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*Review*

# Lipase in biodiesel production

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**Although fossil fuels have been serving mankind for several centuries, their exploration and use bring about several environmental problems. Besides, fossil fuels are neither renewable nor sustainable. There is therefore a need to search for renewable alternatives which are sustainable and environmentally friendly. Biodiesel, chemically known as methyl ester, is a renewable and sustainable biofuel used to run diesel engines and it is mainly produced by transesterification through either chemical or enzymatic catalysis. Enzyme catalyzed transesterification has several advantages over chemical catalysis. However, enzyme production and purification are expensive and the inability to recycle these enzymes has limited their application in large scale bioconversion processes. This review highlights some of the strategies employed to reduce the cost of lipase catalzsed transesterificaion, some important feed stocks used in biodiesel production, methods of processing biodiesel, and advantages of enzymatic transesterification over chemical catalyzed transesterification. The properties and sources of lipase enzymes used in enzyme catalyzed transesterfication are also highlighted and discussed. Factors that affect enzyme catalyzed transesterification are also discussed. It also highlights the possible methods of solving the problems encountered in enzymatic transesterification.**

**Key words:** Biodiesel, transesterification, lipase, enzymes, catalysis, immobilization.

## INTRODUCTION

There has always been an increase in demand for fossil fuels due to an increase in the global human population and industrialization. However, fossil fuels are not sustainable due to their non-renewable nature. There is therefore, an urgent need for renewable, sustainable and environmentally friendly alternative sources of fuels. Various types of bio-energies have been extensively investigated as alternatives to fossil fuels (Ogbonna, 2013; Ogbonna et al., 2013). These include bioethanol (Ogbonna et al., 2001; 2010, 2013; Ogbonna and Okoli, 2010, 2013), biogas (Onwosi and Okereke, 2009; Okeh et al., 2014), microbial fuel cells (Oyiwona et al., 2016, 2018) and biodiesel (Ogbonna et al., 2015). Biodiesel is a good alternative to fossil fuels and it has most of the required positive attributes of biofuel. However, biodiesel is currently more expensive than the fossil diesel and it is very important to drastically reduce the cost of its production. This can be achieved by using cheap carbon

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sources (Ogbonna and Ogbonna, 2018), developing cheap energy crops, screening for efficient oleaginous microalgae (Ogbonna and Ogbonna, 2015), developing efficient culture systems (Eze et al., 2017), reduction in the cost of oil production by coupling to waste treatment (Ogbonna et al., 2018), or reducing the cost of oil extraction and transesterification of the oil into biodiesel. Although various chemicals are used as catalysts for transesterification, they are often expensive, not easily available and not environmentally friendly. On the other hand, lipases are environmentally friendly biocatalysts and crude lipases can easily be produced and used even in rural areas without purification (Nwuche and Ogbonna, 2011; Nwuche et al., 2013). This paper reviews the potentials of using lipases as catalysts in lipid transesterification, as a means of moving the production sites to the farm and thus drastically making the production cost cheaper than what it is now.

## RAW MATERIALS FOR BIODIESEL PRODUCTION

Several feed stocks such as edible and inedible plant oil, waste oil, animal fat and microbial oil (that is oil from oleaginous microorganisms) are used to produce biodiesel (Table 1). The cost and availability of a feedstock are important factors in the choice of raw materials. The cost of feedstock is directly proportional to the overall cost of biodiesel (Braga et al., 2015). All oil feedstocks can be used for enzyme catalyzed transesterification and the composition of feedstock differs based on the oil source and level of refining. Therefore, selection of an appropriate and cheap feedstock is essential in biodiesel production.

### Plant oils

Several edible plant oils have been reported by several researchers as feedstock for biodiesel production. They include palm oil (Talukder et al., 2011), rapeseed oil (Jeong and Park, 2008), soybean oil (Yu et al., 2010), and canola (Jang et al., 2012). Increased quality of edible oil is an advantage as a raw material in biodiesel production by enzymatic transesterification. However, one major drawback is the economic viability of biodiesel from edible oil since the cost of refined vegetable oil is high. Furthermore, there is a big gap between production and demand, the food versus fuel debate and all these make the whole process un-economical. According to Leung et al. (2010) large scale planting of these oil plants would bring about felling of many trees and food crises. These problems could be solved by using alternative raw materials such as inedible plant oil, condemned oil and oil from microorganisms for biodiesel production. Inedible plant oils have been employed for some decades by different researchers and industrialists as alternative raw material for biodiesel production. Such plants include *Jatropha curcas* (Juan et al., 2011), rubber (Sampath et al., 2013), and *Calophyllum inophyllum* (Arumugam and Ponnusami, 2014). Puneet and Sharma (2016) stated that biodiesel produced from these inedible plant oils meets specification of biodiesel according to the standard organizational requirement. Depending on the region, production of these oils does not impede food production (Puneet and Sharma, 2016). Furthermore, wastes (for example seed cake) left after oil extraction can be used as organic manure to enrich soil for agriculture or as a medium for cultivating microorganisms that produce enzymes and other useful metabolites (Nwuche et al., 2013).

