Chapter 15
Immobilized Lipase for Industrial Biodiesel Production

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Abstract The advent of biodiesel is set to usher in a new era of adaptive and environment-friendly fuel with depleting petroleum reserves. With the progressive developments, biodiesel is set to replace traditional petroleum diesel in most vehicles by 2040. Biodiesel produced from vegetable oil and alcohols by chemical catalysis poses a major threat, most notably in the form of health and environmental hazards. High yield, low cost, nonhazardous, and sustainable production of biodiesel is still a major challenge in this field. For an eco-friendly process, enzymes, primarily lipases, from various sources were tested as catalysts. The high costs associated with lipolytic transesterification were overcome by using immobilized enzymes with higher stability, reusability, and better convertibility. With its wide array of options, lipase has the most attractive prospects for commercially and industrially feasible biodiesel production in the coming decades. This chapter primarily focuses on the present status and prospects of immobilized lipase for biodiesel production. This study, therefore, seeks to emphasize the ultimate need of producing high-quality, cost-effective, readily extractable, sustainable, and environment-friendly biodiesel that strictly complies with ASTM standards and proper utilization of its by-products for different industrial applications.

Keywords Biodiesel · Enzyme · Lipase · Renewable · Sustainable

15.1 Introduction

Today, the energy crisis has become a global challenge. We heavily rely on fuels in day-to-day life, especially to transport goods and people. Petroleum and natural gas are primary energy resources to fulfil worldwide demand. Most industries use diesel engines for their respective production processes. Various modes of transport consume significant amounts of gasoline and diesel. However, the increasing demand

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cannot be met by the production and supply of domestic crude oil. Fossil fuels take a long time to form, and therefore, the corresponding fossil oils are nonrenewable energy sources. Projections show that fossil fuels will be completely consumed in just another 65 years, especially owing to the development and application in developing countries (Huang et al. 2011).

Additionally, burning fossil fuels leads to emissions which glaringly contribute to the pressing matters of air pollution and global warming (Agarwal 2007; Chowdhary et al. 2020; Chowdhary and Raj 2020). Among various alternative and renewable fuels, biodiesel reduces the dependency on fossil fuels and shows promising results in terms of its environment-friendly nature (Huang et al. 2011). Chemically, biodiesel is a mixture of fatty acid monoalkyl esters and can be used in any diesel engine with little or no modifications (Gog et al. 2012). Characteristics of biodiesel are ultimately similar to petro-diesel, and biodiesel-diesel blends with different proportions are stable (Agarwal 2007).

15.1.1 Historical Background

In 1893, Rudolf Diesel used peanut oil as fuel for his engine. In remembrance of that eventful day, International Biodiesel Day is celebrated on 10th August every year. With time, as diesel fuels and engines evolved together, during the 1930s and 1940s, vegetable oils were only used as emergency fuels. Escalating crude oil prices and depleting fossil oil resources grab our attention once again toward vegetable oils to produce biodiesel (Ma and Hanna 1999).

15.1.2 Advantages of Using Biodiesel

Biodiesel is a nontoxic and biodegradable fuel. It is produced from renewable sources and emissions of particulate matter and greenhouse gases (CO, CO2, and SOx) are extremely low after its combustion (Tan et al. 2010). The degradation of biodiesel is much faster than petro-diesel. The exhaust has considerably less smoke compared to a diesel with a better smell, higher lubrication greater cetane number (Li et al. 2012).

With consistent price surges and adverse environmental impact owing to toxic emissions by diesel, many countries are making new guidelines to use biodiesel. Biodiesel can be produced from locally available feedstocks and therefore provides energy security. A higher amount of oxygen (about 10 wt%) facilitates complete combustion of the fuel with a reduced number of pollutants. With a high flash point of around 150 °C, biodiesel is safe for transportation and storage. It is a sound alternative as it can be readily blended with petroleum-based fuels as well as used in pure form, with little or no modifications to existing engines (Guldhe et al. 2014). Cancer-causing elements such as sulfur and PAH (polycyclic aromatic hydrocarbons) are almost absent in emitted smoke (Pourzolfaghar et al. 2016; Bünger et al. 2000; Huang et al. 2011).

