Leser *et al.*[14] for reverse micelle protein separation using silica. The subsequent recovery process took place into an aqueous solution at pH 8, with 60–80% recovery of proteins maintaining 80–100% specific activity. Following the same trend, Gupta *et al.*[15] recovered proteins and amino acids from reverse micelles by dehydration with molecular sieves. The hydrophilic products precipitated as a solid powder, being relatively free of surfactant.

The main constraints in traditional purification strategies include low yields and long time periods. Alternative new technologies such as membrane processes, aqueous two-phase systems and immuno-purification are gradually coming to the forefront in the purification of lipases. Very few examples of enzyme–substrate affinity purification for lipases exist.[13]

An extracellular lipase from *Aspergillus niger NCIM 1207* has been purified to homogeneity using ammonium sulfate precipitation followed by phenyl sepharose and Sephacryl-100 gel chromatography. This protocol resulted in 149 fold purification with 54% final recovery and specific activity of 1373 IU/mg. One interesting feature of this enzyme is its highly acidic pH optimum. The activity at pH 1.5 was 70% of its original activity but enzyme was stable at pH 8–11.[16] Moreover, the high stability of the enzyme in presence of organic solvents suggests that it has the potential for industrial applications such as flavor and bio-diesel production.

Reverse micelle solvent extraction is of high industrial potential importance as one can retain enzymes of high stability and activity. It is yet to be exploited in biotechnological fields of application.

Reverse micelle

In an aqueous solution, molecules having both polar or charged groups and non polar regions (amphiphilic molecules) form aggregates called micelles. In a micelle, polar or ionic heads form an outer shell in contact with water, while non polar tails are sequestered in the interior. Amphiphilic molecules can form micelles not only in water, but also in non polar organic solvents. In such cases, micelle aggregates are called *reverse micelles* because the situation is reversed with respect to water. In fact, hydrocarbon tails are exposed to the solvent, while the

polar heads point toward the interior of the aggregate to escape the contacts with the solvent.[17]

Reverse micelles are able to hold relatively large amounts of water in their interior. In that way, a "pocket" is formed which is particularly suited for the dissolution and transportation of polar solutes through a non polar solvent. These reverse micelles are proportionally less likely to form on increasing head group charge, since hydrophilic sequestration would create highly unfavorable electrostatic interactions.

The last method is the most commonly employed in purification. Several variables control the solubilization of proteins in microemulsions, including the pH and the ionic strength of the aqueous phase, the size of the protein, size of the reverse micelles, and the nature of the surfactant. The solubilization properties of proteins depend also on the method used to add protein.

Surfactant

Surfactants are classified by the presence of formally charged groups at its head. A non-ionic surfactant has no charged groups at its head. The head of an ionic surfactant carries a net charge. If the charge is negative, the surfactant is more specifically called anionic surfactant; if the charge is positive, it is called cationic surfactant. If a surfactant contains a head with two oppositely charged groups, it is termed zwitterionic surfactant.

The amphiphilic nature of the surfactant molecule causes its self association in a reverse-micellar solution with the polar head of the surfactant directed towards the aqueous core of the micelle and the hydrophobic tail extending into the continuous organic phase. The essentially spherical semi-permeable membranes of reversed micelles have pores which allow small substrates and product molecules to enter and leave the capsule, respectively, but prevent lipases and large molecules from permeating. The size and structure of reverse micelles make them attractive for integrated processes. In order to improve effectiveness of separation and hence reduce costs, the possibility of continuously removing the product(s) formed during reaction has been under scrutiny.

Reverse micelle separation

By choosing an appropriate set of experimental factors (pH, temperature, ionic strength, co-surfactants, and other parameters), it is possible to transfer a protein from a bulk aqueous phase to the water pool of reverse micelles in an organic phase (forward-extraction process) and later recover these proteins in a fresh aqueous phase (back-extraction process).[18] When techniques with organic solvents are limited by protein denaturation and solubilization, the use of liquid–liquid extraction of biomolecules employing reversed micelles is a promising solution.[17,19,20]

Most of the work with water-in-oil (w/o) microemulsions in biological systems has employed AOT as the surfactant. This anionic surfactant has the ability to form reverse micelles in a great number of non-polar organic solvents (oils) incorporating considerable amounts of water and several other polar solvents such as glycerol.[21] Yamada *et al.*[22] carried out the

extraction of *Chromobacterium. viscosum* lipase from aqueous solution was carried out in AOT/span 60 and in AOT/Tween-85-mixed reversed micelle systems and the activity of lipase was at its maximum when the molar ratio of AOT to Tween-85 was 3:2–4:1.

Selective separation and purification of a lipolytic preparation from *Chromobacterium viscosum* were carried out by liquid–liquid extraction using a reversed micelle system.[23] Krieger[19] proposed the usage of AOT reversed micelles for purifying *P. citrinum* lipase, which gave a better activity yield (68%) and the monomer protein.

In order to improve the back extraction process, many investigations have been carried out using various methods. The strategy of improvement can be divided into three categories. One deals with the stripping aqueous phase by pH, species, and concentration of salts, the second deals with the surfactant-organic phase by species and concentration of surfactant or adding various alcohols, and the third deals with the whole system by temperature or pressure.[24] It is very effective to isolate the protein or enzyme in an active state. Reverse micelle is considered as a good development of two-phase systems that have a wide use for carrying out a bioorganic synthesis[25].

