Review paper
UDC 577.152.311

LIPASE PURIFICATION: THE REVIEW OF CONVENTIONAL AND NOVEL METHODS

Eldar Ruslanovich Yagmurov[1*], Grigori Vladimirovich Kozlov[1], Mikhail Alekseyevich Pushkarev[1]

[1]Saint Petersburg State Institute of Technology (Technical University), Moskovsky prospect 26, 198013 St. Petersburg, Russia

[*]e-mail: 95eldar@gmail.com

Abstract

The current review covers recent trends in the literature regarding methods and techniques of lipase purification and extraction from different sources including microbial, plant and mammalian lipases.

However, due to the cost efficiency, a large number of lipases are isolated and purified to homogeneity from fungal and bacterial sources. Nowadays, lipases are considered of great importance among biocatalysts due to their ability to catalyze a wide range of reactions in both aqueous and non-aqueous environments. Lipases are chemo-, regio-, and enantio-specific, they are used in different industries, including food manufacturing, detergents, biodiesel production and pharmaceuticals. The success in lipase purification to homogeneity is largely attributed to the combination of both traditional (conventional) and novel methods of protein purification. Among the conventional methods in this article we highlighted such methods as: (1) precipitation techniques by using ammonium sulfate and some organic solvents, (2) chromatographic methods of gel–filtration, ion–exchange and affinity chromatography, and (3) membrane processes. For the novel methods of lipase purification, we described (1) Recombinant Technologies, (2) Aqueous Two-Phase Systems, (3) Reverse Micelle Systems, and (4) Aqueous Two-Phase Flotation.

Generally, in order to purify the lipase with high purity and high yields, a multiple step procedure is applied. These multiple step systems of lipase purification consist of both conventional and novel methods, the conventional methods comprise of protein precipitation techniques with the precipitation agents such as ammonium sulfate or organic solvents; chromatography (ion – exchange, gel – filtration or affinity chromatography); ultrafiltration and/or other membrane techniques; novel methods of lipase purification include recombinant technologies, where the lipase-encoding gene is recloned into another host cell and expressed with a specific tag; aqueous two-phase systems, aqueous two-phase flotation and reverse micellar systems, which incorporate the usage of two different aqueous systems. The new developments in both conventional and novel methods of lipase purification allow both researchers and industries to purify lipases with higher yields and a lesser amount of purification steps needed. The introduction of recombinant technologies in lipase production had increased the purity of the enzyme as well as its yield while bringing down the cost of the overall procedure. The works presented in this paper describe developing technologies such as ATPS, ATPF, and RME that once maturated would bring about changes in protein purification strategies, that would allow the fastest and cheapest way for industrial lipase production.

Key words: *Lipase, Lipase Purification, Aqueous Two-Phase system, Aqueous Two-Phase flotation, Reverse Micelle system, Recombinant technologies, Immunopurification.*

1. Introduction

Lipases (triacylglycerol acylhydrolases - E.C. 3.1.1.3) are one of the most versatile classes of enzyme; their widespread application could be found in various industries, including but not limited to the: pharmaceutical, food, leather, textile, biofuel and paper production, detergent, cosmetic and fat-processing [1]. The lipases that are used in food industry are mainly performing the reactions of decomposition and modification of biomaterials. Most of the commercially produced lipases are used as flavor improvement components as well as they are used in processing of dairy products

such as: meat, vegetables, fruit, milk product, baked foods, and beer [2, 3]. In pharmaceutical industries lipases offer several advantages over chemical synthetic reactions. These advantages are mild conditions when compared to synthetic reactions that allow circumventing: isomerization, epimerization, racemization, and rearrangement reactions of bioactive substances [4]. In medical applications lipases serve as therapeutic agents in combination with other components. Their main application is treatment of diseases such as: dyspepsia, gastrointestinal disturbances, cutaneous manifestations of digestive allergies, cancer treatment, lipases also serve as diagnostic tool in medicine, thereby justifying the growing demands [5]. Another promising application of lipases is biodiesel production where higher yields are achieved by using lipase over the existing physical-chemical processes [6].

Lipases hydrolyze fats into fatty acids and glycerol at the water-lipid boundary. The distinguished ability of lipases to react on the frontiers of aqueous and non-aqueous phases makes them different from another type of hydrolases such as esterase [7, 8]. The other unique capability of lipases is that they have high chemo-, regio- and enantio- selectivity, which makes them very useful in industries [9]. Lipases like most industrial enzymes are increasingly produced via recombinant DNA technology, however the search for new types of lipases with high selectivity for particular substances is growing [7].

When compared to plant and mammalian lipases, which, in most cases, are very expensive to obtain and purify, microbial lipases are valuable in industrial processes due to: their short generation times, ease of genetic manipulations and purification, wide range of substrate specificity, greater stability; moreover, due to plethora of industrially relevant hydrolytic and synthetic reactions they catalyze both in aqueous and non-aqueous media, that acclaim to the drastic decrease in the cost of lipase production [1, 7, and 10].

Microbial lipases are divided into two distinct categories: lipases that are produced by bacteria and fungus. Lipase-producing fungi have various habitats in nature and can be isolated from diverse sources ranging from oil-contaminated soils or hot springs including compost heaps, coal tips, to industrial wastes. The majority of fungal enzymes are excreted out of the fungi, thus considered extracellular. Major lipase-producing fungi are: *Aspergillus, Ashbya, Acremonium, Mucor, Rhizopus, Penicillium, Geotrichum, Humicola, Fusarium, Rhizomucor, Metarhizium, Eurotrium, Ophiostoma*, etc. [1]. Bacterial lipases, in general, are glycoproteins; however, some extracellular lipases are lipoproteins. The amount extracellular lipases produced by bacteria depend on several factors such as: temperature, the presence of carbon and nitrogen sources, availability of lipids around the bacterial cell, the presence of other types of hydrolases etc. A huge number of bacteria are known as lipase producers however the most studied and used are: *Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium, Pseudomonas, and Staphylococcus* etc. [7].