One desirable feature of these oil plants is that they can survive in environment with limited water supply and poor soil conditions. On the whole, production of bio-energies from crops is feared to have adverse effects on food security through direct or indirect completion with food crops. However, it has been argued that in most African countries, production of energy crops can even boast agricultural productivity and thus help to achieve food security (Ogbonna et al., 2013).

### Waste oils

According to Prafulla (2012), condemned oil generated by oil factories, and other industrial by-products such as waste from palm oil refineries and domestic cooking oil wastes are good oil sources for biodiesel production. Their utilization in biodiesel production leads to environmental sanitation. Yan et al. (2014) stated that the exploitation of waste cooking oil for biodiesel production eliminates environmental and human health risks. Hayyan et al. (2011) reported lipase mediated transesterification as a promising method for production of biodiesel from waste oil since their free fatty acid and water contents are high. The limitations of using cheap raw materials with high fatty acid contents will be mitigated by using biocatalysts which will generally reduce the cost of production. A major drawback in utilization of waste oil is its availability as it depends on the location and scale of oil processing industries.

### Microbial oils

Microbial oils from oil accumulating microorganisms such as oleaginous microalgae, bacteria, fungi, and yeasts have been widely studied as raw materials for biodiesel production because of their high fatty acid contents. These microorganisms have the capability of accumulating over 20% lipids as their dry cell biomass. Microbial oil has many advantages over some other feedstocks. Such advantages include less influence of seasonal changes (Ogbonna et al., 2015), short life

**Table 1.** Substrates used for biodiesel production

| Source of oil | Advantages | Disadvantages | Remarks |
|---|---|---|---|
| Waste oil | They are cheap.<br>Their use leads to reduced environmental pollution | Not widely available<br>Requires pre-treatment to remove impurities and this can increase the cost of production<br>Their supply is tied to industries that use oil<br>Their qualities vary significantly depending on the source | They can be a good source in areas with oil processing/oil-utilizing industries |
| Edible vegetable oils | They are renewable<br>They are environmental friendly.<br>Some are of very good quality. | Their production requires large area of land and energy.<br>Some have high flash point and poor fuel atomization as a result of high viscosity.<br>They are very expensive.<br>Their production is affected by climatic conditions or seasonal variations.<br>Their use for biodiesel production has direct effect on edible oil prices. | Aside from the direct effect on food security, it is extremely difficult (in terms of required land area etc) to produce enough to meet global food and energy needs. |
| Animal fats | Their use is environmental friendly.<br>They are renewable.<br>They provide greater oxidation stability. | Productivity is very low<br>Takes a long time to grow animals for fat production<br>Their supply is very low<br>Performs poorly in cold weather | Compete with food production and it is very difficult to produce enough to meet the global energy demand. |
| Non-edible vegetable oils | They are relatively cheap.<br>They do not compete directly with edible oil<br>They are renewable and available. | Some have higher reactivity due to high content of unsaturated fatty acids.<br>Some have high viscosity which is inhibitory to lipase catalysed trans-esterification. | As in the case of vegetable oils, it is very difficult to produce enough to meet the global energy demand.<br>It competes indirectly with food for land and other agricultural inputs. |
| Microbial oils | Microorganisms have high growth rate, and thus high oil productivity.<br>There are easy culture methods<br>Their cultivation is not affected by seasonal variations<br>They are renewable and require less energy.<br>They can also be used to generate other by-products like single cell proteins.<br>Quality of oil can be controlled by controlling the culture conditions | There are still some technological challenges related to harvesting, extraction and purification | Microorganisms can be the best alternative, especially if oleaginous species are engineered for high growth rates. It is potentially possible to produce enough to meet the global energy demand. |

cycles, and they can be produced from readily available resources and wastes without competing for the land used for food production (Ogbonna et al., 2018). As a means of reducing the cost of producing microbial oil, lignocellulose based carbohydrates and industrial product can be used as media for microbial oil production (Li et al., 2008; Ogbonna and Ogbonna, 2018). Meng et al. (2009), has observed that the oil content obtained from several yeast strains such as *Cryptococcus*, *Lipomyces* and *Rhodotorula* can be as high as 60 to 70% of their dry cell weight. Xu et al. (2012) used waste by-product from biodiesel such as glycerol as the source of carbon for cultivating *Rhodotorula* sp and *Rhodosporidium toruloides* for biodiesel production. According to Saenge et al. (2011), *Cryptococcus curvatus* and *Rhodosporium glutinis* are able to store lipids when unrefined glycerol and ammonium sulphate were used as the source of elemental carbon and nitrogen source while Zhao et al. (2012) reported that the fatty acid content of *Rhotoruloides* obtained by cultivating in a cheap agro waste medium (lignocellulosic hydrolysate) under controlled conditions was similar to that of various plant oils and animal fats.