The amount of CO_2 absorbed by plants is higher than that discharged after the burning of biodiesel. Therefore, biodiesel can markedly reduce CO_2 emission levels and maintain the ecological balance (Lapuerta et al. 2008). Additionally, biodiesel carries very little sulfur and thus, emission of very low SO_2 during combustion (Basha et al. 2009), effectively reducing acid rain that avoids infrastructural and environmental damages in the form of soil, surface and groundwater acidification, corrosion, and loss of vegetation. Nitrogen oxide emissions may slightly increase if the engine mechanism remains the same, but it can be readily controlled using certain software and biodiesel sensors.

Biodiesel is certainly the focus and the need in this century primarily considering the emission reductions, general positive environmental impact, and fast depleting fossil fuels.

15.1.3 Properties of Biodiesel

15.1.3.1 Antifoaming

Due to vegetable origin, biodiesel poses better antifoam properties that facilitate oil refilling more efficiently and reduced loss (Abbaszaadeh et al. 2012).

15.1.3.2 Cetane Number

The cetane number varies from 45 to 70 depending on the fatty acid distribution in original fats or oils, as compared to petroleum diesel, which varies from 40 to 52 (Abbaszaadeh et al. 2012). Cetane number is an index of flammability and therefore, biodiesel has better flammability (Huang et al. 2011).

15.1.3.3 Amount of Oxygen Present

Usually, 11% oxygen is present in biodiesel. The high oxygen content leads to complete combustion and reduced emissions, and the polarity provides features such as solvency, detergency, wet ability, and conductivity. On contrary, petro-diesel does not contain oxygen (Abbaszaadeh et al. 2012). However, this high concentration of oxygen arises a critical question on the oxygen stability of biodiesel and sometimes damage vehicle parts. Biodiesel is typically used as blends with petroleum diesel, and hence it rarely causes such aforementioned problems (Demirbas 2009).

15.1.3.4 Cold Flow Properties

Solidification is a gradual process in diesel as each component has its dew points, whereas pure biodiesel is a much simpler mixture, which leads to rapid solidification that becomes difficult to control (Abbaszaadeh et al. 2012). This is one of the reasons why biodiesel blends are commonly used instead of pure biodiesel.

15.1.3.5 Flash Point and Viscosity

Biodiesel has a high flash point which enables convenient and safe transportation. It also has high viscosity (within the permissible value set by ASTM) and is composed of highly unsaturated fatty acid methyl esters (Park et al. 2008).

15.1.3.6 Lubrification

Biodiesel has good lubricating properties which reduce the water flow rate in the injection pump, cylinder, and engine, thereby extending the useful lifespan of the engine (Huang et al. 2011).

15.2 Biodiesel Production

Biodiesel is produced by transesterification of triglycerides with an acyl acceptor, such as alcohol (Ruzich and Bassi 2010). Methanol is primarily used due to its lower cost compared to other alcohols. Hence, fatty acid alkyl esters (FAAEs) are generally referred to as fatty acid methyl esters (FAMEs). The catalysts are categorized as chemical or enzymatic based on their use (Tan et al. 2010). Sources include various edible (such as palm, rice bran, sunflower, and canola and soybean oils), nonedible (such as *Jatropha*, Datura metel, rubber, and mastwood oils), animal fats and tallow, waste cooking oil, or algal lipid. Cheaper, nonedible feedstocks are used for production instead of edible ones to alleviate the food crisis (Tan et al. 2018).

15.2.1 Biodiesel Production Techniques

Among various techniques (pyrolysis, microemulsions, and transesterification) used for biodiesel production, the transesterification method also facilitates the reduction of oil viscosity on an industrial scale that can be suitably used in the diesel engine.

15.2.1.1 Direct Use and Blending of Oils

Since more than 100 years ago, vegetable oils have been potentially used as fuel alternatives which began with Dr Rudolph Diesel testing peanut oil in his diesel engine. While there are advantages like liquid nature portability, high heat content, availability, and renewability (Ma and Hanna 1999), vegetable oils if used directly in engines cause serious problems and failures. Biodiesel-diesel blends improve the viscosity and avoid such issues in compression ignition engines.

Some of the commonly associated problems include high viscosity, free fatty acid content, high flash point, and heavy smoke generation during use (Athar and Zaidi 2020). Significant engine modifications are required when utilizing vegetable oils that will otherwise reduce engine life and increase maintenance costs (Abbaszaadeh et al. 2012).

15.2.1.2 Microemulsion of Oils

The formation of emulsions is a way to lowering the viscosity of oils. A microemulsion is a colloidal mixture of water, oil, and an amphiphile that is thermodynamically stable (Mubarak and Ahmad 2020). These fuels are sometimes also termed hybrid fuels. Fukuda et al. (2001) reported that although micro-emulsification lower the viscosity of oil, caveats include carbon deposits, incomplete combustion, and uneven injector needle sticking.