According to Nutraceutical Business Review global demand for enzymes is forecast to grow on average by 4.6% by 2020 to $7.2 billion; in 2015, the enzyme market was worth of $6.5 billion. Gains will reflect a continued world economy rebound from the global financial crisis of 2009. Regardless of the increased growth in world demand for enzymes, only around 200 out of 4000 types of enzymes available on the global market. While out of the 200 enzymes only 20 are mass produced, there are great prospects for developing new methods of enzymes production, in our case lipases, in order to suffice a growing need on a global market [11]. However as optimistic as the prognosis might sound there are various complications in the process of industrialization of enzymes; the greatest hindrances are: (1) nowadays it is difficult to find enzymes that have high yield, activity, and stability; and even if the desired enzyme is found, the tedious and costly process of purification and industrialization of proteins will have some unwanted effects [10]; (2) there are several regulations that are imposed by governmental agencies, such as U.S. Food and Drug Administration (FDA) etc., for newly produced enzymes whether they are created for medicinal, food or industrial purposes, which require thorough testing, clinical trials and approvals that are both time consuming and very costly [10].

The aim of this review is to describe the most recent developments in methods of microbial lipase purification, that are represented by traditional techniques of protein salting out methods, usually performed by ammonium sulfate precipitation, different types of chromatography (ion-exchange, affinity chromatography and gel filtration) well as novel techniques such as: recombinant technologies and immunopurification, aqueous two-phase system (ATPS), aqueous two-phase flotation (ATPF), reverse micellar system (RMS) and membrane processes.

2. Existing methods of lipase purification

2.1 Conventional methods of lipase purification

2.1.1 Preparation of lipases for further purification

In general, microorganisms produce lipases extracellularly, therefore the enzyme is usually found in the surrounding liquid culture during the process of fermentation. The first step, that is taken to prepare lipases for further analysis and purification, is the removal of the cells and other insoluble substances from the liquid culture broth. This process is achieved by using microfiltration or centrifugation, in order to concentrate the

obtained liquid, it is subjected to ultrafiltration. Then the enzymes are subjected to extraction, which is usually achieved by using organic solvents, or precipitation by salts or other organic solvents. The most popular method of protein precipitation is precipitation by ammonium sulfate which is used about 60% of the time, whilst 35% use ethanol, acetone or hydrochloric acid. The precipitation steps are usually followed by a series of chromatographic steps. Precipitation techniques often have a much higher yield (approximately 87%) when compared to other techniques such as chromatography (60 - 70%) [7, 12].

2.1.2 Chromatographic steps for lipase purification

Current literature regarding the lipase purification and characterization contains a myriad of chromatographic methods for enzyme purification purposes. However, a single chromatographic step for the protein purification is not enough, hence the combination of different types of chromatographic methods is required [7]. Presently, three main chromatography methods for protein purification purposes exist: (1) the most frequently used method is ion exchange chromatography; (2) gel filtration is the second most heavily employed method of chromatographic methods in protein purification; however, this method is limited due its small capacity in protein loading and it is usually used in final steps of protein purification; and (3) affinity chromatography with its commonly used method of lipase purification - hydrophobic interaction due to the presence of hydrophobic surfaces that surround the active sites of lipases [12]. The hydrophobic adsorbents usually used contain following functional groups: buthyl, methyl, octyl, and phenyl [7, 12].

Another commonly exploited method of affinity chromatography is adsorption chromatography, with the most popular adsorbent hydrooxyapatite. Despite the high costs, affinity chromatography methods usually have purification factors of 2 to 10 for each step it is used [7]. Kumar *et al.*, [13] purified lipase obtained from *Bacillus safensis* DVL-43 by using Phenyl-Sepharose CL-4B hydrophobic interaction chromatography, the concentrated ammonium sulphate fraction (20 - 70%) was applied onto a column of Phenyl-Sepharose CL-4B (2 cm × 6 cm; Sigma) pre-equilibrated with 1.0 M ammonium sulphate dissolved in 50 mM phosphate buffer (pH 7.0). The bound lipase was eluted by applying a negative linear gradient of 1.0 M to 0 M ammonium sulfate in 50 mM phosphate buffer (pH 8.0). The specific lipase activity was reported 45.9 U/mg with the yield 24.1% and purification fold 11.5.

Sharon *et al.*, [14], reported lipase by *Pseudomonas aeruginosa*. The protein was purified by ammonium sulfate precipitation with the yield of 74%, the salted-out protein was applied to hydrophobic hydroxyapatite column chromatography the purification factor was 11. Wu *et al.*, [15], purified lipase from *Rhizomucor miehei* 42-fold, the activity observed was 32% and molecular weight was 31.6 kDa. The purification was completed by using three following methods: ammonium sulfate precipitation, phenyl Sepharose fast-flow hydrophobic interaction chromatography and DEAE Sepharose fast-flow anion exchange chromatography. *Pythium ultimum* lipase was discovered by Mozaffar *et al.*, where the enzyme was purified by ammonium sulfate precipitation with the high yield of 81%, and then by diethylaminoethyl-Sepharose CL-6B, and by Sephacryl S-200 chromatography. The purified lipase had a molecular mass of 68 kDa determined by SDS-PAGE, however, gel filtration chromatography had indicated that the molecular weight of the lipase was 270 kDa, which suggested that the enzyme had tetrameric structure [16].

Above mentioned chromatographic steps are under consistent advances in search of new breakthroughs. As for example, Li *et al.*, [17], synthesized thermo-sensitive N-alkyl substituted polyacrylamide affinity polymer PNNB. The polymer was used for purification of lipase from the crude medium. The optimized condition was observed under pH 7.0, the absorption temperature was 35 ^{0}C, 120 min. adsorption time, and 0.5 mg/mL initial concentration of lipase. The specific activity of lipase was observed as 506 U/mg, and the enzyme recovery achieved was 82%. Cunha *et al.*, [18], reported that the lipase separation was coupled with its immobilization from *Penicillium simplicissimum* by selective adsorption on hydrophobic supports on three different hydrophobic matrices butyl-agarose (low hydrophobicity), phenyl-agarose (medium hydrophobicity), and octyl-agarose (high hydrophobicity). After three steps enzyme had 85% of specific lipolytic activity. Golaki *et al.*, [19], studied the lipase from the bacterium *Cohnella* sp. A01. The enzyme was purified by a two-steps anion exchange chromatography on DE52 resin with variable pH from 5.5 to 4.3. The yield of lipase was 38% and a purification factor of 13.4. The purified lipase had a molecular weight determined by SDS-PAGE of 29.5 kDa. Gurruraj *et al.*, [20], purified thermostable, organic solvent tolerant lipase by ammonium sulfate precipitation with the yield of 70%. The dialyzed protein sample was then loaded onto a DEAE Sepharose anion exchanger with the yield of 36% and purification fold of 2.2, the specific activity was 83 U/mg. The molecular mass and the isoelectric point (pI) were 41.196 kDa and 7.14, respectively.

