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Minireview

Enzymatic transesterification for biodiesel production

Shweta Shah, Shweta Sharma and M N Gupta*

Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110 016, India

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Biodiesel consists monoalkyl esters of long chain fatty acids. It is produced from vegetable oils or fats either by chemical transesterification or by lipase-catalyzed transesterification with methanol or ethanol. Biodiesel is a green fuel and can be used as a blend with diesel or alone. Either way, it does not require any modification in engine design or storage facilities. The enzymatic process offers several advantages over the chemical routes. The handicap of increase in process cost because of the cost of the enzyme can be overcome by using efficient production process for enzyme and using reusable derivatives of enzymes, such as immobilized enzyme. Numerous strategies available in the area of non-aqueous enzymology can be exploited during the enzymatic alcoholysis for biodiesel production. Some of the technical challenges and their possible solutions are also discussed.

Keywords: Biodiesel, transesterification reactions, lipases, oil-extraction, enzymes in organic solvents

Introduction

The need for clean energy source is necessary because of the carcinogenic particulate emissions from diesel engines, which cause pollution and 'global warming'. The depleting reserves of petroleum-based products have also made scientists look for renewable sources of energy. The concept of biodiesel addresses these twin issues. Biodiesel is an efficient, clean, nontoxic and biodegradable energy alternative to petroleum fuels[1]. Even though "diesel" is part of its name, there are no petroleum or other fossil fuels in biodiesel. "Biodiesel" means a monoalkyl ester that[2]:

- is derived from domestically produced vegetable oils, renewable lipids, rendered animal fats or any combination of those ingredients; and
- meets the requirements of ASTM PS121, the provisional specification for biodiesel.

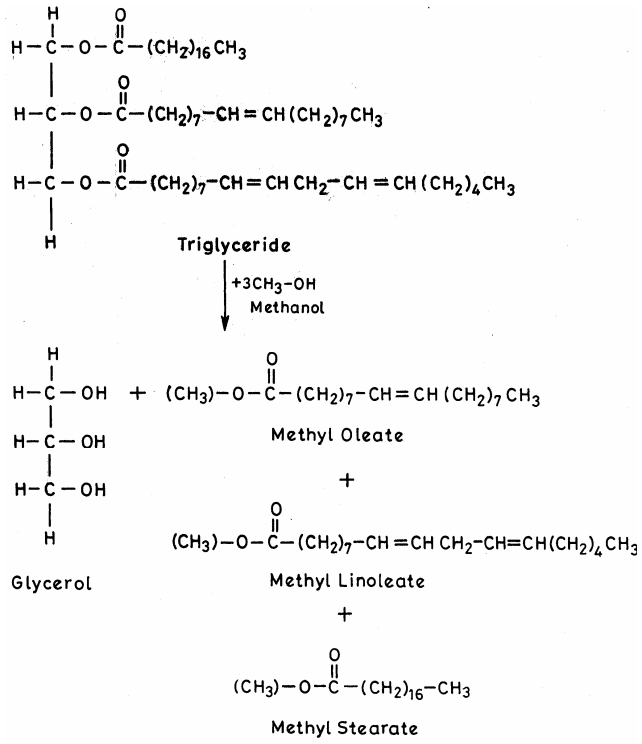
The ASTM standard[3], Indian standard[4] and standard D1NV51606[5] have been specified in the literature.

In fact, the use of vegetable oils (e.g. from palm, soybean, sunflower, peanut, olive etc.) as such as alternative fuels for diesel engine dates back to almost a century[6]. The problem with direct use of such oils arise because of their higher viscosity and lower ignition quality as compared to diesel. The problems are more severe in the case of direct-injection engines than in the less efficient engines having precombustion engines. In the case of direct engines, very dilute blends of oils in diesel can be used[6]. Early approach to improve the usefulness of the vegetable oils consisted of pyrolysis of the oils. The pyrolysates had lower viscosities and higher cetane numbers than the parent vegetable oils[7]. Later work, of course, showed that the production of fatty acid methyl esters from the vegetable oils is a far more satisfactory approach. This conversion of the oil into the esters is essentially a transesterification reaction (Scheme 1). It is these fatty acid methyl esters or ethyl esters, which are collectively called biodiesel[8]. These transesterification reactions can be catalysed by acid or alkali[8]. Lately, considerable work has been carried out on production of biodiesel by using lipases as catalysts. The present review looks at these efforts after discussing the advantages associated with replacing chemical catalysts with enzymes in the context of biodiesel production.