Fungal species produce unique lipids that are polyunsaturated fatty acids such as docosahexanoic acid, linoleic acid, eicosapentaenoic acid and arachidonic acid. Such fungi include *Aspergillus oryzae*, *Mortierella isabellina*, *Humicola langinosa* and *Mortierella vinacea* (Azocar et al., 2010). Furthermore, microalgae such as *Chlorella, Cylindrothecia, Nitzschia, Botryococcus, Schizochytrium, Scenedesmus incrassatula* utilize carbon dioxide and energy from the sun for lipid production under specified growth conditions. Advantages of using microalgae for biodiesel oil production include high oil contents, use of carbon dioxide and solar light as the carbon and energy sources, ability to grow all the year round, and ability to grow on waste materials (Ogbonna et al., 2015; 2018). In bacteria, the lipids accumulate in lipoids as polyhydroxyalkanotes and can hardly be separated.

One major limitations of microbial oil is that it requires cell disruption before oil extraction. Furthermore, the proportion of polyunsaturated fatty acids differs from that of most plant oils and this predisposes such biodiesel to oxidation during storage (Meng et al., 2009).

## METHODS OF PROCESSING BIODIESEL

Biodiesel is produced by hydrolysis, pyrolysis or transesterification. Transesterification is the most commonly used method. Transesterification is the process whereby the alkoxy (organic group) of an ester is exchanged with the organic group of an alcohol which converts the triglyceride in oil to fatty acids, alkyl ester and glycerol. Chemical or biological agents can serve as catalysts.

### Chemical catalysis

Chemical catalysis involves the use of alkali or acids in transesterification processes. The alkali-catalyzed process is currently carried out using sodium or potassium hydroxides as a result of their low price and high biodiesel yields. Alkali-catalyzed process gives a higher conversion of triglycerides with a short reaction time but a major limitation of this process is its sensitivity towards free fatty acids in oil which leads to soap formation and low reaction yield as a result of an excess water produced. Furthermore, the resulting soap could lead to formation of emulsion which makes downstream processing (recovery of the biodiesel) difficult with low ester yield. Acid-catalyzed process using acids such as hydrochloric acids and phosphoric acids is insensitive towards free fatty acids but it has a major limitation of slower reaction rate, corrosive to equipment and a longer reaction time. Chemical catalysis, in general, has many limitations - the side reactions affect both the yield and the quality of the resultant biodiesel; it is energy and capital-intensive; the recovery and purification of the catalyst and the glycerol are difficult; transesterification is difficult, they result in high free fatty acids; and treatment of alkaline wastewater can be very expensive (Fukuda et al., 2001).

### Biological catalysis

Biological catalysis is the use of enzyme in biological reactions. Lipase enzymes are used in transesterification as they offer several environmental and economic advantages. Lipases (glycerol ester hydrolases, E.C 3.1.1.3) are enzymes that breakdown ester bonds especially long chain triglycerides (TAG) to generate free fatty acids, diglycerides, monoglycerides as well as glycerol. These triglyceride-hydrolyzing enzymes have been under study for over 300 years. The activities of some microbial lipases have been observed in *Bacillus prodigiosus*, *Bacillus pyocyaneus* and *Bacillus fluorescens*. Lipases are divided into three groups depending on their specificity as follows: 1,3-specific, fatty acid specific and non-specific lipases. A 1,3-specific lipase discharges fatty acids from position 1 and 3 of a glyceride and breaks down the ester bond in these positions (Ribeiro et al., 2011). Furthermore, in some specific conditions, lipases are involved in other catalysis such as acidolysis, alcoholysis, aminolysis, esterification and transesterification. Lipase enzymes have several applications such as in medicine, disease therapy, food industries, wastewater treatment and in biodiesel production.

As shown in Table 2, lipases can be obtained from the pancreas of mammals such as cattle, pigs, hogs and from plants like castor seeds, rapeseed, paw paw seed and microorganisms (Akoh et al., 2007). Animal and

**Table 2**. Sources of lipases used for transesterification.

| Source | Major characteristics | Advantages | Remarks |
|---|---|---|---|
| Higher plants | Mostly produced from plant seeds. Have a pH range of near neutrality to slightly alkaline. (6.0- 8.5) | Do not necessarily need to be purified to perform catalysis | Not commercially used as they are limited in availability |
| Bacteria | Have a broad pH range with high thermal stability and activity | They are cheap to cultivate and widely available | Industrially used in some processes |
| Fungi | More active at near neutral to slightly acidic pH | They are cheap to cultivate and widely available | Commercially used and usually sold in immobilized forms (Braga et al., 2015) |
| Yeasts | More active at near neutral to slightly acidic pH | They are cheap to culture and widely available | Commercially used and allows the use of waste industrial materials of low quality and high content of free fatty acids (Aarthy et al., 2014) |

