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Review Article

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Techniques for Immobilization and Detection of Lipase: A Review

Vahid Hakimzadeh, Aghdase Sadeghi

Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran

Abstract Lipases from bacterial, fungal, and animal sources have been purified to homogeneity with very few of them being contributed from plants. Plant lipases are mostly found in energy reserve tissues, for example, oilseeds. They act as biocatalysts which are attractive due to their high substrate specificity, low production cost and easy pharmacological acceptance due to their eukaryotic origin. Hence plant lipases represent better potential for commercial applications in organic synthesis, food, detergent and pharmacological industries. However, low expression, uneconomical fold purity and the plethora of difficulties related to their recombinant expression has been limiting their commercial applicability and posing challenges to many researchers. This review provides an overview of recent progresses in improving immobilized lipase (IL) -catalyzed biodiesel production, focusing on mid- and down-stream processing such as immobilization of lipase, simulation and techno-economic evaluation. The immobilization of lipase is a costly process. Most of the commercial ILs are prepared by adsorption of free lipase on polymeric materials. During the course of the last two decades, this area has rapidly expanded into a multidisciplinary field. The review is looking forward to explain different strategies, several prime controlling factors of enzyme immobilization on polymeric membranes. Lipases acts in the presence of interfaces, has attracted membrane researchers and biotechnologists to synthesize variety of polymeric membranes as efficient carriers.

Keywords Lipase, Detection, Immobilized, Enzyme

1. Introduction

For several years now, the enzymes market is growing regularly. Indeed, enzymes are used more and more in various applications of different fields such as pharmaceutical, cosmetic or food industry. In the past years, a better understanding of enzymes functionalities and catalytic behaviours, together with the progress of molecular engineering has led to new applications for various types of enzymes as for example proteases, acylases, oxidases, amylases, glycosidases, cellulases or lipases [1].

Lipases (triacylglycerol hydrolase, E.C. 3.1.1.3) are the enzymes that catalyze hydrolysis of triacylglycerides into free fatty acids and glycerol at the lipid water interface [2]. These hydrolytic reactions can be reversed under micro aqueous environment, such as in organic solvents, leading to esterification and transesterification. Lipase research has largely been focused on investigating the broad substrate specificity and regio-, chemo- and chiral-selectivity [3, 4]. Owing to their unique properties, lipases are frequently used in various industrial sectors viz. detergent formulations, oleochemical industry, biofuels, food and dairy, agro-chemical, paper manufacturing, nutrition, cosmetics and pharmaceuticals [5, 6]. Lipases are well known industrial biocatalyst due to their ability to carry out multitude of bioconversion reactions.

However, under certain conditions, they are also able to catalyze synthetic reactions [7]. The most reported of the reactions carried out by these enzymes are hydrolysis, acidolysis, alcoholysis, aminolysis, esterification and inter-

esterification [8]. Currently, lipases are a popular choice as a biocatalyst because they can be applied to chemo-, regio- and enantioselective hydrolyses and also in the syntheses of a broad range of compounds [9]. These enzymes are considered to have great potential as biocatalysts in numerous industrial processes, such as the synthesis of food ingredients [10], their use as additives to detergents [11] and to obtain enantiopure drugs and other refined products [12].

Lipases with new specificities are needed and the engineering of cloned enzymes as well as the isolation of new lipases from natural sources is therefore of increasing potential value. Reliable, convenient, and sensitive assays to detect a true lipase activity in cellular homogenates are required. As with all reactions catalysed by enzymes, activity measurements can be carried out using various physicochemical methods (by monitoring the disappearance of the substrate or the release of the product) [13]. Recently, few more lipase producing yeasts have been reported such as *Rhodosporidium* babjevae from fresh water of Archipelago, Svalbard [14]; *Candida boidnii* from spent olive fruits of the *Algerian variety* [15] and *Rhodotoryla slooffiae*, *Rhodotoryla muciloginosa*, *Candida davisiana, Cryptococcus diffluens, Cryptococcus uzbekistanensis, Cryptococcus albidus* and *Wickerhamomyces anomalus* from petroleum sludge [16].