Yu *et al.*[3] used AOT reverse micelles in isooctane for the separation of yeast-lipase by a two-step procedure. Under the optimized conditions of forward extraction using 250 mmol/L AOT, 0.05 mol/L KCl in the initial aqueous phase, stirring time 10 min and room temperature, 100% of the lipase was extracted. Under optimized conditions of back extraction, pH 8.0 and 0.5 mol/L KCl in the second aqueous phase, 3% (v/v) ethanol, stirring time 15–30 min, and room temperature, 68% of the lipase and 45% of activity recovery were obtained.[3]

The activity of lipase in modified AOT reverse micelles strongly depended on dimethyl sulfoxide (DMSO) concentration, surfactant concentration and molar ratio of water to surfactant (W_0). The enzyme entrapped in AOT/DMSO reverse micelles was very stable, retaining over 87.5% of its initial activity after 40 days, whereas the half-life in simple AOT reverse micelles was 33 days. This increased stability of lipase suggested that AOT/DMSO reverse micelles provided a better microenvironment for enzymes than that of simple AOT reverse micelles.[26]

The inactivation of enzyme due to strong interaction with AOT molecules is a severe problem. To overcome this problem, the reverse micelle system was modified by adding short chains of polyethylene glycol 400 (PEG 400). This modified system was used to extract *Mucor javanicus* lipase from the aqueous phase to the reverse micelle phase. The maximum specific activity of lipase extracted in PEG 400 modified reverse micellar system was three-fold higher than that in the PEG-free system.[27]

Mixed reverse micelles with ionic and non-ionic surfactants have been developed to decrease the protein deactivation in ionic reverse micelles and to increase the extraction yield and selectivity of proteins. Furthermore, affinity-based reverse micelles composed of nonionic surfactant coupled with affinity ligands have been investigated for the separation of proteins with high selectivity and purification factor, which can provide a mild microenvironment and keep high activity of proteins.[18]

The increase in surfactant concentration from 0.10 to 0.20M was found to increase the extraction efficiency of protein from 54.37% to 68.49% and activity recovery of lipase from 61.50% to 85.49%. The increase in the activity recovery of lipase with the increase in surfactant concentration up to 0.20M was explained based on the fact that the increase in surfactant concentration resulted in the increase in number of surfactant aggregation and reverse micelles, which in turn enhanced the extraction process. The maximum activity recovery, extraction efficiency and purification factor were 82.72%, 40.27% and 4.09-fold, respectively. SDS–PAGE profile further confirmed the purity of extracted lipase using reverse micelles.[28]

The Winsor II microemulsion, formed by the addition of iso-butanol or 2-phenylethanol as the organic solvent, favored the stripping of the nonionic surfactant into the O_m phase, whereas the lipase was left in the excess aqueous phase. Winsor II microemulsion extraction with 2-phenylethanol as the organic solvent accounted for the high lipase recovery (above 80%) and good non-ionic surfactant removal were achieved.[29]

A sustainable development of the various applications using RMS faces the imperative needs for environmental-friendly and biodegradable constituents. Non-ionic RMS is extremely sensitive to the process parameters. Ionic liquids can be used as co-surfactants. Substantially enhanced activity of surface active enzymes was measured when imidazolium-based ionic liquids were added to classical quaternary ammonium surfactants to form mixed cationic reversed micelles.[30] The AOT / isooctane RMS is known for its stability and fine dispersion with an enormous intrinsic water/oil interfacial area.

Simple and efficient downstream processing systems are developed; such as liquid–liquid extract with AOT/isooctane reversed micelles. Mutagenesis and directed evolution are valuable tools for improving the properties of the lipases such as thermostability, tolerance towards organic solvents and specificity that would remarkably expand lipases biotechnological applications.[1]

Following is the tabulation of reverse micelle extraction of lipase from different sources using different combinations of surfactant and solvents:

Chang and Rhee reported the relative activity of lipase in reverse micelles formed by various surfactants in isooctane and clearly demonstrated that the activity in AOT (100%) is much higher than in CTAB (11.8%) and in phosphatidyl choline derivatives (20–27%).[32]

Surfactant + Solvent	pH	Recovery/Yield	Activity	Reference
AOT + Ethanol	9	75%	-	Vincente et al.[24]
AOT + Isooctane	8	60-80%	80%	Leser et al.[13]
AOT + Isooctane	6	26.4%	68%	Krieger et al.[20]
AOT + Isooctane	8	68%	45%	Yu et al.[3]
CTAB + TRPO	7	70%	-	Shen et al., 2005
CTAB + Isooctane	Forward-9 Backward-7	40.27%	82.72%	Nandini et al.[28]

CONCLUSION

This review has pointed out several features of reverse micelle, their formation and some important features of liquid–liquid extraction of enzymes by reverse micelles. The most researched applications of RMS as protein purification method concern hydrolytic enzymes which have the widest variety of uses in food, detergent, textile, pharmaceutical, diagnostics, and fine chemical industries. The enzyme lipases is one such example. Widely used organisms are the *Aspergillus* and *Penicillium* species and their production were optimized using Response Surface Methodology. Conventional methods of extraction like centrifugation, filtration, etc gave less yield and activity. Reverse Micelle Extraction of the lipase was found to account for much better yield and improved activity. The purification factor was increased by many folds. Surfactants were accompanied by co-surfactants to prevent denaturation of enzyme and also to stabilize the reverse micelles. Widely used anionic surfactant was AOT along with isooctane. Taking into account of the stability and activity of the enzymes, it is better to opt for reverse micellar separation, an area which is yet to be exploited by the biotechnology industry.

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