Sourcing starting material

One of the two reactants in the transesterification is methanol or ethanol. As ethanol can be obtained from renewable sources, it is the preferred starting material. The degradation of starch by chemical or enzymatic method produces glucose; its fermentation by whole cell catalysis produces ethyl alcohol. At both steps, free as well as immobilized biocatalysts have been

*Author for correspondence
Tel.: 91-11-26591503; 91-11-26596568
Fax: 91-11-26581073
Email: mn_gupta@hotmail.com



Scheme 1—Biodiesel production from vegetable oils/fats by transesterification

used. The fat or oil can be obtained normally from seed or kernel from variety of plant sources.

Unfortunately, there is a gap between the production and demand in the case of edible oil. Thus, a reasonable approach has been to search for oils, which are not used for edible purpose. *Jatropha*, a tropical plant has been extensively studied for production of biodiesel[9]. Its oil contains some toxic substances, which makes it unfit for human consumption. It may also be added that some people feel that using Jatropha oil for biodiesel production diverts it from its existing uses for soap making, lighting and motive power[10].

The oils from tobacco[11], mango kernel[12], palm[8,13], soybean[7,14], sunflower[15,16], safflower[17], cottonseed[18], rapeseed[19,20], Jatropha[21] and beef tallow[22] have been used for biodiesel production. Its preparations from waste or spent palm oil[23] and restaurant grease[24] are also worth mentioning. Shimada *et al.*[25] have mentioned, "In Japan, 400000 tons of waste edible oils are discharged yearly. In this regard, several local governments in Japan have started collecting used frying oils from households and have converted them to biodiesel fuel for public transportation". It is an example, which is worth emulating.

Extraction of oil/fat from plant sources

Generally, three main approaches for extraction of oil/fat have been used: hydraulic pressing, expeller pressing and solvent extraction[26]. While pressing and expelling can be used for oilseeds containing more than 35% oil, solvent extraction with hexane can be employed irrespective of the oil content[27]. However, the main problem with solvent extraction has been the production of volatile organic compounds, which harm the ecosystem. During the last several years, environmental concerns have led scientists to focus on development of methods, which do not utilize organic solvents. Aqueous oil extraction (AOE) consists of breaking the oil bodies mechanically in the presence of water and collecting the floating oil from the surface[27]. The yields of oil, understandably, are generally quite poor, but not insignificant. This encouraged the development of aqueous enzymatic oil extraction (AEOE), wherein enzymes like cellulase, hemicellulase and proteases are used to free oil bodies enmeshed in complex chemical structures by hydrolyzing the latter[28,29]. In our lab, AEOE has given high yields of 86 and 78% in the case of peanut and rice bran, respectively[28,29].

Recently, we have also described a novel approach of oil extraction by three-phase partitioning[30]. It was found that a mixture of salt and organic solvent mixed with homogenous plant material led to the formation of three phases: lower aqueous/water phase, interfacial precipitate containing mostly protein and upper organic phase, which contained oil. The oil can be recovered from the organic solvent by evaporation or freezing the mixture[31]. The technique has given promising results with oil from soybean[30].

Benefits of biodiesel

In the past decade, biodiesel has been gaining worldwide popularity as an alternative energy source because of its many benefits.

1. *Environmental benefits*

Biodiesel is the only alternative fuel in the US to complete EPA (Energy Policy Act) tier I health effects testing under section 211(b) of the clean air act, which provides the most thorough inventory of environmental and health effects attributes that current technology will allow[32]. Unlike other "clean fuels", such as compressed natural gas (CNG), biodiesel and other biofuels are produced from renewable agricultural crops that assimilate carbon dioxide from the atmosphere to become plants and vegetable oil. Bio-

diesel reduces the emission of carbon monoxide, ozone-forming hydrocarbons, hazardous diesel particulate, acid rain-causing sulphur dioxide, smoke and soot. It also lowers impact on marine environment by reducing toxicity[32].

2. Biodiesel is the only alternative fuel that runs in any conventional, unmodified diesel engine. Also, it does not require any changes in storage facilities, which exist for storing petroleum-based diesel[32].

3. Biodiesel can be used alone or mixed in any ratio with petroleum diesel fuel[33]. The most common blend is a mix of 20% biodiesel with 80% petroleum diesel, or "B20". Biodiesel has positive performance attributes such as increased cetane number, high fuel lubricity, and high oxygen content, which may makes it a preferred blending stock with future ultra-clean diesel[34].

4. Biodiesel is safe to handle and transport because it is "as biodegradable as sugar, 10 times less toxic than table salt", and has a high flash point of about 300 F, compared to petroleum diesel fuel, which has a flash point of 125 F[34].