microbial lipases have wider application and industrial uses than most plant lipases. Microbial lipases are mostly used because of their stability and high yields. They can be modified genetically with ease, are available every season and above all, microorganisms grow very rapidly, leading to high productivities (Hasan et al., 2006; Nwuche et al., 2013). According to Akhil et al. (2010), the most attractive qualities of lipases are their ability to use monoglycerides, diglycerideides and triglycerides as well as free fatty acids in transesterification. Lipases are highly active with high reaction rates, they are not inhibited by the products and they have the ability to generate a high yield in non-polar media. Lipases are re-usable, and are resistant to both temperature and alcohol. Several microbes have been reported to produce lipases used in biodiesel production. Such organisms include: filamentous mould genera such as *Aspergillus, Rhizopus, Thermomyces, Fusarium* and some yeasts including *Candida* spp*., Saccharomyces cerevisiae and Rhodotorula* (Nwuche and Ogbonna, 2011; Nwuche et al., 2013). Some bacterial species such as *Bacillus,* and *Pseudomonas* are also good lipase producers (Fjerbaek et al., 2009). Studies by Luo et al. (2006) have shown that bacteria such as *Pseudomonas florescens* (Lip B68) and *Bacillus pumilus* have been commonly used because their lipases can tolerate high pH and temperature.

Lipases are grouped into two based on their storage sites by the producer organism as follows:

### *Extracellular lipases*

These are lipases produced by the microorganisms that are recovered and purified from the cultivation broth. In industrial production of extracellular lipases, bacteria, fungi and yeasts are preferred as they are able to catalyze the same reaction. They are soluble enzymes that disperse in solution and can move freely during the catalytic reactions. Extracellular lipases have several advantages such as easy and low preparation cost but their utilization is limited by poor operation stability, high cost of separation and purification techniques and they can only be used once because they are inactivated easily (Yan et al., 2014). Freeze drying the solution containing the enzyme can also prevent carrying over water into the reaction medium. In addition, large scale application of lyophilized enzymes poses some health risks since inhalation of these enzymes cause allergy in some workers (Nielsen et al., 2008; Freire et al., 2011). The most widely utilized extracellular lipases are: Novozyme 435 from *Candida antarctica,* Lipozyme RM IM from *Rhizomucor miehei* and Lipozyme TL IM from *Thermomyces lanuginosus* (Robles-Medina et al., 2009).

### *Intracellular lipases*

These are enzymes that remain inside or attached to the cell wall of the producer organism. One big issue in biological catalysis is the expensive nature of the enzymes. In order to avoid the exorbitant purification steps required for extracellular lipases, whole cells are used as biocatalyst. This eliminates the downstream processing operation and promotes the recycling of enzymes. Although, intracellular enzymes tend to solve some of the problems of extracellular lipases, it is still limited in large scale industrial production of biodiesel.

## Some positive properties of lipases

The following properties of lipases make them good catalysts for transesterification of lipids to biodiesel:

### *Specificity*

Three major groups of lipases are recognized based on the position where they attack a triglyceride molecule namely: 1,3 specific, 2 specific, or non-specific (Ranganathan et al., 2008). 1,3 Specific lipases attack the ester linkages at the end of the triglyceride molecule. It rarely touches the middle ester bond. Lipases that are position 2 specific specifically act on the middle ester bond of the triglyceride molecule while non-specific lipases attack the ester bonds at any position (Ranganathan et al., 2008). Lipases that recognize and act on 1,3 linkages of a triglyceride are synthesized by some genera of filamentous fungi including *Rhizomucor, Rhizopus, Thermomyces*, *Aspergillus* (Robles-Medina et al., 2009). Some yeasts and bacteria such as *Candida and Pseudomonas* species can synthesize lipases that are very position specific and as such very applicable in transesterification of lipids to biodiesel (Fukuda et al., 2008). Lipases that are not specific are not useful since they can attack any of the ester linkages on a triglyceride molecule.

According to Fukuda et al. (2008), spontaneous migration of acyl moieties from position 2 of the triglyceride molecules to either position 1 or 3, brings about increase in the product yield. Akoh et al. (2007) and Robles-Medina et al. (2009) reported that in an attempt to encourage acyl movement and bring about an increase in the reaction products, utilization of aqueous immobilization supports and addition of silica gel into the reaction mixture were good strategies.

### *Stability*

For any lipase to be used in transesterification of lipids to biodiesel, it must be stable under the reaction temperature and pH (Moreira et al., 2007; Zheng et al., 2009). Naturally, every biological molecule, including enzymes is more active and stable *in vivo* than in culture vessels such as bioreactors due to the harsh environmental conditions obtainable in culture vessels. As a result of these hash culture conditions like high temperature, by-products of reaction, impurities and rough surfaces of the bioreactors, enzymes are deactivated or inhibited more when used industrially than *in vivo.* Other enzyme deactivating and destabilizing by-products during the transesterification process include lower chain alcohols, glycerol, water content and high alcohol to oil ratios (Marchetti et al., 2007; Robles-Medina et al., 2009). Mateo et al. (2007) and Illanes et al. (2008) pointed out that cell immobilization has been proven to improve and increase lipase stability.

