



Review Paper

Green biodiesel production: a review on feedstock, catalyst, monolithic reactor, and supercritical fluid technology

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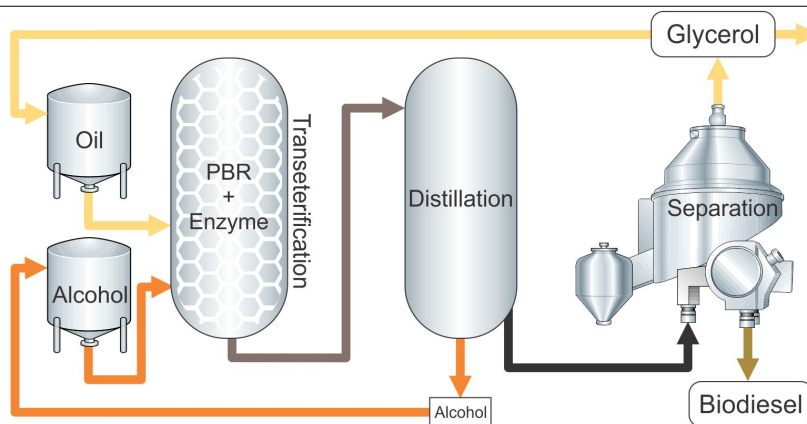
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HIGHLIGHTS

- Supercritical carbon dioxide (SC-CO₂) for continuous biodiesel production has been reviewed.
- The potential of SC-CO₂ system to be applied in enzymatic reactors has been discussed.
- Monoliths as support matrices for enzyme immobilization have been reviewed.

GRAPHICAL ABSTRACT



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ABSTRACT

The advancement of alternative energy is primarily catalyzed by the negative environmental impacts and energy depletion caused by the excessive usage of fossil fuels. Biodiesel has emerged as a promising substitute to petrodiesel because it is biodegradable, less toxic, and reduces greenhouse gas emission. Apart from that, biodiesel can be used as blending component or direct replacements for diesel fuel in automotive engines. A diverse range of methods have been reported for the conversion of renewable feedstocks (vegetable oil or animal fat) into biodiesel with transesterification being the most preferred method. Nevertheless, the cost of producing biodiesel is higher compared to fossil fuel, thus impeding its commercialization potentials. The limited source of reliable feedstock and the underdeveloped biodiesel production route have prevented the full-scale commercialization of biodiesel in many parts of the world. In a recent development, a new technology that incorporates monoliths as support matrices for enzyme immobilization in supercritical carbon dioxide (SC-CO₂) for continuous biodiesel production has been proposed to solve the problem. The potential of SC-CO₂ system to be applied in enzymatic reactors is not well documented and hence the purpose of this review is to highlight the previous studies conducted as well as the future direction of this technology.

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Abbreviations

APPI	Atmospheric pressure photoionization
<i>C. antarctica</i>	<i>Candida antarctica</i>
CALB	<i>C. antarctica</i> lipase B
CaO	Calcium oxide
CCFS	Closed cross flow structures
CLEA	Cross linking enzyme aggregate
CRL	<i>C. rugosa</i> lipase
EPA	Environmental Protection Agency
FAME	Fatty acid methyl ester
FFA	Free fatty acid
<i>J. curcas</i>	<i>Jatropha curcas</i>
KOH	Potassium hydroxide
M-SILPs	Monolith-supported IL phases
MPPM	Microporous polymeric matrix
NaOH	Sodium hydroxide
NREL	National Renewable Energy Laboratory
OCFS	Open cross flow structures
PBR	Packed bed reactor
PEDS	Polyethoxydisiloxane
PEG	Polyethylene glycol
polyHIPE	poly-High internal phase emulsion
ROL	<i>Rhizopus oryzae</i> lipase
RSM	Response surface methodology
RVO	Renewable Volume Obligation
SC-CO ₂	Supercritical carbon dioxide
SCF	Supercritical fluid
STR	Stirred tank reactor
<i>T. lanuginosus</i>	<i>Thermomyces lanuginosus</i>
TEOS	Tetraethoxysilane
TMOS	Tetramethoxysilane
Zr	Zirconia