2.1.3 Membrane Processes

Membrane processes are found to be increasingly involved in the purification of different biomolecules. Membrane systems are very advantageous due to their selectivity and highly developed surface area per volume [21]. They are very suitable for working with

biological molecules due to their ability to be operated in comparatively low temperature and pressure ranges while involving no changes of phases, thus minimizing the possibility of protein denaturation and deactivation, or decomposition of enzymatic products [22]. The most renowned examples are ultrafiltration and microfiltration as well as filtration of fermentation media, purification of buffers and proteins, which have become routine methods. Another, more exciting applications of membrane processes have been introduced more recently such as membrane bioreactors, membrane chromatography, and membrane contactors, and as for today are not incorporated in the enzyme purification steps as needed [23].

Among described above methods the most prominent method for lipase purification by using membrane processes is membrane chromatography, which is a platform technology (synonymous membrane absorber technology) that can meet the selectivity and throughput requirements of enzyme purification [24]. Membrane materials tested for chromatographic applications include: cellulose, polysulfone, polyamide, hydrazide, and composite membrane such as blend of polyethersulphone and polyethylene oxide coated on all surfaces with a covalently bound layer of hydroxyethylcellulose [25]. The basic membrane absorber is composed of functionalized macroporous membranes with ligands coupled to the inner specific surface area. Thanks to their pore structure, the mass transfer rate is not limited by pore diffusion as in resin-based chromatography and is primarily convection driven [23]. The technique was conceived about twenty years ago, when Bastida *et al.*, [26] first reported that a great number of bacterial lipases can be immobilized on different hydrophobic supports such as octyl-agarose gels in a rapid and strong fashion, while Cunha *et al.*, [18], reported a modification of the described method that allowed *Penicillium simplicissimum* lipase separation while it was coupled and immobilized by selective adsorption on hydrophobic supports on three different hydrophobic matrices. The lipase was first applied to the butyl-agarose matrix (low hydrophobicity), then to phenyl-agarose (medium hydrophobicity), and subsequently to octyl-agarose (high hydrophobicity). Upon completion of all three steps enzyme had 85% of specific lipolytic activity. A great protein amount was retained on butyl-agarose support, the retained protein was concluded to be an impurity. The phenyl-agarose and octyl-agarose supports presented a reduced amount of proteins in comparison with butyl-agarose, meaning that the selectivity of hydrophobic matrices was increasing with each step.

Further development in lipase purification on industrial scale led to new developments in this type of purification. Beutel *et al.*, [27], who purified *Staphylococcus carnosus* lipase with two purification strategies, the first strategy involved the use Sartobind-phenyl membrane and the second approach was based on strong cation exchanger CEX mixed-mode prototype, which combined electrostatic (sulfonic acid groups) with HICs. For HIC–phenyl (Sartobind-phenyl) membrane, the optimal conditions for specific binding of lipase from cell lysate was observed at pH 7.0 with 0.5 M $(NH_4)_2SO_4$. The yield was 89%, residual activity 92% and a purification factor 3.2. For mixed-mode CEX membrane prototype, the optimal binding was observed at pH 5.0, the yield was 93% of lipase and residual activity was 98% while purification factor of 7.8.

2.2 Novel methods of lipase purification

2.2.1 Recombinant technologies in lipase purification

Immunoaffinity chromatography or immunopurification is a type of affinity chromatography when a protein of interest is purified by applying an antibody-antigen principle [4]. Immunopurification as of today is considered one of the strongest and most selective methods of protein purification, with the purification factor ranging from 1000- to 10000-fold in one single step procedure. This method could be used for protein separation when other existing methods the desired level of separation [28].

Most protein immunopurifications techniques consist of either monoclonal antibodies or affinity-purified polyclonal antibodies [29]. Two important factors should be considered before proceeding to immunopurification: (1) whether the suitable monoclonal antibody is available for the desired protein; and (2) whether the contaminants present in the analyte and whether their composition and concentration are known [29]. Altought the immunopurification is considered very efficient and selective, the cost and availability of antibodies for specific proteins is enormous, not to mention the amount of work it would require for a lab staff to find the right antibody [4]. Therefore, the easier and cheaper methods of using already existing antibodies and antigens represented by the fusion of the target protein and a specific tag are being applied during the process of protein purification [30]. Affinity tags are highly efficient tools for protein detection, characterization, and purification. An epitope is a short sequence of amino acids that typically serves as the antigenic determinant, or the region to which an antibody binds [31]. Thus, epitope tagging is a technique in which a short sequence (i.e., an epitope) is added to a protein of interest by recombinant DNA methods [31, 32].

Specifically constructed purification tags can facilitate very efficient purification of recombinant proteins, producing high levels of yields and purities in a few general steps [32]. These tags are usually fused to the N-terminus or C-terminus of a target protein and

commonly allow their partner proteins to be selectively captured and purified through association with a tag-specific antibody, affinity resins, or by selective tag-dependent precipitation or aggregation [31]. The tags that are mostly used in lipase purification are generally: Polyhistidine tag, or His-tag, which is a polyamino acid construct, the polyhistidine (poly-His) tags are the most widely used affinity tags for purifying recombinant proteins. Advantages of the polyHis tag include its low immunogenicity and small size (0.84 kDa) - with composition ranging from 3 to 10 His tags in series. In addition, many proteins function with the polyHis tag positioned at either the N-, or C-terminus and purification methods can be carried out under both native and denaturing conditions [31 - 34]. The following examples show the application polyHIS-tag in the lipase purification. Rivera *et al.*, [35], for the first time functionally expressed *Carica papaya* lipase 1 from its genomic sequence (CpLip1) by cloning the sequence into pGAPZαB plasmid and incorporating it into *Pichia pastoris* as a host system. The recombinant protein contained a C-terminal extension including a His6 tag which allowed its purification by HisTrap (Fast Flow) FF (High Performance) HP 5 mL column affinity chromatography. The purification factor was reported as 7-fold with lipase activity of 25 U/mg in the purified fraction. Also, the authors reported the homology of papaya lipase with microbial lipases. The biochemical characterization of purified lipase showed that CpLip1 hydrolyzed preferentially long-chain triglycerides, with an optimal pH of 8.5 and an optimal temperature of 35 ^{0}C. Another study conducted by Memarpoor-Yazdi *et al.*, [36], cloned and expressed thermo-halophile GDSL lipase-encoding gene from *Rhodothermus marinus*, in *Escherichia coli*. The optimum conditions for enzyme expression were 0.1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and expression period of 12 h at 28 ^{0}C. The extracted recombinant enzyme from the supernatant of cell lysates contained a His-tag sequence in its N-terminal which was subjected to Ni-NTA affinity column chromatography using imidazole gradient for purification with a purification factor of about 6.4-fold and a yield of 53.8%. The enzyme had a molecular mass of 40 kDa and revealed the highest hydrolytic activity (1055.3 U/mg) towards p-nitrophenyl butyrate (C4) at 70 ^{0}C, pH 8.5 and retained 78.6% of its initial activity after 60 min. incubation at this temperature [36].