### *Reusability (Recovery and reuse)*

Recycling and reusability of lipase is a key and important factor when biodiesel is produced by the enzymatic process since high cost is an important drawback in the use of lipase for biodiesel production. In an effort to make the process cheap, the biocatalyst has to be recycled while at the same time keeping them active and stable. Bhushan et al. (2008) stated that immobilizing the enzyme is an intelligent strategy that could be used to maintain operational stability, enzyme activity and selectivity and at the same time allows the enzyme to be studied under harsher environmental conditions. Immobilized enzymes can also be easily separated from the reaction mixture without filtration as obtainable in a packed bed bioreactor. Moreover, it could lead to more favourable economic benefits. Fukuda et al. (2009) and Robles-Medina et al. (2009) stated that the method of microbial cultivation and the strength of the immobilization material are crucial factors that determine how long an enzyme can be used.

## Advantages of using lipases in biodiesel production

Lipases can function in both biphasic and monophasic media. They are robust and versatile enzymes, have high activity and can easily be separated during downstream processing. They are also thermostable and tolerate short chain alcohols. The glycerol produced during transesterification has minimal impurities and water content, making product separation very easy. The use of lipases for biodiesel production is eco-friendly, requires mild temperature with high selectivity and they are very specific towards substrate. The enzymes require lower alcohol to oil ratios, tolerate high water content of oil and increase biodiesel yield by avoiding soap formation. Use of lipase requires less energy than chemical catalysis and waste water treatment is minimal while high-grade glycerol can be produced.

## Limitations of using lipase in transesterification

Although there are several advantages of using lipase in bioconversion of lipids to biodiesel, there are some limitations. The enzymes can be very expensive and their activities are often lost during the process. The enzymes become unstable after a single use and the reaction rates are generally low due to enzyme inhibition by the reactants, products and by-products (Gog et al., 2012).

## Challenges in enzymatic transesterification

The rate at which transesterification proceeds and the

**Table 3.** Conditions that affect transesterification.

| Factor | Range | Optimum | Remark |
|---|---|---|---|
| Temperature | 20-70$^{o}$C | 50-60$^{o}$C | Depends on the source of enzyme, type of oil etc. (Puneet and Sharma, 2016) |
| pH | 2.5 - 11 | 7.5 -9.0 | This also depends on the source of enzyme, stability and the solvents |
| Water activity | Minimum water | Appropriate amount of water | Optimum water content should fall between minimum water to prevent hydrolysis and maximum to ensure the active conformation of biocatalyst. It varies from system to system. (Azocar et al., 2011) |

overall biodiesel yield is affected by several factors such as choice of alcohol, solvents utilization, pre-treatments of the biocatalyst, the molar ratio of alcohol to oil, water activity and reaction temperature (Table 3).

### *Type of alcohol and alcohol to oil molar ratio*

Several polar compounds such as methanol, ethanol, propanol, isopropanol, 2-propanol, n-butanol and isobutanol have been considered to be acceptable acyl/ alkanoyl (RCO) group acceptors in transesterification (Braga et al., 2015). Ethanol has been known to be a good alternative polar solvent in enzymatic biodiesel production since it is less toxic than methanol. José et al. (2011) noted that ethanol could change the secondary structure of *Candida antartica* (CALB) lipase by lowering the α-helix contributions while the β-sheet structure was increased.

Alcohols are more soluble in non-polar solvents like vegetable oil than aqueous solvents due to their number of carbon atoms, thereby making them less toxic to lipase enzymes (Chen, 2012). Although methanol and ethanol are less soluble in oil, the alcoholysis reaction usually employ given amounts of these alcohols above the stoichiometric ratio, since at least three moles of alcohol are needed for complete conversion of the oil into their corresponding esters.

Hernandez-Martin and Otero (2008) and Véras et al. (2011) pointed out that lipases exhibit more activity and stability in substrates with high free fatty acid (FFA) content than in refined oils since alcohols are more soluble in FFA than in ordinary fat. Watanabe et al. (2007) observed the denaturation of immobilized *Candida antarctica* lipase in acid oil molar ratio at methanol: oil acid ratios higher than 8:1. They suggested different strategies to reduce enzyme denaturation which include addition of alcohol in batches, substitution of the acyl acceptor with a less harmful substrate than alcohols, pre-loading alcohol before silica gel to supply the substrate gradually and use of co-solvents in order to keep the concentration of alcohol below the limits of solubility in oil and prevent the deactivation of enzymes (Lee et al., 2011)

### *Glycerol inhibition*

Glycerol is one of the by-products of biodiesel which ironically inactivates the lipases used as biocatalysts in transesterification. By nature, glycerol is insoluble in oils and it can be adsorbed onto immobilized enzyme surfaces thereby reducing enzyme activity and stability (Talukder et al., 2009). Glycerol also increases the viscosity of the reaction medium and thus reduces mass oxygen transfer in agitated batch cultures. According to Hama et al. (2011), glycerol also reduces the volume of water required for lipase activity. Hernandez-Martin and Otero (2008) reported that free glycerol mixes with methanol in the reaction process and forms a secondary liquid phase that is almost not mixable with the oil. Extraction of the methanol from the organic phase by the glycerol decreases the substrate in the reaction medium, and reduces the conversion efficiency of lipase. Halim and Kamaruddin (2008) noted that adding organic co-solvents in the medium reduces the negative effects of glycerol and alcohol on lipase activity. When methanol is used, it is better to add it step by step because ethanol exerts less inhibitory effect on the lipase than methanol. Lee et al. (2008) reported a 98.92% conversion by stepwise addition of methanol and a 65% conversion when methanol was added at a time in a batch methanolysis of olive oil. Bernardes et al. (2007) also reported a similar result in the transesterification of soybean oil using ethanol and Lipozyme RM IM. According to Fjerbaek et al. (2009), the choice of lipase also has an effect on inhibition. Greater resistance to alcohol inhibition have been displayed by *Pseudomonas* lipases than lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei*.