The environment where these organisms are isolated occasionally confers some char-acteristics to the enzymes. Lipases isolated from thermophilic, halophilic, alkaline and acidic sources tend to be thermostable, salt, alkaline and acid tolerant, respectively. Bacillus sp. isolated from soil obtained around edible oil and fish oil processing plants have acidophilic characteristics, with an optimum pH of 1 [17]. Lipase from *Acinetobacter* sp. EH28, which was isolated from oil spill sites, is thermostable with good tolerance to alkaline and organic sol-vents [18]. In the case of salt tolerance, Ozcan *et al*. [19] reported that archaeal strains have halophilic properties. Thus, lipases are employed in various processes of industrial relevance, as shown in Fig. 1.

Lipases with high activity in aqueous media tend to have lower activities of up to four or five orders of magnitude innon-aqueous media. Several factors may contribute to this effect, including disruption of tertiary structure due to changes in medium hydrophobicity, limited conformational flexibility, desolvation of the active site resulting in limited enzyme-substrate binding and interfacial denaturation of the enzyme due to interfacial tension [20,21].

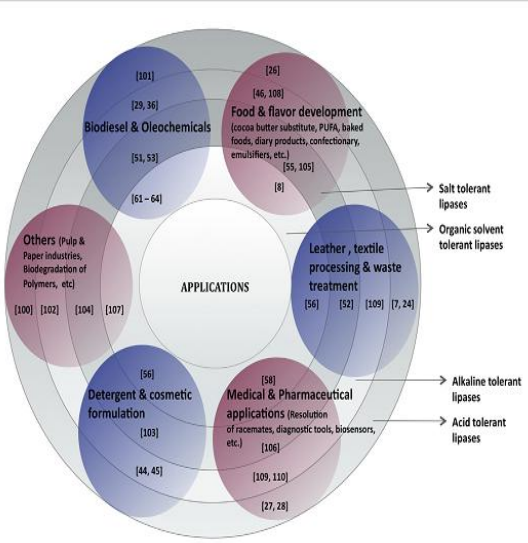

Figure 1: Application of lipases in different solvent systems. Each line within the main circle represents a particular solvent system, and the number(s) in parenthesis indicate reference(s) [22].

2. Immobilization of lipase

The first immobilized enzyme was reported by Nilson and Grifflin more than a century ago [23], while Chibata and co-workers developed the first industrial immobilized enzyme, *i.e., Aspergillus oryzae aminoacylase* for synthetizing racemic D–L amino acids [24]. In recent years, production of biodiesel using immobilized lipases has

attracted great interest. Significant progresses have been made on both of the immobilization techniques and process development for IL-mediated biodiesel production. An immobilized enzyme is defined as the enzyme physically confined to a certain defined region while retaining its most catalytic activity [25]. Similar to other immobilized enzymes, ILs show many advantages over FLs for the large-scale application in biodiesel production [26], such as easy recovery and reuse, higher adaptability for continuous operation, less effluent problems, greater pH and thermal stability, and higher tolerance to reactants and products. However, the current ILs still show several draw-backs for industrial applications, including: (1) loss of enzymatic activity during immobilization; (2) high cost of the carriers; (3) low stability in oil–water systems; and (4) requirement of novel reactors for well mixing and maximizing oil-to-biodiesel conversion.

2.1. Techniques for immobilization of lipase

2.1.1. Overview of lipase immobilization

Various techniques have been developed for lipase immobilization (Fig. 2) as intensively reviewed in some recently published papers [27, 28]. Generally, these techniques can be classified into three types: carrier bonding, cross-linking and entrapment. Depending on the type of interactions between enzymes and carriers, these techniques can be further classified into irreversible and reversible immobilization techniques [24].

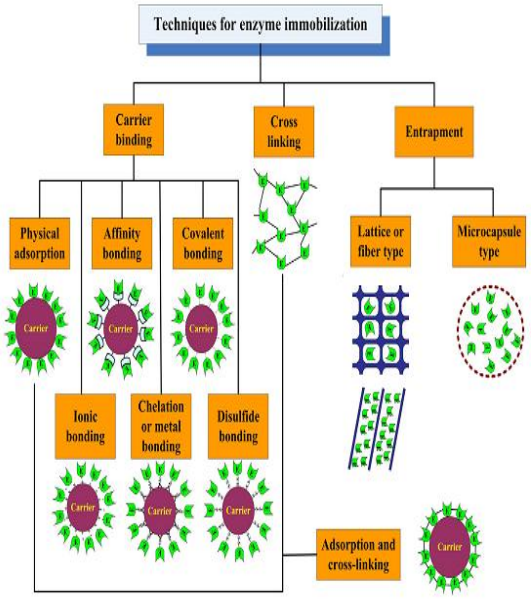

Figure 2: Various techniques for enzyme immobilization (adapted from [24, 26, 28]).