In fact, biodiesel is a proven fuel with over 30 million successful US road miles, and over 20 years of use in Europe[32]. It is the only alternative fuel that can actually extend engine life because of its superior lubricating properties[34].

5. *Energy security benefits*

Many countries, which have to import oil and other petroleum products become dependent upon other nations. Their economy, thus, is extremely vulnerable to any adverse effect of changes in political climate. Biodiesel, produced from domestic sources, generates self-reliance in a crucial area.

Biodiesel production

Biodiesel is produced from any fat or oil through a refinery process called transesterification (Scheme 1). Presently, the industrial production of biodiesel fuel is performed by alcoholysis of waste oil using alkaline catalysts. A by-product, glycerol, thus contains the alkali, and hence has to be treated as a waste material. Acid-catalysed transesterfication is another route for biodiesel production, which is more suitable for glycerides that have relatively high free fatty acid content and more water. Aksoy *et al.*[35] reported that it was necessary to perform transesterification under an acidic condition when the oil composition was a low-grade material containing sulphur etc.

Problems associated with chemical transesterification of vegetable oil

- High reaction temperature[36].
- Soap formation due to the presence of free fatty acids in oil in case of base-catalyzed transesterification reduces the ester yields[37].
- A major economic disadvantage in chemical alcoholysis is that the purification of glycerol (produced as a secondary product) is very difficult[16].
- Homogeneous catalyst removed with glycerol layer cannot be reused.
- For diesel fuel, ethyl ester is preferred because ethanol can be produced from biomass and is less toxic, but conventional alcoholysis with ethanol gives low yields[21].

In view of the above disadvantages associated with the chemical transesterification for biodiesel production, the enzymatic alcoholysis of oil/fat is considered desirable. Table 1 compares chemical and enzyme-based methods for biodiesel production[36].

Enzymatic alcoholysis

To overcome problems associated with chemical catalysis for production of biodiesel, enzymatic processes using lipases have been developed. Alcoholysis by lipases is considered one of the most effective reactions for the production of biodiesel. Lipases (E.C. 3.1.1.3) hydrolyze triglycerides and can be isolated from microorganisms, plants, mammals etc.

The lipase-catalyzed reactions can be classified as follows[38]:

(i) *Hydrolysis*

$$R^1COOR^2 + H_2O \rightarrow R^1COOH + R^2OH$$

Table 1—Enzymatic and non-enzymatic methods for biodiesel fuel production[36]

Conditions	Alkali-catalyzed	Lipase-catalyzed
Reaction temperature	60-70°C	30-40°C
Free fatty acids in raw materials	Saponified products	Methyl esters
Yields of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

(ii) *Synthesis:* reactions under this category can be further divided:

— Esterification

$R^1COOH + R^2OH \rightarrow R^1COOR^2 + H_2O$

— Transesterification

Alcoholysis

$R^1COOR^2 + R^3OH \rightarrow R^1COOR^3 + R^2OH$

Acidolysis

$R^1COOR^2 + R^3COOH \rightarrow R^3COOR^2 + R^1COOH$

The summary of biodiesel production from various sources is given in Table 2. Biodiesel production, thus involves using enzymes in non-aqueous media.

Non-aqueous enzymology and transesterification of oils and fat

It is now well-established that the enzymes can be used under nearly anhydrous conditions of neat organic solvents as reaction media[39,40]. The enzymes under these conditions do require a minimum amount of water, which is less than monolayer amount of water molecules around the enzyme molecular surface. Enzymes, in fact, become highly thermostable and can be used at high temperatures. Lipases, for example have been found to be biologically active at 100°C in nearly anhydrous organic solvent. The major application of non-aqueous enzymology is synthesis by hydrolases. Thus, in the absence of water, thermodynamic reversibility pushes the enzyme-catalyzed reaction in the reverse gear. A major limitation in the use of enzymes in these media is their low catalytic efficiency. This has been improved by immobilization[41,42], chemical modification[43] and protein engineering[44]. For improving the catalytic efficiency as well, protein engineering has been used[44]. Recently, some workers have described 'salt activation' for improving catalytic efficiency[45]. Ultrasonication[46] and microwave-irradiation[47] have also been used in a limited way. The cost of the enzymes is, in fact, always a major deterrent in adopting their use in any biotechnological process.

The two major approaches, which are viewed as possible partial solutions to the dilemma are: (i) efficient bioseparation strategies; and (ii) enzyme immobilization.