15.2.1.3 Pyrolysis of Oils (Thermal Cracking)

Pyrolysis is the breakdown of a large organic substance into a smaller one via heating with the aid of a catalyst (Mohan et al. 2006). Thermal cracking is a promising technology for biodiesel production using triglycerides (Yusuf et al. 2011). Pyrolysis of triglycerides has been reported to be very suitable for diesel engine applications. Pyrolysis of oil was reported both with and without catalysts (Maher and Bressler 2007). The equipment required is quite expensive. Additionally, while the resulting product is similar toppers-diesel, the removal of oxygen in the process reduces the environmental benefits as a fuel (Abbaszaadeh et al. 2012).

15.2.1.4 Transesterification of Oils

Transesterification (or alcoholysis) of oils (triglycerides) with alcohols provides biodiesel (fatty acid alkyl esters, FAAE) as the major product and the glycerin as a by-product (Abbaszaadeh et al. 2012). The basic mechanism of the reaction is presented in Fig. 15.1 (Barnwal and Sharma 2005).

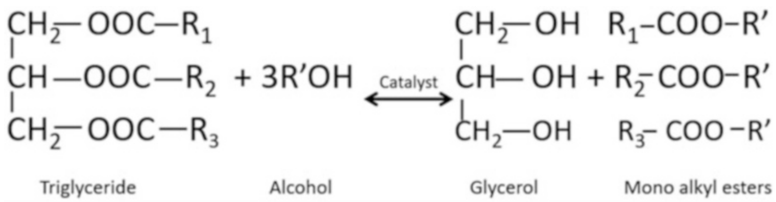

Fig. 15.1 Transesterification reaction. One mole of triglyceride reacts with three moles of alcohol to produce one mole glycerol and three moles of esters (Abbaszaadeh et al. 2012)

Although the reaction can proceed with or without a catalyst (Demirbas 2009), to increase the reaction rate and improve the contact between alcohol and triglycerides, catalysts are often used. Research is being conducted worldwide to produce biodiesel without using any catalysts to overcome the problems associated with catalytic reactions (Abbaszaadeh et al. 2012).

15.3 Catalytic Biodiesel Production

Biodiesel can be produced by homogeneous and heterogeneous catalysts. When both the catalyst and reactants are in the same (liquid) phase, the process is called homogeneous catalytic transesterification, and when they are in a different phase (solid catalyst, liquid reactant), it is known as heterogeneous catalytic transesterification (Abbaszaadeh et al. 2012). The selection of the catalyst is critical to make the biodiesel production process cost-effective (Sharma et al. 2008).

Biodiesel is commercially produced using a homogeneous catalyst. A major factor dictating the type of catalyst to be used is the amount of free fatty acid (FFA) present in the oil. Base-catalyzed reactions are used with feedstock having low FFA content while an acid-catalyzed reaction is more suitable for oils with higher FFA content (Schuchardt et al. 1998). Enzymatic reactions are independent of FFA content, and therefore, used cooking oil can be used as feedstock for biodiesel production using an enzyme as a catalyst (Hsu et al. 2001).

15.3.1 Homogeneous Catalysts

Quality of raw material and purification of the product are two major challenges for the homogenous acid or base catalytic transesterification process (Abbaszaadeh et al. 2012). The following sections describe the two processes in detail.

15.3.1.1 Homogeneous Base Catalytic Transesterification

Biodiesel is generally produced in the presence of base catalysts like sodium and potassium hydroxides and carbonates and by using alkaline metal alkoxides. They are popular in the industry due to high conversion rates, high catalytic activity, and economical availability (Kawashima et al. 2009). However, this process poses problems due to certain parameters like reactant purity, FFA content, and water concentration. The high amount of free fatty acids present in the feedstock reacts with the base catalyst to produce soaps (saponification) that inhibit the reaction and reduce biodiesel yield (Meher et al. 2006; Enweremadu and Mbarawa 2009). For efficient production of biodiesel, both saponification and hydrolysis reactions need to be reduced (Abbaszaadeh et al. 2012).