Other commonly used tags are FLAG epitope tag is a short, hydrophilic octapeptide (DYKDDDDK) and c-myc proto - oncogene product (EQKLISEEDL) [37-39]; the application of FLAG and c-Myc tags in lipase purification were showed by Berryman *et al.*, [40], who proposed an approach of purifying and determining the structure of rat hepatic lipase (rHL) by utilizing epitope-tags. The authors placed myc and flag epitope tags at the C-terminus of the Hepatic Lipase cDNA just 5′ of the stop codon. They have applied the enzyme-linked immunosorbent assay (ELISA) method to identify the structure of the lipase, where the anti-myc monoclonal antibody, 9E10, was chosen as a capture antibody, and the anti-flag-M2 peroxidase conjugate as a detecting antibody. The authors also suggested that these antibodies might serve as a base for immunoaffinity purification. The results showed that the rHL was comprised of two oligomeric species. The molecular mass of the rHL monomer was determined as 76 kDa. The specific activity of the myc/flag tagged recombinant protein was reported as 8696 U/mg by utilizing tri-(3H)oleoylglycerol as a substrate stabilized with gum Arabic.

Kumari *et al.*, [41], obtained lipases lip11 and lip12 from *Yarrowia lipolytica* MSR80 by incorporating lipases with IgG tag. The lipases were cloned and expressed with IgG tag in *Escherichia coli* HB101 pEZZ18 system. The enzymes were purified by IgG-Sepharose affinity chromatography and analyzed by SDS-PAGE. The molecular masses of lipases were 47.51 and 48 kDa respectively; specific activities of 314.352 U/mg and 198 U/mg for Lip11, and Lip12 were observed.

There are also a lot of new type of tags are being tried as a mean of lipase purification, Xing *et al.*, [42], described a lipase A purification scheme that takes advantage of induced protein aggregates. The team tested two amphipathic peptides (18A or ELK16) in search of cleavable aggregates that could be used for protein purification. The lipase A was incorporated into LipA-ELK16 and lipA-18A fusion aggregates. Lipase A released by tag cleavage at a range of pH from 5.6 to 8.5, from the LipA-ELK16 aggregate had a specific lipase activity at 133.4 U/mg, the results were comparable with those reported in the literature and slightly higher than that released from the LipA-18A fusion which had the lipase activity of 72.8 U/mg. The mass of extracted enzyme was reported 21 kDa. It is also interesting to note that lipase A (LipA) in either LipA-ELK16 aggregate showed little hydrolytic activity against the substrate para-nitrophenyl palmitate (pNPP). In a different study, Singh *et al.*, [43], reported a strategy for simultaneous purification and refolding of proteins overexpressed with an intein tag. A recombinant lipase overexpressed in *Escherichia coli* ER2566 with the intein tag and obtained as inclusion bodies were solubilized in buffer containing 8 M urea or cetyltrimethylammonium bromide. The solubilized lipase was precipitated with chitosan and the affinity complex of the polymer with the fusion protein was obtained. The intein tag was cleaved with dithiothreitol and the refolded lipase was obtained in active form. Activity recovery of 80% was observed and the enzyme had a specific activity of 2965 U/mg. The purified lipase showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the molecular mass of 29 kDa.

Pauwels *et al.*, [44], reported a purification procedure of the lipase (LipA) produced by *Burkholderia glumae*. The proposed affinity purification was based on a specific binding scaffold of a lipase specific foldase (Lif) and the intrinsic resistance to chemical denaturation of LipA. The Lif-His scaffold was immobilized onto His-select column. After the immobilization of Lif the liquid broth was applied to Lif-His column and the column was saturated. Upon saturation of the column with lipase, unbound proteins were washed away and the LipA–Lif complex was eluted. The lipA-Lif complex was dissociated by incubation for 1 h in 6 M urea solution. The obtained lipase had molecular mass of 35.4 kDa. The method reported is less labor-intensive, fast, leads to a homogeneous preparation and can be easily scaled up.

2.2.2 Aqueous two-phase system

Lately, the surge in the numbers of publications describing methods of lipases purification such as conventional column chromatography, in addition to some alternative modern approaches such as reverse micellar and aqueous two-phase extraction (ATPE) systems has been observed [45]. Industries today look for purification methods that are cheap, rapid, have a high yield and applicable to large-scale operations [46, 47]. The conventional purification methods of lipase recovery are not only expensive but also result in a longer period and lower yield [48]. Therefore, a desperate need for new and alternative methods of lipase purification such as aqueous two-phase systems will be trending in the lipase research [49].

Phase separation is a general phenomenon that occurs when two solutions of water-soluble polymers are mixed. ATPS is an effective liquid-liquid separation technology that has proven to be effective in biomolecules recovery. ATPS is popular due to its low energy rapid partition of molecules between phases. ATPSs are usually formed by the combination of either polymer/polymer or polymer/salt as phase-forming components [49].

Souza *et al.*, [50], compared an aqueous two-phase system (ATPS) composed of polyethylene glycol (PEG) and potassium phosphate with ultrafiltration and precipitation with acetone and kaolin as conventional methods of lipase purification with an average purification fold of 6.55 and specific lipase activity of 1208.96 U/mg. After lipase purification on polyethylene glycol (PEG)/potassium phosphate ATPS at the optimum conditions (pH 6 and 4 ^{0}C) the purification factor for lipase was greater than 41 with a selectivity of almost 500 for the bottom phase, the specific lipase activity was observed as 7665.27 U/mg. Therefore Souaza *et al.*, proved that the ATPS method could have a greater efficiency than conventional methods of lipase purification.