Table 2—Summary of biodiesel production by transesterification of plant oils/fats

Route	Oil source	Catalyst	Alcohol	Yield (%)
*Chemica*l	Sunflower[7]	KOH	Methanol	96
	Jatropha curcas[21]	KOH	Methanol	92
			Ethanol	88.4
	Crude palm[11]	H_2SO_4	Methanol	78
Enzymatic (Free Enzyme)	Soybean[22]	*Candida rugosa*	Methanol	30
	Palm kernel[10]	*Psuedomonas cepacia*	Methanol	
	Sunflower[7]	*Psuedomonas fluorescens*	Methanol	
			Ethanol	82
	Rapeseed[16]	*Candida rugosa*	2-ethyl-1-hexanol	97
	Mowrah,mango	*Mucor mehei*	C_4-$C_{18:1}$	86.8-99.2
	Kernel, Sal[19]			
	Recycled	*Psuedomonas cepacia/*	Ethanol	85.4
	Restaurant grease[20]	*Candida antarctica*		
Enzymatic (Immobilized)				
	Soybean[13]	*Candida antarctica*	Methanol	97
	Safflower[14]	*Psuedomonas fluorescens*		
	Cottonseed[15]	*Candida antarctica*	Methanol	87.4

Efficient bioseparation strategies

The major component of the production cost of any enzyme is generally the expenditure on the downstream processing operations[48,49]. With this realization, many novel bioseparation techniques have emerged as a more efficient and economical alterations. In classical downstream processing, a non-selective precipitation with salt, organic solvent or a water-soluble polymer (like PEG) is generally used to concentrate the enzyme solution. This is generally followed by a chromatographic step. The introduction of affinity chromatography as a final and polishing step has become de rigour in the last 2-3 decades[48,49]. This kind of protocol is still widely used and serves the purpose, if the enzyme is required in milligram amounts for research needs. For any large-scale application, it is necessary to follow somewhat different sequence[48,49]. One important paradigm shift has been to use affinity interactions in free solution[50-52]. Such an approach has been tried for lipases as well[53]. Using alginate as a smart macroaffinity ligand, it has been possible to selectively precipitate the lipase from *Chromobacterium* and porcine pancreas. The simple, scalable and one-step purification gave 1.7 and 5.6-fold purification with 87 and 75% of the total activity recovered[53]. The other important factors like short process time, low cost of the macroaffinity ligand (alginate is cheap and can be recovered for reuse) and scalability makes this a valuable approach for obtaining adequately purified lipases for production of biodiesel.

Another possible approach, which may be worth trying is the use of expanded bed chromatography[48,49,54-56]. The use of stable fluidized beds in this technique obviates the need for any pre-clarification or concentration step. If the chromatographic media uses an affinity ligand, affinity capture of lipases would be expected to give adequately pure enzyme. The use of these robust, economical and efficient processes and some others of similar nature has been reviewed earlier[54-56]. The adoption of these techniques is considered to be useful in cutting down the cost of downstream processing (and ultimately production costs) of enzymes.

Immobilized enzymes[42,51,57]

Linking enzymes to insoluble matrices by a variety of methods (such as adsorption, covalent coupling and entrapment) provides several advantages. In numerous cases, the enzyme becomes more stable. In the context of transesterification, when the media is predominantly non-aqueous, stability is a major issue. In aqueous media, where enzymes are freely soluble, immobilization provides with a means to recover the biocatalyst by centrifugation or filtration. In anhydrous media, enzyme molecules, though mostly insoluble, tend to clump together. Thus, immobilization is a good way of increasing the surface area of the biocatalyst. Bosley and Peilow[58] have provided a critical review and necessary protocol for immobilization of lipases. Their recommendation includes the use of celite or Accurel™ (macroporous polypropylene). A protocol for lipase immobilization by sol-gel technique is also described. It is also possible to extensively cross-link the enzyme to obtain insoluble chemical aggregates, which often show the advantage of enhanced stability and reusability without increasing the required reaction volume (as these do not have any volume occupying carrier). A recent innovation in this area is CLEC™ (cross-linked enzyme crystal) developed by Altus biologicals (USA)[59]. CLEC™ form of lipases from *Pseudomonas cepacia* (CLEC-PC) and *Candida rugosa* (CLEC-CR) are also available[60,61].