2.1.2. Immobilization of lipase by physical adsorption

Adsorption is a commonly-used method to immobilize lipase. Several non-covalent interactions are involved in this immobilization, including non-specific physical adsorption, bio-specific adsorption, affinity adsorption, electrostatic interaction (also ionic binding), and hydrophobic interaction [29]. Compared with other immobilization techniques, adsorption immobilization is advantageous in the following aspects [28]: (1) mild conditions and easy operation; (2) relatively low cost of carrier materials and immobilization procedure; (3) no requirement of chemical additives during adsorption; (4) easy regeneration of carriers for recycling; and (5) high lipase activity recovery.

2.1.3. Immobilization of lipase by ionic bonding or covalent bonding

In the immobilization process by ionic bonding, the enzymes are bound through salt linkages [24]. The carriers typically contain ion-exchange residues such as polysaccharides and synthetic polymers [26]. Mendes *et al*. [30] used anionic exchange resin MANAE-agarose to immobilize *Penicillium camembertii* Lipase G. They found that the procedure was quite fast, and the immobilization step was completed within 60 min resulting in protein immobilization up to 87% corresponding to 4.5270.18 mg protein g^{-1} carrier. However, the activity of the enzyme is

slightly decreased during this immobilization procedure. The ionic bonding process can be easily performed, but the interactions between lipase and carrier are much stronger than physical adsorption. Compared with covalent bonding method, ionic bonding can be conducted under much milder condition; therefore, the ionic binding method causes little changes in the conformation and the active site of the lipase, retaining lipase activity in most cases. However, the binding forces between enzymes and carriers are less strong than that of covalent binding, and leakage of enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH [26].

2.1.4. Immobilization of lipase by entrapment

Entrapment immobilization refers to the capture of enzymes within a polymeric network or microcapsules of polymers that allows the substrate and products to pass through but retains the enzyme (Fig. 2) [24, 28]. After entrapment, lipase proteins are not attached to the polymeric matrix or capsule, but their diffusion is constrained. Compared with physically adsorbed lipases, entrapment-mobilized lipases are more stable. Entrapment immobilization is relatively simple to perform than covalent bonding while the activity of lipases is maintained. However when entrapped lipases are used for biodiesel production, the conversion rate is relatively low. In addition, the entrapped lipases also show relatively low stability. The recent work by Jegannathan *et al*. [31] showed that *Burkholderia cepacia* lipase can be encapsulated into ê-carrageenan with an encapsulation efficiency of 42.6%. The encapsulated lipase retained 72.3% of its original activity after 6 cycles of hydrolysis of p-NPP. However, when the same type of lipase was used for transesterification reactions, the biodiesel yield decreased to only 40% after 10 cycles [32].

2.1.5. Immobilization of lipase by cross-linking

Immobilization of lipase by cross-linking refers to the process to immobilize the enzyme via the formation of intermolecular cross-linkages. It can be achieved by the addition bi- or multifunctional crosslinking reagents such as glutaraldehyde [26]. This immobilization technique is usually support-free and involves joining enzymes to each other to form a three-dimensional structure [33]. Lipase can be directly immobilized from fermentation broth and recovered as cross-linked enzyme aggregates (CLEAs). The formed CLEAs demonstrate significantly high stability in aqueous solutions within a broad range of pH and temperature values [34].

2.1.6. Commercialization of immobilized lipase for biodiesel production

Although many processes have been developed for immobilization of lipases in lab, only a few techniques have been successfully commercialized. The major roadblock for the technical transfer is the high cost of immobilization steps. For example, the market price of Novozyms® 435 is ~$1000/kg [35]. To reduce cost, the carrier must be easy to synthesize or commercially available at low prices. The immobilization process should be efficient for recovering proteins and retaining their enzymatic activities.