Aqueous two-phase systems have a lot of hindrances during the procedure that could cause the enzyme to lose its activity, reduce the protein recovery. As was investigated by Fernandes Duarte *et al.*, [51], the partitioning and recovery of lipase derived from *Leucosporidium scottii* L117 using ATPS. They evaluated three ATPS: (1) polyethylene glycol (PEG)/phosphate salts, (2) PEG/polyacrylic acid (NaPA) in different molecular weights (1500, 4000 and 8000 g/mol), and (3) Triton X-114 (TX-114)/McIlvaine buffer pH 7.0 in different conditions (2.0% (w/w) of TX-114 at 25.0 and 28.0 ^{0}C). The reported loss of enzymatic activity when using PEG/phosphate and PEG/NaPA systems made a system of Triton X-114/McIlvaine with a purification fold of 12, a viable option for lipase purification. Also, Souza *et al.*, [52], in a different study, reported an aqueous two-phase system (ATPS) formed with cholinium-based ionic liquid - ILs (or salts). The study examined the formation of ATPS based on cholinium-based salts (cholinium chloride, cholinium bitartrate, and cholinium dihydrogencitrate) and tetrahydrofuran (THF) for the purification of lipase from *Bacillus* sp. ITP-001, that was produced by submerged fermentation. The optimum purification conditions were determined to be 40 weight% (wt%) of THF and 30 wt% of cholinium bitartrate at 25 °C. A purification factor of 130.1 – fold, a lipase yield of 90.0% and a partition coefficient of the enzyme for IL-rich phase (K_E = 0.11) and protein contaminants for THF-rich phase (K_P = 1.16) were achieved.

Ramakrishnan *et al.*, [53], purified an MTCC5695 lipase, which partitioned to the bottom phase of polyethylene glycol PEG8000/Na_2HPO_4 ATPS with concentrations (wt%) of 2.11 and 1.98 respectively. The specific lipase activity was observed as 6325.04 U/mg, 75.69% enzyme recovery and a purification factor of 2.881 also were reported. The authors coupled the ATPS with ultrafiltration which led to a purification factor of 5.99-fold and an enzyme recovery of 82.09%. The molecular weight of MTCC5695 lipase was found to be 19.2 kDa. MTCC5695 lipase showed optimal activity at pH 10.8 indicating that it was alkaline in nature. The enzyme showed stability over a pH range of 7.0 - 12.0. The optimum temperature for lipase activity was observed to be 40 ^{0}C, the lipase was stable between the temperature ranges 30 - 70 ^{0}C after which there was a two third reduction of activity to (from 6000 U/mL to 4000 U/mL) at which it remained stable from 80 to 100 ^{0}C.

2.2.3 Reverse micellar system (RMS)

Reverse micelles are a system of bulk organic solvent where an amount of water and amphiphilic molecules (surfactant) are dissolved to form a single optically isotropic and thermodynamically stable liquid solution. The surfactant molecules form a monomolecular layer around the nanometer-sized water droplet with

hydrocarbon tails facing the organic solvent and polar head groups pointing inwards [54].

Because of the electrostatic interactions, the positively charged proteins could transfer from the aqueous phase to the inner core of the reversed micelles, thus effecting a separation. Reversed micellar extraction is an attractive separation method for the large-scale operation because the process could be carried out using the existing liquid–liquid extraction system in the chemical and biochemical industries. Factors affecting the performance of the reversed micelle system are rather complicated, including the: nature and concentration of target protein, pH, and ionic strength of the aqueous phase, extraction temperature, type and concentration of the surfactant, and the processing time [55].

In general, the recovery process is composed of two simple steps, 1) a forward extraction process involving the entrapment of proteins from a raw supernatant containing the enzyme of interest into the water pools of reverse micelles in an inorganic solvent, and a backward-extraction process where the proteins are transferred from the reverse micelles into another aqueous solvent to be recovered [56]. The application of RMS is limited for several reasons. The higher the concentration of surfactant, the harder it is to separate and recover the protein; and the range of organic solvents with which the technique operates efficiently are limited by the constraint to avoid protein denaturation while enabling protein solubilization [56-58].

Nandini *et al.*, [59], investigated lipase purification by applying reverse micellar extraction of lipase with the help of cationic surfactant cetyltrimethylammonium bromide (CTAB). Complex interaction of salt concentration (0.05 to 0.15 M), surfactant concentration (0.10 to 0.30 M), and pH (6.0 to 9.0) for forward extraction, as well as, salt concentration (0.5 to 1.5 M) and pH (6.0 to 9.0) for backward extraction were studied with the help of response surface methodology. Optimum purification conditions, such as salt concentration 0.16 M, surfactant concentration 0.20 M, and pH 9.0 for forward extraction, as well as, salt concentration 0.80 M and pH 7.23 for backward extraction, the mentioned optimal conditions have lipase recovery of more than 78% and a purification factor of the lipase of more 4.0. The report has demonstrated that response surface methodology can be used for optimization of the conditions for reverse micellar extraction of lipase.

The micellar systems reported by Fernandes Duarte *et al.*, [51], had the best results for lipase extraction with enzyme activity balances ranging between 84.7% and 113.05%. Lipase was preferentially partitioning into the micelle-rich phase with the partition coefficient of lipase K_{Lip} = 7.76%, enzyme recovered in bottom phase (REC_{Bot}) = 93.85% and purification factor = 1.2 at the temperature 25.03 °C, and the pH 5.1.

Gaikaiwari *et al.*, [60], have purified *Pseudomonas sp.* CSD3 by applying reverse micellar extraction of surfactant AOT (Aerosol OT (bis 2-ethylhexyl) sodium sulfosuccinate) - isooctane system. The lipase was purified 15-fold with 80% recovery and 2.5-fold concentration with specific lipase activity of 23.2 U/mg. The forward reaction consisted of crude lipase, 25 mM AOT and isooctane, the mixture was incubated at 25 ^{0}C for 30 min. under constant stirring conditions, backward extraction using 0.05 M NaCl in 50 mM Tris–Cl buffer, pH 7.0 and 15% isopropanol (1:1) at 25 ^{0}C for 30 min.

2.2.4 Aqueous two-phase flotation (ATPF)

ATPF, in general, represents an adsorptive bubble separation technique, in which, the enzyme (surface-active compound) in aqueous phase is located on the bubble surface of the rising, through the body of liquid phase, nitrogen gas stream and then when the bubble ruptures at the top phase where the polymer layer is, the lipase is released [61].

The mass transfer of bubble adsorption in ATPF offers more efficient separation, softer mixing and higher concentration coefficients with very low amounts of polyethylene glycol (PEG) usage. The objective of this paragraph is to demonstrate how the new method of ATPF is used nowadays to separate proteins [61, 62].