15.3.1.2 Homogeneous Acid Catalytic Transesterification

The acid catalytic process, though less commonly used, can be used as an alternative to the base catalytic process if cheap feedstocks are used. Sulfuric acid, sulfonic acid, and hydrochloric acid are commonly used as a catalyst. In acid-catalyzed transesterification, acid is first mixed with alcohol and then the oil is mixed with the acidified. In this process, the alcohol acts both as a solvent and as an esterification reagent for a single-step process (Cerveró et al. 2008). Usage of excess alcohol adversely affects the reaction times. Acid catalysts are specially used where the FFA content in the feedstock is high. The disadvantages are numerous: corroded equipment increased waste due to neutralization, low recycling, long reaction times, higher temperatures, and inefficient catalytic activity (Goff et al. 2004).

15.3.2 Heterogeneous Catalytic Transesterification

The high energy consumption and costly separation in the homogenous catalysis led to the advent of heterogeneous catalysts. Their usage does not yield soap (Wang and Yang 2007), and as they are in a different phase, separation and reuse become easy. The product recovery step is eliminated, ensuring higher efficiency and significant cost reduction. The heterogeneous process reduces the risk of releasing dangerous hazardous and flammable chemicals due to leakage. With the absence of the energy-intensive purification step thereby avoiding waste generation, additional environmental benefits are observed (Abbaszaadeh et al. 2012).

15.3.2.1 Heterogeneous Solid-Base Catalytic Transesterification

The heterogeneous solid catalysts facilitate easy separation in a fixed bed reactor system. Some inexpensive and readily available solid-base catalysts such as calcium oxides, hydrotalcite, magnesium oxides, zeolites, alkaline earth metals, etc. are extensively used. The low solubility of the metals in the substrate makes them suitable for industrial biodiesel production (Abbaszaadeh et al. 2012).

15.3.2.2 Heterogeneous Solid-Acid Catalytic Transesterification

Although heterogeneous solid-acid catalysts have lower activity, they have been widely used for biodiesel production. Commonly used solid-acid catalysts include Nafion-NR50, sulfated zirconia, and tungstate zirconia. They bear advantages such as low susceptibility to FFA content, single-step process, purification step elimination, easy separation, and reduced corrosion (Abbaszaadeh et al. 2012).

15.4 Feedstocks Used in Transesterification

Biodiesel is produced from various edible oils such as rapeseed oil, soybean oil, palm oil, cottonseed oil, sunflower oil, etc. *Jatropha* and mahua are nonedible oils, which are used in areas where edible oils are not abundant. Algal oils, especially microalgae, are most prospective as they carry the highest potential oil yield: 250 times more per acre as compared to soybean and can be quickly grown (Athar and Zaidi 2020).

Oil composition and yield are important for selecting feedstock. Edible oils are more than 95% of total feedstocks used for biodiesel production. Tallow, poultry, and lard fats have been investigated for potential utility while recycled oils, grease, and waste oils have also been used as feedstock. The current challenges in the industrial application of microalgae are economical cultivation, biomass harvesting, and efficient lipid extraction techniques (Guldhe et al. 2014). About 70% of the biodiesel production cost is attributed to the substrate (Abbaszaadeh et al. 2012), and hence due care needs to be taken during the selection of feedstock.

15.5 Modern Catalysts for Biodiesel Production

Chemically catalyzed processes are energy-intensive and inefficient in terms of separation and recovery. Additionally, acidic and alkaline wastewater is generated that requires extra energy and processing while acid catalysts also corrode equipment (Guldhe et al. 2014).

Efforts are being made to reduce and recycle wastes. This has led to biological alternatives such as lipases replacements for chemical catalysts, owing to their properties such as substrate and functional group specificity, chemobiological activity, and enantioselectivity (Quayson et al. 2020). Enzymatic production of biodiesel is economical and efficient. It produces less waste and requires milder operating conditions (Meunier et al. 2017).

15.5.1 Biocatalytic Transesterification

Biocatalysts are naturally occurring lipases with potential usage in biodiesel production. Ease of product removal, moderate process conditions (Abbaszaadeh et al. 2012), and reusability especially with immobilized lipase (Ribeiro et al. 2011) are some of the advantages of transesterification using lipase over chemical catalysts. Enzymatic reactions do not depend on feedstock's FFA content, and thus used cooking oils can also be used as raw material (Hama et al. 2004). However, inhibition of lipase by short-chain alcohols, high cost, and presence of by-products and other impurities (Ribeiro et al. 2011) is a major concern for reaction.