3. Food and dairy industries

In food and dairy industries, yeast and fungal lipases with high specificity have multiple uses including modification of fats and oils, synthesis of fatty acid esters, structure lipids, low calorie lipids and milk fat. Fats and oils are major constituents of diet, therefore low cholesterol and polyunsaturated fatty acid enriched oils and fats are needed to make them healthier. In this respect, a million tons of fats and oils undergo chemical processing such as hydrolysis, esterification, acidolysis, interesterification and transesterification [36].

These chemical processes require high temperatures and pressures and often introduce impurities due to these requirements [37, 38]. However, lipases require milder conditions such as lower temperatures, near neutral pH and are also regio- and chemo-selective for different fatty acids. Among all lipolytic conversions, esterification and interesterification properties of lipases are widely used to form value added products such as production of di- or mono-glycerides with special composition and specialty fats. Lipases used for this purpose include those from C. antarctica (Novozym 435), R. miehei (RM1M) [39, 40]. Both the enzymes are commercially available in soluble and immobilized form having high activity and stability under diverse conditions. In addition to this, many other modifications are performed to increase the nutritive value of oils and fats such as enriching oils with PUFA and oleic acid, synthesis of structural triacylglyceride, cocoa butter substitute, human milk fat substitute, directed interesterification of non-hydrogenated solid fats and synthesis of saturated triacylglycerides [36, 39, 40]. Besides

this, nearly all lipases display some degree of carboxylic acid selectivity. For example, lipase from G. *candidum* which mainly reacts with fatty acids having cis-9 configuration by factor of 20 or more and 100:1 oleic acid vs stearic acid. Similarly, *C. rugosa* and *R. delemar* lipase can discriminate erucic acid and c-linolenic acid and docosahexanoic acid during esterification, respectively [40]. This kind of property of lipase fascinates oil chemists to perform desired modification in fats and oil. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production. These requirements are influenced by the type and concentration of the carbon and nitrogen sources, culture pH and growth temperature [41]. Lipidic carbon sources generally seem to be essential to obtain a high lipase yield, although a few authors observed that the presence of fats and oils was not statistically significant for enzyme production [42]. The use of agro-industrial residues as the substrate could result in a reduction in the costs of lipase production, considering that the culture medium usually represents 25–50% of the total production costs [43]. Table 1 lists the different residues that could be used for microbial lipase production.

Table 1: Different agro-industrial residues for microbial lipase production in solid-state fermentation

Microorganisms	Agro-industrial residue used as the nutrient	Ref.
A. niger	Wheat bran, soapstock and stearin	Damaso *et al*. [44]
Aspergillus sp.	Wheat rawa, corn steep liquor	Adinarayana *et al*. [45]
Penicillium restrictum	Babassu oil industrial waste	Palma *et al*. [46]
Penicillium simplicissimum	Soybean meal	Vargas *et al*. [47]
Rhizomucor pusillus	Olive oil cake and sugar cane	Cordova *et al*. [48]
Rhizopus rhizopodiformis	bagasse	
Bacillus coagulans	Solid waste from melon	Alkan *et al*. [49]
Candida rugosa	Coconut oil cake	Benjamin and Pandey [50]

4. Enzyme production in a bioreactor

Kar *et al*. [51,52] investigated lipase production by Y. *lipolytica* in a 20-L batch reactor and different scaled-down apparatuses, which had been designed to reproduce the hydrodynamic phenomena encountered in large-scale equipment. Lipase production under oscillating dissolved oxygen tension significantly influenced the lipase gene expression. Furthermore, considering the nature of the substrate (lipidic) and the capacity for protein excretion and biosurfactant production of Y. *lipolytica*, intensification the oxygen transfer rate is accompanied by an excessive formation of foam, which was controlled using a mechanical foam control method [53]. This system was designed to induce the formation of a persistent foam layer in the bioreactor that led to the segregation of microbial cells, inducing a reduction in lipase yield. Fickers *et al*. [54] reported the development of a process for extracellular lipase production in a 2,000-L bioreactor that yielded a lipase activity of approximately 1,100 U/mL after 53 h of fermentation. This finding demonstrates that the lipase production was adequately scaled-up and that its yields were comparable to those of the bench scale process. Furthermore, batch, fed-batch and continuous operations in a stirred-tank bioreactor to produce lipase were investigated by Deive *et al*. [55] and the authors demonstrated the success of the continuous process by regular enzyme production, good operational stability and mathematical models that closely approximated the experimental results.