Tan *et al.*, [62], reported a newly constructed aqueous two-phase flotation (ATPF) composed of PEG and sodium citrate. The micellar system was used for direct purification of thermostable lipase 42 from recombinant *Escherichia coli* BL21(DE3) pLysS. The optimum purification condition of lipase by PEG 8000/sodium citrate ATPF were determined as tie-line length (TLL) of 25.4, volume ratio (V_R) of 0.3, crude loading (CL) of 20% (w/w) at pH 7 with average flotation time (F_t) of 10 min. and gas nitrogen flow rate (F_R) at 20 mL/min. Lipase was successfully purified using ATPF up to 4.05-fold with a separation efficiency of 99%. In a different study conducted by Preshna Mathiazakan *et al.*, [63], the lipase was recovered and purified from the fermentation broth of *Burkholderia cepacia* using an alcohol/salt ATPF system on a pilot scale. The parameters of ATPF, including the concentration of crude lipase feedstock, types of alcohol and salt, concentrations of alcohol and salt, volumes of buffer solution and alcohol, were investigated for their effects on the partitioning behavior of lipase in ATPF. ATPF comprised of 1-propanol and ammonium sulfate was successfully established for feasible and cost effective separation of *Burkholderia cepacia* ST8 lipase from the liquid fermentation broth. The alcohol/salt ATPF system had a purification factor of 12.2, with a separation efficiency of 93% and a selectivity of 40. Also, the team had conducted a comparison between small-scale and large-scale ATPF production, which showed that this technique could be used for industrial scale processes.

Show *et al.*, [61], described an aqueous two-phase flotation (ATPF) consisting of thermo-sensitive ethylene oxide-propylene oxide (EOPO) copolymer and ammonium sulfate. The developed system was applied for a direct recovery of lipase derived from the fermentation broth of *Burkholderia cepacia* strains ST8. Under the optimal conditions of ATPF, [3900 g/mol molecular mass of EOPO, 250 g/L concentration of ammonium sulfate, pH 6, 10 mL of initial volume of EOPO phase, 50% (w/w) of concentration of EOPO 3900, 200 mL of total volume of aqueous phase system, 40% (w/w) of loaded crude feedstock, 30 mL/min of nitrogen flow rate at 60 min. flotation time; the average separation efficiency and purification fold are 76% and 13%, respectively. The same author continued his work with ATPFs and reported a novel, efficient and low-cost method for recovery of biomolecules - recycling hydrophilic organic solvent/inorganic salt ATPF. The ATPF consisted of 2-propanol and potassium phosphate was developed for sustainable separation, concentration and purification of *Burkholderia cepacia* ST8 lipase from the liquid fermentation broth. The optimum conditions for this recycling ATPF were determined to be 40 mL volume of 50% (w/w) 2-propanol, 1.0 L of 250 g/L of potassium phosphate, pH 8.5, 100% (volume/volume (v/v)) of crude feedstock, 30 mL/min of nitrogen flow rate for 30 min in 8 cm radius of colorimeter tube with a G4 porosity (5 - 15 µm) sintered glass disk. A purification factor of 14 and a lipase yield of 99 were achieved in the optimized ATPF.

3. Conclusions

- Usually, in order to start the process of lipase purification one must ask three important questions: 1) What quantity of enzyme and level of enzyme purity is required? 2) What is the source of lipase (microorganisms, cell culture or plant cells) and the type of biomass, nutrient and other chemicals used? 3) What equipment is available? Generally, in order to purify the lipase with high purity and yield, a multiple step procedure is applied. The multistep systems of lipase purification consist of both conventional and novel methods. The conventional methods comprise of: protein precipitation technique by ammonium sulfate or organic solvents, chromatography (ion - exchange, gel - filtration or affinity chromatography), ultrafiltration or other membrane techniques. Novel methods of lipase purification include: recombinant technologies, where the lipase-encoding gene is recloned into an another host cells and expressed with a specific tag, aqueous two-phase system, aqueous two-phase flotation and reverse micellar systems, which incorporate the usage of two different aqueous systems.

- The new developments in both conventional and novel methods of lipase purification allow both researchers and industries to purify lipases with higher yields and a lesser amount of purification steps needed. The introduction of recombinant technologies in lipase production had increased the purity of the enzyme as well as its yield while bringing down the cost of the overall procedure. The works cited here also describe developing technologies such as ATPS, ATPF, and RME that once maturated would bring about changes in protein purification strategies, that would allow the fastest and cheapest way for industrial lipase production.

- Worth noticing is the fact that there is no "ready - to - use" strategy of lipase application exists, the selection of purification steps and the lipase production depends on the source of lipase as well as its desired homogeneity and yield. Nevertheless, the examples of novel purification methods such as Recombinant technologies, ATPs, ATPF and Micellar systems cited here, are being dynamically developed, which in turn would bring a more standardized strategy for lipase purification that could be applied for commercial use.

4. References

[1] Singh A. K., Mukhopadhyay M. (2012). *Overview of fungal lipase: a review*. Applied biochemistry and biotechnology, 166, (2), pp. 486-520.

[2] Farahat S. M., Rabie A. M., and Faras, A. A. (1990). *Evaluation of the proteolytic and lipolytic activity of different Penicillium roqueforti strains*. Food Chem., 36, pp. 169-180.

[3] Tecelão C., Rivera I., Sandoval G., Ferreira-Dias S. (2012). *Carica papaya latex: A low-cost biocatalyst for human milk fat substitutes production*. European Journal of Lipid Science and Technology, 114, (3), pp. 266-276.

[4] Joseph B., Ramteke P. W., Thomas, G. (2008). *Cold active microbial lipases: Some hot issues and recent developments*. Biotechnology Advances, 26, (5), pp. 457-470.

[5] Arpigny J. L., Jaeger K. E. (1999). *Bacterial lipolytic enzymes: classification and properties*. Biochemical Journal, 343, (1), pp.177-183.

[6] Ribeiro B. D., de Castro A. M., Coelho M. A. Z., and Freire D. M. G. (2011). *Production and Use of Lipases in Bioenergy: A Review from the Feedstocks to Biodiesel Production*. Enzyme Research, doi: 10.4061/2011/615803.

[7] Saxena R. K, Sheoran A., Giri B., Davidson W. S. (2003). *Purification strategies for microbial lipases*. Journal of Microbiological Methods, 52, (1), pp. 1-18.

[8] Vakhlu J., Kour A. (2006). *Yeast lipases: enzyme purification, biochemical properties and gene cloning*. Electronic Journal of Biotechnology, doi: 10.2225/vol9-issue1-fulltext-9.

[9] Schmid R. D., Verger R. (1998). *Lipases: Interfacial Enzymes with Attractive Applications*. Angewandte Chemie (International Edition), 37, pp. 1608-1633.