15.6 Lipase

Lipase is the catalyst used for transesterification, biodiesel purification, ease of by-product removal, low energy consumption, low waste generation, etc. owing to its high specificity, selectivity (Narwal and Gupta 2012), and stability in nonaqueous medium (Villeneuve et al. 2000). Biodiesel production using lipase requires less alcohol and does not lead to any side reactions. The cost of preparation of the enzyme is a pressing issue but it can be resolved by immobilization which significantly reduces the cost and increases reusability (Narwal and Gupta 2012; Chandra and Chowdhary 2015). Lipase catalyzes the transesterification reaction in a two-step process: it hydrolyzes the ester bond present in fatty acids, converting triglycerides into diglycerides, and then the alcohol acts as an acyl acceptor forming an ester (Bhan and Singh 2020).

15.6.1 Sources of Lipase

Lipases are isolated either from plants or animals and can be produced by microbes (Sanchez et al. 2018). Among plants, the enzyme can be extracted from the seeds of castor bean and canola (Ribeiro et al. 2011), maize and barley, and the latex of papaya (Bhan and Singh 2020). Porcine and human pancreatic lipases are mostly investigated. Lipase can be found in the energy reserve tissues of many plants

(Ribeiro et al. 2011). Presently, sources like oil mill effluents, hot springs, shrimps, paper mill wastewater, etc. have been identified (Quayson et al. 2020).

15.6.2 Notable Properties of Lipases

The lipase specificity makes them a good candidate for biodiesel production. Lipases are grouped based on the position of free fatty acids they attack in a triglyceride molecule. 1,3-Specific lipases act on the ester linkages at the end; position 2-specific ones attack the middle ester bond, while nonspecific lipases can attack ester bonds present at any location and are generally not useful for that very reason (Ranganathan et al. 2008). Lipases generally are pH stable and thermally stable which are important factors in the transesterification process. High costs involved with lipases are countered by techniques to recover them and increase reusability while ensuring high activity and stability (Chioke et al. 2018).

15.6.3 Immobilized Lipase

The high cost of lipase is the major challenge for industrial biodiesel production using an enzyme. Arrangements need to be made to enhance prolonged activity, and the lipase has to be solvent and substrate tolerant (Meunier et al. 2017). Lipases are attached to solid carriers or modified into aggregates. This technique is known as lipase immobilization. It allows the lipase to withstand various inhibitory factors besides lowering the costs involved through reusability (Quayson et al. 2020).

Several studies have been done to screen and immobilize the lipases. The immobilized lipase was found to react slowly but ended up being more reusable, thermally stable, resulted in higher conversion, and was unaffected by inhibition when compared to free lipase (Meunier et al. 2017).

15.6.4 Enzyme Immobilization Methods

Certain popular lipase immobilization techniques include adsorption, entrapment, encapsulation, covalent bonding, and cross-linking (Tan et al. 2010). The methods can be categorized under physical and chemical processes, as per the interaction between the enzyme and support. The physical methods comprise of interactions through weaker bonds such as van der Walls interactions and hydrogen bonds, facilitating reversible interactions. On the other hand, the chemical methods render the process irreversible on account of stronger interactions through covalent bonds (Filho et al. 2019).

15.6.4.1 Physical Methods

15.6.4.1.1 Adsorption

While adsorption immobilization is primarily a physical process, it simultaneously involves chemical interactions such as Hydrogen bond, Van der Waals forces or acid-base bonding. It is the most commonly used technique owing to its reversibility. Surface area and porosity are two important parameters, and hence carrier particles like activated carbons, silica gels, acrylic resins, polyurethane foams (Quayson et al. 2020), textile membrane, Toyonite 200-M, polypropylene EP 100, celite, hydrotalcite, and anion resins are generally used (Jegannathan et al. 2008).

For successful adsorption, temperature, pH, ionic strength, and agitation rate need to be optimized. The advantages of this method are simplicity, fewer reagent requirements (Quayson et al. 2020), ease of preparation, low costs, and unlike entrapment and cross-linking. As the enzyme is present mostly on the surface, the process is not limited by internal mass transfer resistance (Jegannathan et al. 2008).

The conversion of waste cooking oil to biodiesel using adsorbed lipase was reported to be more than 80%. Among the most frequently used lipases, Novozym 435 and *Candida* sp. 99–125 are industrially important and are capable of converting vegetable oil with yield more than 90% and 87%, respectively (Meunier et al. 2017). They show excellent reaction ability in the presence of t-butanol. Leaching of lipases is a major drawback here. Lack of long-term attachments reduces reusability (Sheldon 2007). Due to weak interaction forces, enzymes are desorbed from matrix surfaces frequently, and that creates a serious challenge for its commercial use (Jegannathan et al. 2008).