5. Detection and measurement of lipolytic activity

Several methods have been developed for the measurement of lipase activity in crude or purified lipase preparations. Rates of lipase reaction can be measured by determining either (a) the rate of disappearance of the substrate, the triglyceride (b) the rate of production of fatty acids or (c) the rate of clarification of emulsion [56].

5.1. Egg yolk as substrate

The lipase acts on the lipid moiety of lipo-protein (lipovitellenin) in egg yolk, resulting in alterations in the solubility of lipovitellenin. Modified Egg Yolk Agar, based on an egg yolk medium was developed by McClung and Toabe (1947) for the isolation and presumptive differentiation of clostridia based on lecithinase and lipase production and proteolytic activity [57]. Egg-yolk and lecithin LB-plates were used for screening of *E. coli* clones expressing lipase

variants with phospholipolytic activity [58]. Lipase activity of *Gardnerella vaginalis* was detected in 21 of 31 strains tested (68%) using egg yolk agar but using the 4-methylumbelliferyl-oleate MUO spot test only 12 of 31 strains tested positive (39%) [59].

5.2. Released glycerol from triacylglycerol

Direct determination of free glycerol is not commonly performed, since all three acyl chains of a triacylglycerolmolecule are rarely released by a single lipase, and therefore the initial hydrolysis rates cannot be determined. For estimating the released free glycerol, periodic oxidation of free glycerol, which leads to the formation of formaldehyde can be assayed spectroscopically [60].

5.3. Acoustic wave conductance

A surface acoustic wave sensor system for assaying the activity of pancreatic lipase has been proposed by Ge *et al*. (1995) [61]. The assay of this enzyme is based on the change in conductance of the solution caused by the release of a fatty acid, using triolein as a substrate.

5.4. Chromatographic methods

Various chromatographic techniques can be used to detect lipids as well as FFAs released from TAGs, namely florisil columns or silicic acid columns, thin-layer chromatography or gas–liquid chromatography [62].

5.4.1. HPLC methods

A high-performance liquid chromatographic (HPLC) assay for determining lipase activity was developed by Maurich *et al.* (1991) using β-naphtyl laurate, palmitic and lauric esters of p-nitrophenol as substrates. β-naphthyl laurate is incubated with enzyme and the formation of naphthol is quantified by reversed phase HPLC [63]. HPLC method was used for assaying phospholipase A2 (PLA2). The procedure is based on heptane–isopropanol–H_2SO_4 extraction of fatty acids released by the enzyme in the presence of margaric acid as an internal standard, and precolumn derivatization with 9-anthryldiazomethane. The derivatives of naturally occurring longchain fatty acids were accurately determined by reverse-phase HPLC with ultraviolet detection at 254 nm [64]. HPLC was used for the measurement of the product, (R)- and (S)- 2-hydroxy octanoic acid, when methyl or butyl esters of 2-hydroxy octanoic acid used as a substrate [65].

5.4.2. Gas chromatographic methods

GC may be used to separate and quantify the hydrolytic products of lipase [66]. This method is generally preferable to HPLC, primarily because HPLC suffers from the low sensitivity of available detectors. Transesterification activity and the industrial potential of a novel lipase prepared from Acinetobacter ventiatus RAG-1 were evaluated. Production of 1-octyl butyrate by lipase-catalyzed transesterification of vinyl butyrate with 1-octanol in hexane was monitored by gas chromatography [67].

5.5. Interfacial tension monitoring

As lipases are active at interfaces, it is possible to monitor enzyme activity by following changes in surface pressure. Cleavage of a monomolecular substrate film at the air–water interface causes the substrate to leave the interface, leading to reduced surface density and reduced surface pressure, which can be quantified [68].