[10] Tan C. H., Show P. L., Ooi C. W., Ng E. P., Lan J. C. W., Ling T. C. (2015), *Novel lipase purification methods – a review of the latest developments*. Biotechnology Journal, 10, pp. 31-44.

[11] Ferreira-Dias S., Sandoval G., Plou F., Valero F. (2013). *The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries*. Electronic Journal of Biotechnology, 16, (3), pp?

[12] Houde A., Kademi A., Leblanc D. (2004). *Lipases and their industrial applications: an overview,* Appl. Biochem. Biotechnol., 118, (1-3), pp. 155-170.

[13] Kumar D., Parshad R., Gupta V. K. (2014). *Application of a statistically enhanced, novel, organic solvent stable lipase from Bacillus safensis DVL-43*. International Journal of Biological Macromolecules, 66, pp. 97-107.

[14] Sharon C., Furugoh S., Yamakido T. H., Ogawa I., Kato, Y. (1998). *Purification and characterization of a lipase from Pseudomonas aeruginosa KKA-5 and its role in castor oil hydrolysis*. Journal of Industrial Microbiology and Biotechnology, 20, (5), pp. 304-307.

[15] Wu X. Y., JÄÄskelÄinen, S., Linko W. Y. (1996). *Purification and partial characterization of Rhizomucor miehei lipase for ester synthesis*. Applied biochemistry and biotechnology, 59, (2), pp. 145-158.

[16] Mozaffar Z., Weete J. D. (1993). *Purification and properties of an extracellular lipase from Pythium ultimum*. Lipids, 28, (5), pp. 377-382.

[17] Li Y., Gao F., Wei W., Qu J. B., Ma G. H., Zhou W. Q. (2010). *Pore size of macroporous polystyrene microspheres affects lipase immobilization*. Journal of Molecular Catalysis B: Enzymatic, 66, (1-2), pp. 182-189.

[18] Cunha A. G., Fernández-Lorente G., Gutarra M. L., Bevilaqua J. V., Almeida R. V., Paiva L. M., Fernández-Lafuente R., Guisán J. M., Freire D. M. (2009). *Separation and immobilization of lipase from Penicillium simplicissimum by selective adsorption on hydrophobic supports*. Applied biochemistry and biotechnology, 156, (1-3), pp. 133-145.

[19] Golaki B., Aminzadeh S., Karkhane A., Yakhchali B., Farrokh P., Khaleghinejad S., Tehrani A., Mehrpooyan S. (2015). *Cloning, expression, purification, and characterization of lipase 3646 from thermophilic indigenous Cohnella sp. A01*. Protein Expression and Purification, 109, pp. 120-126.

[20] Gururaj P., Ramalingam S., Nandhini Devi G., and Gautam P. (2016). *Process optimization for production and purification of a thermostable, organic solvent tolerant lipase from Acinetobacter sp. AU07*. Brazilian Journal of Microbiology, 47, (3), pp. 647-657.

[21] Russotti G., Goklen K. E. (2001). *Crossflow membrane filtration of fermentation broth*. In: Wang W.K. (Ed.), Membrane separations in biotechnology, Marcel Dekker, New York, USA, pp. 85-160.

[22] Zeman L. J., Zydney A. L. (1996). *Microfiltration and ultrafiltration. Principles and applications*. Marcel Dekker, New York, USA.

[23] Charcosset C. (2006). *Membrane processes in biotechnology: An overview.* Biotechnology Advances, 24, (5), pp. 482-492.

[24] Ghosh R. (2002). *Protein separation using membrane chromatography: opportunities and challenges*. J. Chromatogr. A., 952, pp. 13-27.

[25] Charcosset C. (1998). *Purification of proteins by membrane chromatography,* J. Chem. Technol. Biotechnol., 71, pp. 95-110.

[26] Bastida A., Sabuquillo P., Armisen P., Fernández-Lafuente R., Huguet J., and Guisán, J. M. (1998). *A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports*. Biotechnol. Bioeng., 58, pp. 486-493.

[27] Beutel S., Villain L., and Scheper T. (2016). *Industrial Application of Membrane Chromatography for the Purification of Enzymes.* In: Hilterhaus L., Liese A., Kettling U. and Antranikian G. (Eds.), Applied Biocatalysis: From Fundamental Science to Industrial Applications, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

[28] Greenfield E. A. (2014). *Antibodies*. Cold Spring Harbour Laboratory Press, New York, USA.

[29] Bandmann N., Collet E., Leijen J., Uhlen M., Veide A., and Nygren P. A. (2000). *Genetic engineering of the Fusarium solanipisi lipase cutinase for enhanced partitioning in PEG- phosphate aqueous two-phase systems*. J. Biotechnol., 79, (2), pp. 161-172.

[30] Wood D. W. (2014). *New trends and affinity tag designs for recombinant protein purification*. Current Opinion in Structural Biology, 26, pp. 54-61.

[31] Young C. L., Britton Z. T., Robinson A. S. (2012). *Recombinant protein expression and purification: A comprehensive review of affinity tags and microbial applications*. Biotechnology Journal, 7, pp. 620-634.

[32] Xie H., Guo X. M., Chen H. (2009). *Making the most of fusion tags technology in structural characterization of membrane proteins*. Mol. Biotechnol., 42, pp. 135-145.

[33] Derewenda Z. S. (2010). *Application of protein engineering to enhance crystallizability and improve crystal properties*. Acta Crystallogr. D Biol. Crystallogr., 66, pp. 604-615.

[34] Chaga G. S. (2001). *Twenty-five years of immobilized metal ion affinity chromatography: past, present and future*. J. Biochem. Biophys. Methods, 49, pp. 313-334.

[35] Rivera I., Robles M., Mateos-Díaz J. C., Gutierrez-Ortega A., Sandoval G. (2017). *Functional expression, extracellular production, purification, structure modeling and biochemical characterization of Carica papaya lipase 1*. Process Biochemistry, 56, pp. 109-116.

[36] Memarpoor-Yazdi M., Karbalaei-Heidari R. H., and Khajeh K. (2017). *Production of the renewable extremophile lipase: Valuable biocatalyst with potential usage in food industry*, Food and Bioproducts Processing, 102, pp. 153-166.

[37] Hopp T. P., Prickett K. S., Price V. L., Libby R. T. March C. J., Cerretti D. P., Urdal D. L., Conlon P. J. (1988). *A short polypeptide marker sequence useful for recombinant protein identification and purification*. Bio-Technology, 6, pp. 1204-1210.