15.6.4.1.2 Entrapment

Entrapment detains lipases within a polymeric matrix. Unlike other techniques where the lipase is bound to the carrier surface, entrapped lipase can freely move inside the polymer network. Collagen, alginate, agar, and zeolites are preferably used as a matrix for entrapment. Silica sol-gels are quite cheap, achievable at room temperature, and have been successfully employed (Quayson et al. 2020).

The entrapment method has advantages similar to adsorption as it is fast, inexpensive, easily executed, and generally occur at room temperature (Tan et al. 2010). The entrapped lipase is found to be more stable than adsorbed one. The major drawback related to entrapped lipase is due to poor diffusion of the substrate, which in turn reduces conversion rate (about 70%) (Jegannathan et al. 2008). Precise process control is still a bottleneck associated with both entrapment and encapsulation methods (Orçaire et al. 2006).

15.6.4.1.3 Encapsulation

The enzyme is confined within a two-layered porous membrane (Jegannathan et al. 2008), which allows the interaction of substrates and products. Unlike adsorption, encapsulation shields the enzyme from its direct contact with the medium, thereby reducing potential inactivation. Additionally, it provides stability to the enzymes for relatively long periods and extraction of enzymes from the medium becomes unnecessary (Filho et al. 2019).

It also avoids leaching by providing a cage. At the same time, the problem arises due to the blocking of pores by enzyme aggregates that in turn increase mass transfer resistance of the substrate. A smaller-sized encapsulated enzyme needs to be used to overcoming this problem to avoid clogging (Jegannathan et al. 2008).

15.6.4.2 Chemical Methods

15.6.4.2.1 Covalent Bonding

Covalent bonds are formed during reactions between the carriers and some amino acids of enzymes such as cysteine, lysine, or aspartic and glutamic acid residues (Filho et al. 2019). After addition, functional groups are activated for attachment of the amine residue. Silanization and grafting are certain techniques used with aldehydes and epoxy groups being the common activation reagents. This method is often used along with other immobilization techniques (Quayson et al. 2020). Covalent immobilization provides enzymatic stability and ensures rigidity in structure. This rigidity provides irreversibility and the enzyme remains unchanged against any form of denaturing agents such as heat, extreme, and organic solvents (Filho et al. 2019).

15.6.4.2.2 Cross-Linking

Enzyme molecules are cross-linked using reagents such as glutaraldehyde. Such enzyme aggregates are immobilized matrix-free preparations (Jegannathan et al. 2008). It increases stability in enzymes that do not require support for binding. The primary function of cross-linkers is the protection of the enzyme from the external environment. Major observed advantages of cross-linked enzymes are high activity and stability, low cost of production due to support exclusion, and a versatile procedure (Filho et al. 2019).

Cross-linked enzymes were found to accelerate the reaction rates and a 92% yield has been obtained. A drawback in the case of cross-linked enzyme aggregates (CLEA) is that their size is in the same range as substrate particles in heterogeneous systems, thereby creating difficulties in removing enzymes from the product, limiting continuous usage (Jegannathan et al. 2008).

15.6.5 Factors Affecting Biodiesel Production Using Immobilized Lipase

Biodiesel production through lipase-immobilized transesterification is notably influenced by factors such as the lipase source, feedstock type, immobilization methods, (overmentioned factors covered in earlier sections) solvent and alcohol type, temperature, water content, and the molar ratio of the used alcohol.

15.6.5.1 Solvents

The presence of a solvent lowers the viscosity, thereby leading to better interaction between the lipase and the substrate, consequently increasing mass transfer (Sankaran et al. 2016). Organic solvents are generally not suitable for enzymatic reaction due to toxicity, adverse effects on the environment, solvent flammability, and further requirements for its removal. Solvent-free systems have therefore been developed to increase process efficiency (Narwal and Gupta 2012). Hydrophobic organic solvents improve activity by allowing aggregation of water molecules around the enzyme and hence are favored in comparison to other organic solvents. Most recently, researchers have found supercritical CO2 and ionic liquid to be alternative solvents that can enhance the production of biodiesel (Sankaran et al. 2016).