5.6. Lipoprotein lipase ELISA kit

In acute pancreatitis, lipase levels can rise 5 to 10-fold within 24 to 48 h. BioAssay Systems' colorimetric lipase assay is based on an improved dimercaptopropanol tributyrate (BALB)method, inwhich SH groups formed fromlipase cleavage of BALB react with 5,5□ Œ-dithiobis(2- nitrobenzoic acid) (DTNB) to form a yellow colored product. The color intensity, measured at 412 nm, is proportionate to the enzymeactivity in the sample. The assay can be directly applied to serum, plasma, saliva, urine and other biological samples (Lipase QuantiChrom. Assay Kit from BioAssay Systems) [69].

5.7. NMR method

NMR could be used for quantitative analysis of lipase activity in biphasic macroemulsions [70].

5.8 Atomic force microscopy

Nielsen *et al*. (1999) suggested that atomic force microscopy (AFM) to be helpful in providing the picture of the kinetic of lipid degradation by lipases. The enzymatic hydrolysis of mixed bilayers of acylglycerols/phospholipids by Humicola lanuginosa lipase (HLL), was also investigated using AFM [71, 72].

5.9. Infrared spectroscopy

A continuous assay for measuring lipase-catalyzed hydrolysis of TAGs in reverse micelles using Fourier transform infrared spectroscopy (FTIR), was developed byWalde and Luisi (1989) for measuring the lipolysis of various substrates (trioctanoylglycerol, vegetable oils) [73].

5.10. Detection by electron microscopy

Fatty acids released in animal tissues by lipases can be detected by electron microscopy. This technique involves the incubation of tissues with triolein and then exposed to lead salts to form insoluble soaps, and finally processed for electron microscopy. Larger precipitates have been obtained by using Tweens instead of triolein, but the former are not specific lipase substrates [74].

6. Conclusions

Lipases are important enzymes that are widely studied for industrial applications, and different microbial lipase sources have been amply reported. The possibility of using these enzymes in nearly anhydrous organic solvents (and other low water media) has been an important driving force behind this. Now, with the discovery of catalytic promiscuity, with their role extended beyond that of hydrolases, we are witnessing second renaissance in their applications.

Lipases have been used in different hydrolytic and synthetic reactions, and the solvent systems determine the favored type of reaction. Tolerance, stability and compatibility to different reaction media are the key requirements for lipases. For lipases to be utilized in waste treatment, leather processing, flavor synthesis, biodiesel production, and detergent formulation, their unique properties and tolerance will aid in selecting and determining their effectiveness. The isolation of microbial strains from extreme environments assists in producing lipases with unique characteristics. Advances in biotechnology associated with cloning, expression, and mutagenesis as well as directed evolution contribute to conferring different properties to microorganisms. Thus, the ability of lipases to be stable in acid, alkali, salt, and organic solvents without deteriorating in ILs and detergents contributes immensely to their wide industrial application.

Lipases are versatile enzymes that can be used for various kinds of biocatalyzed reactions. Owing to their regioselectivity, their mild reactions conditions, they can be often considered as more interesting than classical chemical catalysts. Besides their application in oils and fats processes, these enzymes have proved to be very attractive for others lipase-catalyzed reactions with non-natural substrates. In particular they appear to be very effective for the synthesis of molecules involving the grafting of a lipophilic moiety or a hydrophilic one.

In such reactions, various parameters and strategies can be modulated in order to improve reaction yields and kinetics. Among these parameters, appropriate choice of reaction medium, control of water activity and water content of the systems, nature of acyl donor, subtract ratio appear to be the keys for optimized reaction rates and conversion yields. Recently, some new applications of lipases have been described in the field of the modifications of natural compounds such as phenolic acids or polyphenols. These biocatalyzed reactions intend to modify the hydrophilic/lipophilic properties of the initial molecules to obtain new products with multi-functional properties combining for example, microbial, antioxidant and emulsifying properties.

Although the literature on such lipase-catalyzed reactions is still scarce, it is expected to be further extended and give access to new products and bioactive molecules. Of course, a lot of work and studies are still to be done for a potential industrial application of such lipase-catalyzed reactions. The gap between the feasibility studies on the lab scale and the corresponding industrial development is very large. Notably, improvements in reactions yields or enzyme performances must be carried out. However, it appears evident that lipase applications will be more and more extended allowing the synthesis of very specific compounds with added values in various fields of food, pharmacy or cosmetic industry.

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