### *Water content*

Water is very essential in lipase catalyzed transesterification. The presence and amount of water in

the reaction mixture affects both the reaction rate and product yield. Water is essential in maintaining the specific three dimensional structure of the enzyme (Lu et al., 2009). Since lipase activity depends on the interfacial area, lipase enzyme needs a given volume of water to retain its active conformation for effective reactions catalysis. A certain amount of water is required in the medium to form oil/water interface. However, excessive water may bring about an increase in oil hydrolysis and FFA content. It may also lead to the lipases clumping together which will lead to a decrease in their activity. In case of immobilized lipase, Robles-Medina et al. (2009) argued that excess water closes the openings of the immobilization material and thus decreases the contact of the enzyme with the reaction medium. Therefore, the water content should be low enough to avoid oil hydrolysis but high enough to ensure active conformation of the enzyme. The amount of water required depends on the system, the substrate, origin of the enzyme, method of immobilization, the stability of the enzyme and the type of alcohol used. Fjerbaek et al. (2009) reported that lipases from *Candida rugosa*, *Pseudomona cepacia* and *Pseudomonas fluorescens* cannot catalyze any transesterification reaction in the absence of water. They also pointed out that the optimum water requirement for lipases from the above three organisms ranges between 1 and 20%. *Candida antarctica* shows the highest dislike for water (Fjerbaek et al., 2009) while *Rhizopus oryzae* lipase was found to be active when the water content was between 4 and 30% (Fukuda et al., 2008).

Some researchers have reported that the rates of bioconversion can be improved if the water generated during the esterification/transesterification processes is removed. This is applicable especially when feedstocks with high free fatty acids (FFAs) contents are used. Several methods are used to remove water from the medium but using molecular sieves as adsorbents is the most popular method (Correa et al., 2011). Molecular sieves are ideal tools since they do not adsorb hydrocarbons and alcohols (Azócar et al., 2011). On the other hand, Tamalampudi et al. *(*2008) reported that adding water into the reaction mixture can increase the ester yields in transesterification reactions since many interfacial areas are formed between oil and water and these are required for enzyme activity. As reported by Tan et al. (2006) in methanolysis catalysed by *Candida sp*., the triglycerides are first hydrolysed into free fatty acids which are subsequently converted into methyl ester. Thus, in the early stage of the reaction, high water content contributes to the hydrolysis of oil, and in the later stage of the reaction, inter- esterification plays an important role.

### *Temperature*

Enzymatic reactions are strongly influenced by temperature. Most enzymes are denatured at extreme temperatures but lipases are known to have a fairly large thermal stability (Marchetti et al., 2008).

Transesterification is rarely influenced by temperature fluctuations and can occur at temperatures between 20 and 70°C. However, most lipases are thermally stable with the optimal temperatures ranging between 30 and 60°C. Enzyme immobilization brings about increase in the optimum temperature for a given lipase. However, some factors such as lipase stability, alcohol to oil molar ratio and the type of organic solvent used influence temperature optima. It has been observed that enantioselectivity increases with decrease in temperature in ammoniolysis-based kinetic resolution of 2-amino-4-phenylbutyric acid methyl ester catalysed by *Thermomyces lanuginosus* lipases*.*

## Solution to the challenges encountered in biologically catalyzed transesterification

As discussed above, bioconversion of lipids to biodiesel is still faced with several challenges which must be solved before efficient production of biodiesel can be achieved. Possible solutions to some of these challenges include:

### *Immobilization*

Immobilization is the confinement of enzymes in a matrix or within a restricted space. Biocatalysts have been used immensely in various sectors of biotechnology because of their high substrate specificity, ease of production and environmentally friendliness. However, use of enzymes for large scale production is restricted by the cost, low stability and inability to recycle the enzymes. The structural instability of some of these bioctalysts during any biochemical reaction is always a challenge. In an attempt to overcome these challenges, immobilization of the enzymes has been exploited. Immobilization makes it possible to use the same enzymes over and over in many experimental cycles with easy downstream processing and high productivity (Rodrigues et al., 2013). With a suitable immobilization protocol, the enzymes are protected from fluctuation in the operational conditions such as pH and temperature, leading to high productivity. Based on the interaction between the enzymes and the carriers, immobilization could be irreversible or reversible (Brena and Batista-Viera, 2006). In irreversible immobilization, the enzymes cannot be separated from the carrier without destroying the biological activity of the enzyme or the carrier. Covalent bonding, entrapment and cross-linking are the most commonly used procedures for irreversible immobilization. In reversible immobilization, enzymes can be removed from the support material under gentle conditions without destroying them. Physical adsorption and non-covalent bonding like affinity bonding and chelation bonding are used in reversible immobilization. Each immobilization technique involves different levels of complexity, enzyme activity and