Fruitful observations were noted during biodiesel production using cottonseed oil methyl ester. t-Butanol, a partially polar solvent, was found to be more efficient than the other organic solvents. The transesterification reaction was catalyzed by pancreatic lipase, resulting in conversion rates up to 80%. The product was blended with petroleum diesel in 1:4 ratios to B20, which was readily suitable as per ASTM standards (Chattopadhyay et al. 2010).

15.6.5.2 Alcohol Type

Reaction yields are higher when ethanol is used as compared to methanol. This can be reasoned in a way that lipases are more tolerant toward ethanol or lipases have a better effect on long-chain alcohols than short-chain ones. Another reason for preferring ethanol could be that it is less hazardous and can be made from plenty of renewable resources. Economically, methanol is most commonly used for the commercial production of biodiesel. Other alcohols that can readily function as alkyl donors besides methanol and ethanol are propanol and butanol (Narwal and Gupta 2012).

15.6.5.3 Temperature

The activity is found to increase with an increase in the temperature. In most cases, enzymes get denatured when exposed to extreme temperatures but most lipases are thermally stable with high optimal processing temperatures (Marchetti et al. 2008). Immobilization further increases thermal stability. The optimum temperature for a reaction is in turn influenced by other factors such as intrinsic stability of the lipase, alcohol-to-oil molar ratio, and the type of solvent (Chioke et al. 2018).

15.6.5.4 Effect of Water Content

The amount of water is important in biodiesel production and following the observations has been noted. Enzyme activity is low without water. Therefore, for the enzyme to function properly, a very small amount of water is required. A gradual increase in the water content enhances the enzyme activity and a considerable increase is observed in the produce. After a certain stage, the activity decreases with an increase in water content (Narwal and Gupta 2012). Water strongly influences the stability besides the catalytic activity of the lipase. More than an essential amount of water can fiddle with the process and influence the equilibrium. Water can improve lipase activity by increasing the interfacial area (Tan et al. 2010). However, excessive water content weakens the enzyme support through gradual flooding of the pores, making it difficult for the substrate to access the enzyme and reducing activity (Sankaran et al. 2016). It increases flexibility in the lipase and can lead to certain unintended side reactions such as hydrolysis (Tan et al. 2010).

15.6.5.5 Inhibiting Lipase Inactivation Caused by Short-Chain Alcohols

Polar short-chain alcohols render lipases inactive and it becomes a major hurdle in biodiesel production. Researchers have found the following three solutions to overcome this problem: (1) stepwise methanol addition, (2) change in acyl acceptor, and (3) using different solvents for production (Tan et al. 2010).

15.6.5.5.1 Methanol Stepwise Addition

The strategy of adding methanol stepwise has been used for many years and is a primary choice as it leads to high yields (>87%) under simple operating conditions like two- or three-step methanol addition. The major advantage here is that a significantly high yield can be obtained without inactivating the lipase. However, on large scale, this method can become quite complicated (Shimada et al. 1999; Soumanou and Bornscheuer 2003; Chen et al. 2006; Lu et al. 2007).

15.6.5.5.2 Acyl Acceptor Alterations

This method seeks to avoid lipase inactivation due to methanol by replacing it with methyl acetate or ethyl acetate. The yield observed here is even higher than the methanol stepwise addition. (>90%) Additionally, glycerol is not produced here but the low reaction rate and high cost of the acyl acceptor become huge obstacles for industrial production (Xu et al. 2003).

15.6.5.5.3 Solvent Engineering

Suitable solvent selection can be used to improve methanol solubility and inhibit the inactivation caused by insoluble methanol. t-Butanol has been thoroughly studied and found to be a good solvent with yields >80%. Glycerol deposits are readily avoided but recovering the solvent becomes difficult and adds to the cost thereby adversely affecting the yield and limiting industrial application (Ha et al. 2007; Iso et al. 2001; Royon et al. 2007).

15.6.6 Applications of Immobilized Lipase

Microlipases are most sought after in industries these days. The market for microbial lipases is expected to rapidly expand in the future. Their versatility is readily demonstrated by a plethora of industrial applications such as in food, soap, detergent, oil, fat, cellulose, paper, textile, leather, cosmetics, and last but not the least, biodiesel which will drive the future. New technologies are continuously developing for an increase in production and conservation for its reusability.

In the food industry, lipases are useful for increasing the organoleptic characteristics of various products. They are used for the enhancement of flavor in flavored dairy products such as cheese, milk, and butter, increase in shelf life in baking, development of aroma in beverages, improvement of mayonnaise quality, process of cocoa butter, and others. Additionally, sensors based on lipase and immobilized lipases are used for food quality checks, especially for detecting the presence of triacylglycerols. Such sensors can also detect pesticides and other such environmental pollutants.