Effect of presence of solvent on biodiesel production

Biodiesel production using enzymes from oil and alcohol has been tried in the presence and absence of solvent. Methanolysis of tallow oil, using *Mucor mehei* lipase in hexane has led to 77.8% ester yield[19]. Lately, solvent-free transesterification reaction is favoured by many workers, since it is more economical. Also, the toxicity and flammability of organic solvents, and product recovery without further organic solvent evaporation favours the use of solvent-free reaction[62]. Öznur *et al*.[18] reported alcoholysis of cotton seed oil in a solvent-free medium, using immobilized *Candida antarctica* lipase with 92% of the total ester yield. The use of *Rhizopus oryzae* lipase (whole cell biocatalyst) for the synthesis of methyl esters (MEs) in a solvent-free system led to 71% MEs after 165 hr reaction at 37°C with stepwise addition of methanol[63]. Table 3 lists and compares the ester yields obtained by alcoholysis with various lipases in the presence and absence of solvent. For alkali-catalyzed transesterification, the glycerides and alcohol must be substantially anhydrous because water produces soap. The soap reduces the catalytic efficiency by consuming the catalyst and, increases viscosity, gelation,

Table 3—Effect of presence and absence of solvent on enzyme-based biodiesel production

Alcohol	Oil source	Solvent	Lipase	Yield	References
Methanol	Tallow	Hexane	*Mucor miehei*	94.8	16
	Tallow	None	*Mucor miehei*	19.4	16
	Cotton seed	None	*C. antarctica*	92	15
	Restaurant grease	None	*Thermomyces lanuginosa (immobilized)*	4	20
	Restaurant grease	None	*C. antarctica (immobilized)*	27	20
Ethanol	Tallow	Hexane	*Mucor miehei*	98	16
	Tallow	None	*Mucor miehei*	65.5	16
	Restaurant grease	None	*Thermomyces lanuginosa (immobilized)*	87	20
	Restaurant grease	None	*C. antarctica (immobilized)*	76	20
Propanol	Restaurant grease	None	*Thermomyces lanuginosa (immobilized)*	87	20
	Restaurant grease	None	*C. antarctica (immobilized)*	79	20
Isopropanol	Tallow	Hexane	*C. antarctica*	51.7	16
	Tallow	None	*Mucor miehei*	90.3	16
	Restaurant grease	None	*Thermomyces lanuginosa (immobilized)*	61	20
	Restaurant grease	None	*C. antarctica (immobilized)*	59	20
Butanol	Restaurant grease	None	*Thermomyces lanuginosa (immobilized)*	90	20
	Restaurant grease	None	*C. antarctica (immobilized)*	56	20
2-Butanol	Tallow	Hexane	*Mucor miehei*	83.8	16
	Tallow	None	*Mucor miehei*	96.4	16
	Restaurant grease	None	*Thermomyces lanuginosa (immobilized)*	97	20
	Restaurant grease	None	*C. antarctica (immobilized)*	89	20

thereby causing difficulty in glycerol separation. Nelson *et al.*[19] have reported the solvent-free lipase-catalyzed methanolysis of oils and fats to be sensitive to water. In contrast, Hsu *et al.*[64] reported water activity (a_w) to be an important parameter for methyl ester synthesis. Water activity <0.5 led to 98% conversion to methyl esters. The essentiality of presence of water has also been demonstrated, while using *Rhizopus oryzae*[62], *Candida rugosa*[62], *Psuedomonas fluorescens*[17, 62] and *P. cepacia*[62] for transesterification.

Some biochemical challenges

While lipases catalysed transesterification reactions have been extensively used for production of drug intermediates, biosurfactants and designer fats, the alcoholysis of the fat/oil has several complex factors, which need to be taken into account for a viable process development:

Inhibition by alcohols

Short chain alcohols like methanol and ethanol are known to inactivate enzymes. Shimada *et al.*[25] and Belafi-Bako *et al.*[65] have suggested stepwise or continuous addition of methanol. In the case of latter work, the highest conversion (97%) was obtained by continuous addition strategy as compared to the protocol when necessary methanol was added in eight steps (94% conversion).

Inhibition by glycerol (and its recovery)

Belafi-Bako *et al.*[65] reported that while alkyl esters did not inhibit the biotransformation, the second product glycerol did inhibit both rate and extent of conversion. Their solution was use of a membrane reactor to continuously remove glycerol in order to relieve product inhibition. It was found that 85 mL L^{-1} flow rate at 50°C with a flat sheet membrane gave good results and 100% glycerol recovery rate was achieved after about 150 min.

Conclusion

The production of biodiesel starting with (i) a local or regional source of oil/fat, which is either non-edible or where availability exceeds its consumption; and (ii) waste or spent cooking oil seem an attractive approach for obtaining "green fuel". As in many biotechnological processes, the key is to develop self-reliance in production of the biocatalyst. Unfortunately, as often happens, lopsided emphasis on more fashionable areas in biology has resulted in neglect of the art and science of obtaining enzymes.

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