conversion efficiency. Technically, immobilization improves the technological properties of the enzyme. Therefore, the choice of immobilization technique is determined by the nature of process, costs and desired properties (Nasratun et al., 2009). Immobilization of lipases stabilizes the enzyme molecules in open conformation, promotes their hyper activity and makes them stable under different experimental conditions. Immobilization increases enzyme tolerance to organic solvents, heat, shear stress, easy product purification and increases the shelf life of the biocatalyst (Zhao et al., 2015). Immobilization can also be achieved by entrapment in polymer gels such as carrageenan and alginate (Ogbonna et al., 1989a). Lipase PS from *Burkholderia cepacia* has been encapsulated within K-carrageenan biopolymer for the transesterification of lipid from palm oil. At the optimal condition, triglyceride conversion of up to 100% was obtained and the immobilized lipase was stable (Kenthorai et al., 2010). Nadir et al. (2009), used lipase confined in microporous polymer to transesterify lipids from sunflower, soybean and waste cooking oil. In their experiments, they reported that the lipase so immobilized retained their activity after ten repeated batches at 25ºC with 24 h batch duration. Immobilization can also be done by adsorption onto resins or fibrous materials (Ogbonna et al. 1994; 1996). Lipase from *Candida sp.* 99-125 has been immobilized by physical adsorption onto macro porous resins by Gao et al. (2006). The immobilization effect was found when the coupling procedure was performed in the presence of heptane. Lipase adsorbed onto non-polar resin NKA could effectively catalyse biodiesel production via methanolysis of soybean oil. Cristobal et al. (2011) reported that immobilized *Thermomyces lanuginosus* could be used as a catalytic enzyme to produce second generation biofuel. Nasratun et al. (2009) compared the use of free and immobilized *Candida rugosa* lipase using chitosan beads as carrier and methanol as the organic phase to catalyse transesterification of cooking oil to biodiesel. The results they obtained were 72.25 and 76.5% conversion for immobilized and free enzymes, respectively. The interesting thing about immobilization is that the enzymes could be easily separated from the culture broth and could also be reused in another batch of experiments. Meng et al. (2011) reported stepwise catalysis of soybean oil namely: hydrolysis and esterification with immobilized *Yarrowia lipolytica* lipase and they reported 85% transesterification after 3 h. They also reported that the enzymes could be reused in about 25 batches without loss in enzyme activity and biodiesel yield. Although immobilization has several advantages, it is still limited by the cost of preparation and the negative effect of mass transfer. One method of reducing mass transfer limitation in polymer entrapped biocatalysts is to reduce the diameters of the beads (Ogbonna et al., 1989b; 1991). Huang et al. (2010) investigated co-immobilization of Novozyme 435 and Lipozyme T4-1M instead of a single enzyme in order to reduce the cost of biodiesel production by transesterification. They pointed out that the two lipases to be co-immobilized must have complementary position specificity. In their study (Huang et al., 2010), there was 97.2% conversion yield and 20 times recycling without loss in enzyme activity and stability when two enzymes were co-immobilized. Co-immobilization has been proven to increase productivity, stability and re-useability (Hadjer et al., 2017). Use of agricultural waste residues as support materials in an attempt to reduce the cost has been reported by Pairat et al. (2014). They carried out immobilization of *Acinetobacter baylyi* lipase on agricultural waste residues as support material and the lipase showed remarkable solvent stability and reusability. Agricultural fibrous materials such as loofa sponge have also been found to be a cheap, and easily available support materials for immobilization (Ogbonna et al., 1994; 1996).

### *Nano immobilization*

Currently, the use of nanostructures such as nano tubes, nanopores, nanosheets etc. for immobilization of biocatalyst is gaining importance in industrial biotechnology as a means of salvaging the limitations in transesterification. This deals with the use of nanomaterials in immobilization. Nano particles offer several advantages due to their unique size and physical properties as enzymes show high stability in a wide range of temperatures and pH values, reusability and thermostability. Advantages of nano-immobilization are: easy synthesis in high solid content without toxic reagents and surfactants, possibility of co-immobilization of multi enzyme systems, reduced mass transfer limitations of substrate and inhibitors, homogeneous coating and well-defined particles with convenient adjustment of the particle size, high surface area and mechanical strength. Nano-structured tin dioxide (Nano-$SnO_2$-CrL) has been used as support for immobilization of *Candida rugosa* lipase (Guncheva et al., 2011). Also, Nano-sized $Fe_3O_4$ particles have been used for immobilization of *Mucor lavanicus* lipase with reusability of about 10 times at 55°C with only 10% loss of activity (Meng et al., 2014).