[38] Manstein D. J., Schuster H. P., Morandini P., Hunt D. M. (1995). *Cloning vectors for the production of proteins in Dictyostelium discoideum*. Gene, 162, pp. 129-134.

[39] Evan G. I., Lewis G. K., Ramsay G., Bishop J. M. (1985). *Isolation of monoclonal antibodies specific for human c-myc protooncogene product*. Mol. Cell Biol., 5, pp. 3610-3616.

[40] Berryman D. E., Mulero J. J., Hughes L. B., Brasaemle D. L., Bensadoun, A. (1998). *Oligomeric structure of hepatic lipase: evidence from a novel epitope tag technique*. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1382, (2), pp. 217-229.

[41] Kumari A., Verma V. V., Gupta R. (2012). *Comparative biochemical characterization and in silico analysis of novel lipases Lip11 and Lip12 with Lip2 from Yarrowia lipolytica*. World Journal of Microbiology and Biotechnology, 28, (11), pp. 3103-3111.

[42] Xing L., Wu W., Zhou B., Lin Z. (2011). *Streamlined protein expression and purification using cleavable self-aggregating tags*. Microbial Cell Factories, doi: 10.1186/1475-2859-10-42.

[43] Singh P. K., Gupta M. N., (2008). *Simultaneous refolding and purification of a recombinant lipase with an intein tag by affinity precipitation with chitosan*. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1784, (11), pp. 1825-1829.

[44] Pauwels K., Gelder P. V. (2008). *Affinity-based isolation of a bacterial lipase through steric chaperone interactions*. Protein Expression and Purification, 59, (2), pp. 342-348.

[45] Nadar S. S., Pawar R. G., Rathod V. K. (2017). *Recent advances in enzyme extraction strategies: A comprehensive review*. International Journal of Biological Macromolecules, 101, pp. 931-957.

[46] Duong-Ly K. C., Gabelli S. B. (2014). *Chapter Seven - Salting out of Proteins Using Ammonium Sulfate Precipitation*. In: Lorsch J. (Ed.), Methods in Enzymology, 541, pp. 85-94.

[47] Boominathan R., Mishra P., Chand S. (1995). *Isolation of lipase from the culture filtrate of Humicola lanuginosa*. Bioseparation, 5, pp. 235-239.

[48] Linke D. (2014). *Chapter Eleven - Explanatory Chapter: Choosing the Right Detergent*. In: Lorsch J. (Ed.), Methods in Enzymology, 541, pp. 141-148.

[49] Lee S. Y., Khoiroh I., Ling T. C., Show P. L. (2017). *Enhanced recovery of lipase derived from Burkholderia cepacia from fermentation broth using recyclable ionic liquid/polymer-based aqueous two-phase systems*. Separation and Purification Technology, 179, pp. 152-160.

[50] Souza R. L., Lima R. A., Coutinho J. A. P., Soares C. M. F., Lima, Á. S. (2015). *Novel aqueous two-phase systems based on tetrahydrofuran and potassium phosphate buffer for purification of lipase*. Process Biochemistry, 50, (9), pp. 1459-1467.

[51] Duarte A. W. F., Lopes A. M., Molino J. V. D., Pessoa A., Sette L. D. (2015). *Liquid-liquid extraction of lipase produced by psychrotrophic yeast Leucosporidium scottii L117 using aqueous two-phase systems.* Separation and Purification Technology, 156, (2), pp. 215-225.

[52] Souza R. L., Lima R. A., Coutinho J. A. P., Soares C. M. F., Lima Á. S. (2015). *Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification*. Separation and Purification Technology, 155, pp. 118-126.

[53] Ramakrishnan V., Goveas L. C., Suralikerimath N., Jampani C., Halami P. M., and Narayan B. (2016). *Extraction and purification of lipase from Enterococcus faecium MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization*. Biocatalysis and Agricultural Biotechnology, 6, pp. 19-27.

[54] Melo E. P., Aires-Barros M. R., Cabral J. M. S. (2001). *Reverse micelles and protein biotechnology*. Biotechnol. Annual Rev., 7, pp. 87-129.

[55] Chen Y. L., Su C. K., Chiang B. H. (2006). *Optimization of reversed micellar extraction of chitosanases produced by Bacillus cereus*. Process Biochemistry, 41, (4), pp. 752-758.

[56] Yu Y. C., Chu Y., and Ji J. Y. (2003). *Study of the factors affecting the forward and back extraction of yeast-lipase and its activity by reverse micelles*. J. Colloid Interface Sci., 267, pp. 60-64.

[57] Levashov A. V., and Klyachko N. L. (2001). *Reverse micellar systems*. In: Vulfson E. N., Halling P. J., Holland H. L. (Eds.), Methods in Biotechnology, Enzymes in Nonaqueous Solvents: Methods and Protocols 15, Humana Press, Totowa, New York, USA,, pp. 575-586.

[58] Dekker M., Hilhorst R., Laane C. (1989). *Isolating enzymes by reversed micelles*. Anal. Biochem., 178, pp. 217-226.

[59] Nandini K. E., Rastogi N. K. (2010). *Separation and purification of lipase using reverse micellar extraction: Optimization of conditions by response surface methodology*. Biotechnology and Bioprocess Engineering, 15, (2), pp. 349-358.

[60] Gaikaiwari R. P., Wagh S. A., Kulkarni B. D. (2012). *Efficient lipase purification using reverse micellar extraction*. Bioresource Technology, 108, pp. 224-230.

[61] Show P. L., Tan C. P., Anuar M. S., Ariff A., Yusof Y. A., Chen S. K., Ling T. C. (2011). *Direct recovery of lipase derived from Burkholderia cepacia in recycling aqueous two phase flotation*. Sep. Purif. Technol., 80, pp. 577-584.

[62] Tan J. S., Abbasiliasi S., Lin Y. K., Mohamed M. S., Kapri M. R., Kadkhodaei S., Tam Y. J., Rahman R. N. Z., Ariff A. B. (2014). *Primary Recovery of Thermostable Lipase 42 Derived From Recombinant Escherichia coli BL21 in Aqueous Two-Phase Flotation*. Separation and Purification Technology, 133, pp. 328-334.

[63] Mathiazakan P., Shing S. Y., Ying S. S., Kek H. K., Tang M. S. Y., Show P. L., Ooi C. W., Ling T. C. (2016). *Pilot-scale aqueous two-phase floatation for direct recovery of lipase derived from Burkholderia cepacia strain ST8*. Separation and Purification Technology, 171, pp. 206-213.