Lipase and phospholipase sensors find use in the medical industry in the form of diagnostic tools for detecting triglycerides levels, phospholipids, and cholesterol in the blood. The biosensor has additionally shown great results in determining tributyrin in human serum and will be potentially useful in critical applications ahead by providing quick, effective, safe, and inexpensive results. Immobilized lipases can improve the biodiesel production process by increasing the yield, improving the stability of the enzyme, and effectively reducing costs through reusability (Filho et al. 2019). The process continues to be the field attracting the most research interest.

15.7 Non-catalytic Biodiesel Production

Catalytic production of biodiesel is commonly used on an industrial scale, but it comes with certain major drawbacks such as treatment of FFA and triglycerides during the reaction, production of wastes, generation of wastewater (Aransiola et al. 2014), necessary purification of product, and removal of unreacted catalysts (Abbaszaadeh et al. 2012). Since the non-catalyzed procedures are inherently absent of such disadvantages simply due to catalyst absence, they are automatically explored.

Some types of non-catalytic production techniques are the supercritical fluid method, Biox process, microwave process, and membrane technology. Transesterification using supercritical alcohol is one of the most explored non-catalytic ways of producing biodiesel. This reaction allows a good conversation with a faster reaction rate as it is insensitive to free fatty acid and promotes both hydrolysis and esterification reactions simultaneously with transesterification (Athar and Zaidi 2020). Biox process utilizes a cosolvent which is usually tetrahydrofuran (THF), generating a single-phase oil-rich system. In the microwave process, polar ends of the molecules continuously oscillate due to microwave irradiation causing collision and friction between them, thereby leading to localized superheating. *Membrane technology t*akes advantage of the immiscibility of methanol and oil by combining the reaction and membrane-based separation for better purification (Xuan Tan et al. 2018).

15.8 Comparison of Production Techniques

Chemical catalysis has many limitations such as high energy and capital requirement, difficult recovery and purification, high FFA content, adverse effects of the side reactions on biodiesel yield and quantity, and expensive treatment of wastewater. Lipases are contrarily well off in the overmentioned issues. They can function both in monophasic and biphasic media. They have high activity, are thermally stable robust and versatile enzymes, and can tolerate short-chain alcohols, and their separation is easily done using downstream processing. The glycerol produced through transesterification using lipase enzyme contains low impurities and water content facilitating ease of product separation.

Most notably, lipases are eco-friendly and are selective as well as specific. They can tolerate high water content in the oil and improve biodiesel yield by avoiding saponification. They require low alcohol to oil molar ratios and lesser energy than chemical catalysts with minimal wastewater generation and lead to the production of high-grade glycerol (Chioke et al. 2018). The glycerol produced is quite pure and requires low pretreatment for usage in drug and food products. Additionally, biodiesel production using chemical catalysts requires high temperatures for optimum activity, while lipases can function properly even at room temperatures (Quayson et al. 2020).

Lipases are also associated with certain limitations. They can be very expensive and need to be immobilized for reusability as they become unstable after one-time use. The reaction rates in lipase catalyzed processes are low due to enzyme inhibition which requires further investment to overcome (Gog et al. 2012).

15.9 Conclusion

Shortly, *Jatropha* oil, microbial oil, and microalgae are all set to become the primary feedstocks for biodiesel production. Ethanol is found to be the best acyl acceptor, but the quantity used should be within the solubility limit, so that it is not present in a separate phase. Stepwise addition is therefore highly used. Immobilized lipase-catalyzed reactions are the biggest research attraction but have been consistent due to the high production cost. The conventional biodiesel production methods using chemical catalysts like acids and bases have various drawbacks and requirements which are overcome by replacing them with enzymes, particularly lipases. Enzymatic production overcomes such difficulties and offers advantages like simplified separation and waste reduction, besides being environment-friendly. The enzyme needs to be immobilized to overcome costs and make the process economical by increasing the stability and reusability of the enzyme by many folds.

Each immobilization technique has its respective advantages and disadvantages, and therefore, the selection of a suitable technique is important for making effective usage of the enzyme. Some are relatively easy and cheap but activity and stability cannot be overlooked. Production of biodiesel using enzymatic methods has immense potential especially owing to immobilized lipase. Efforts are being made to further cut down costs and increase enzymatic activity and stability.

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