Nano immobilization has some limitations of high cost of the fabrication process, low effectiveness in large scale application and difficulty in the separation of the reaction medium. Dussan et al. *(*2010) used a magnetic nanostructure prepared by co-precipitating $Fe^{2+}$ and $Fe^{3+}$ ions in a sodium hydroxide solution for lipase immobilization. Also, immobilization of *Burkholderia sp*. lipase on a ferric silica nanocomposite has been studied by Tran et al. (2012). In transesterification of olive oil with methanol, the immobilized lipase produced fatty acid methyl esters with 90% conversion rate. Akash et al. (2015) carried out conversion of jatropha oil to fatty acid

methyl esters using lipase immobilized carbon nanotubes. Immobilized lipase showed a high catalytic efficiency and a regeneration time of 10 without loss of activity or any adverse effect. Jiang and Gang (2012) reported improved stability and activity of lipase from *Candida rugosa* immobilized on glutaraldehyde activated nano-fibrous material. Lidija et al. (2017) observed an improved thermostability and reuseability potential of *Candida rugosa* lipase when adsorbed onto titanic acid as nano biocatalyst. Mohadese et al. (2016) reported high reuseability of lipase from *Candida antarctica, Thermomyces langinosus* and *Rhizomucor miehei* immobilized on silica nanoparticles. Saima et al. (2017) reported an improved catalytic activity of *Penicillium notatum* lipase when immobilized in nanoscale silicone polymeric film.

### *Whole cell biocatalyst*

Instead of undergoing the costly enzyme purification process, a lipase producing microorganism can be cultivated on either solid or liquid medium containing cheap substrate, harvested and used as biocatalyst. This approach has been adopted by some researchers including Christopher et al. (2014) to reduce the cost of producing biodiesel by transesterification. Liu et al. (2014) reported a catalytic synthesis of biodiesel in n-heptane using lipase produced by solid state cultivation of *Burkholderia cepacia* LTEB11. It has been demonstrated that lipase productivity can be increased and the cost of production reduced by using mixed-solid substrate for cultivation (Benjamin and Pandey, 1998).

### *Hydroesterification*

This has been reported by several authors as an important method of overcoming some of the challenges posed by a high percentage of free fatty acids in biodiesel production. This process can be carried out by supercritical hydrolysis and esterification, enzymatic chemical hydroesterification and enzymatic hydro-esterification. In the first stage of hydroesterification, mono and tri acyl glycerols are hydrolysed to free fatty acids and glycerol and then the free fatty acids are separated and esterified to biodiesel (De Sousa et al., 2010). Although, the hydroesterification process has some advantages of being eco-friendly, it is energy intensive.

### *rDNA technology*

Although several solutions have been proposed for the challenges in biodiesel production, a recombinant DNA technology, protein engineering and directed evolution can be used to overcome most of such limitations. These can be used to improve lipase specificity, thermostability, activity and improved quality of the lipase produced. High level expression of a thermostable and methanol tolerant lipase from *Proteus sp.* in *Escherichia coli* has been reported. The recombinant strain was able to produce biodiesel in the presence of high water concentration. The recombinant *Escherichia coli* strain was also alternatively used as whole cell biocatalyst (Gao et al., 2009). Furthermore, Adachi et al. (2013) developed a robust recombinant whole cell thermostable and solvent tolerant biocatalyst from *Aspergillus niger* (rBTL) for biodiesel production. The *Aspergillus niger* strain rBTL had an increased tolerance toward organic solvents such as dimethyl carbonate, ethanol and acetone. Challenges in lipase production could also be solved by metabolic engineering by heterologous protein production of which codon optimization and selection of promoters responsible for lipase production is necessary in these microorganisms. Also, deletion of competing pathways, genome editing using CRISPR/Cas 9 mediated genome editing, blockage of competing pathways and over expression of specific gene responsible for lipase production are used to improve the process efficiency.

### Other methods

Several other methods have been employed by researchers to curb the limitations such as the use of temperature sensitive polymer. Currently the use of temperature sensitive polymers has been reported as a means of protecting enzymes against heat inactivation caused by irreversible aggregation among lipase molecules. Qian et al. (2013) reported an improved thermal stability of lipase in water/oil microemulsions by temperature sensitive polymers as the lipase was observed to form complex with the polymers at high temperature in a confined space of the water in oil microemulsion.

Treatment of the reaction mixture with additives such as calcium chloride, milk powder, gum arabic, and maltodextrin, has been reported also as a means of improving the property of the lipase. Hao et al. (2017) carried out thermostability of *Yarrowia lipolytica* lipase by treating with p-cylodextrin as an additive. Use of organic solvents has also been reported to reduce viscosity of the reaction mixture in biodiesel production, thereby facilitating mass transfer and increase productivity. These solvents prevent the inactivation of enzymes by high concentrations of methanol/ethanol and glycerol. One of the most commonly used solvents is tert-butanol. Currently, supercritical fluids and ionic liquids are used as they offer several advantages such as high thermal stability, vapour pressure, low toxicity and viscosity (Tang et al., 2013). As different lipases from different sources have various process conditions specific to it,

optimization of process condition for biodiesel production could also lead to increased productivity of lipase. Some major substrates used for biodiesel production are summarized in table 1 while the conditions that affect biodiesel transesterification and the sources of lipases used for transesterification are summarized in Tables 2 and 3, respectively.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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