1. Introduction

Fossil fuel usage has become a major concern in the last decade due to the diminishing oil reserve and environmental effects associated with it. The world's oil production capacity is expected to decline within ten decades (Crookes, 2006). Alternative energy has been extensively researched to mitigate this problem focusing on its renewability, economic aspects, and environmental impacts. Biodiesel is one of the prominent candidates which have attracted numerous researchers from all over the world. One of the predominant advantages of biodiesel is that it can be used in existing engines, vehicles, and infrastructure with practically no changes (Du et al., 2008). The biodiesel properties are similar to those of petrodiesel fuel (Ranganathan et al., 2008). However, engines fueled with biodiesel emit significantly fewer particulates, hydrocarbons, and less carbon monoxides than those engines operating on petrodiesel thus reducing the greenhouse gas effect (Sheehan et al., 2000; Yee et al., 2009). Biodiesel is much safer than petrodiesel with a flash point of over 266 °F compared to about 126 °F for regular No. 2 diesel (ASTM standard D6751). They were 105 biodiesel production plants in operation as of early 2007 with a total production capacity of 864 million gallons and additional 1.7 billion gallons coming from plants under construction according to U.S. National Diesel Board. In 2013, The U.S. Environmental Protection Agency (EPA) set the Renewable Volume Obligation (RVO), in an annual rule making under a congressional mandate that called for the country to use 36 billion gallons of renewable fuels in transportation by 2022 (Schnepf and Yacobucci, 2010).

The quality of biodiesel is primarily determined by its fatty acid composition hence varies among different biodiesel feedstocks (Knothe, 2008; Ramos et al., 2009). It was reported by the U.S. Department of Energy in early 2014 that the price of biodiesel (B99-100) was approximately \$4.28/gal compared to petrodiesel with only \$3.89/gal (U.S. Department of Energy, 2014). The high price of biodiesel is directly associated with the cost of biodiesel feedstock. It is estimated that the raw material alone contributes to 75–90% of the total biodiesel production cost (Parawira, 2009). Since the production cost is directly proportional to the cost of raw material, a quest to

select the best biodiesel feedstock is increasingly becoming a topic of interest in the development of an economically viable biodiesel production platform. Enzymatic transesterification of lipids using lipases is considered as highly effective since the glycerol can be recovered easily and economically (Vicente et al., 1998; Fukuda et al., 2001; Jegannathan et al., 2008). However, the major setback for its commercial application in biodiesel production is the high-price of lipase. In order to solve this problem, lipase can be immobilized onto a solid support to allow repeated use in batch or continuous reaction processes (Balcão et al., 1996; Hama et al., 2007). The choice of a support matrix for enzyme immobilization is based on several factors, namely the loading capacity, ease of regeneration, kinetic stability, functional groups availability, microbial resistance, mechanical strength, and cost (Urban et al., 2012). There are various support materials available such as porous beads (Gao et al., 2006) and monoliths (Orçaire et al., 2006; Eeltink et al., 2007).

The advancement of technology in biodiesel production has led to the development of an enzymatic monolithic reactor. In comparison with packed bed reactor (PBR) and stirred tank reactor (STR), integrating enzymes on a monolithic reactor facilitates better mass transfer and lower bed pressure drop, besides permitting higher substrate flow rate thus enhancing biodiesel productivity (Santos and Costa, 2009). Chemical catalysts, both homogenous and heterogenous have been widely used to catalyze the conversion of oil into biodiesel. However, both types of chemical catalysts are often associated with the corrosion effect and formation of soap in the reaction medium containing high free fatty acid (FFA) or water (Canakci and Gerpen, 1999), leading to the development of non-catalytic supercritical transesterification. This catalyst-free reaction is much simpler and environmentally-friendly due to the absence of toxic chemicals.

Supercritical biodiesel production, employed at temperatures and pressures above the critical value of fluid provides improved mass transfer rate while promoting esterification and transesterification at high rates (Kondoh and Fukuda, 2008). Biodiesel synthesis in supercritical carbon dioxide (SC-CO₂) thus provides an alternative to the expensive supercritical methanol-based method. SC-CO₂ can be operated at mild temperatures and pressures. The integration of enzymatic monolithic reactor with supercritical carbon dioxide is now becoming a cutting-edge research in the field of biofuel. An extensive review highlighting this technology in biodiesel production is also less published.

This article aims to review the potential of integrating enzymatic reactors with SC-CO₂ for biodiesel production using microalgae as the feedstock. The different generations of biodiesel feedstock are firstly discussed. The catalysts used for the transesterification of oil into fatty acid methyl ester (FAME) are highlighted in the next section followed by strategies for lipase immobilization and detailed description of SC-CO₂ enzymatic-based monolith reactor. Previous published works employing this technology for biodiesel production are also highlighted. This review also provides perspectives and challenges associated with the commercialization of this technology.

1.1. 1st and 2nd generation biodiesel feedstocks

Several generations of feedstocks have been used for biodiesel production as shown in Figure 1. 1st generation biodiesel feedstocks are mainly produced from plants such as soybean, jatropha, palm oil, and canola oil (Ong et al., 2011) or animal fats (Ma et al., 1998). The economic viability and sustainability of the 1st generation feedstock however has come under scrutiny. Environmentalists claim that the expansion of oil crop plantations for large scale biodiesel production has resulted in deforestation in countries such as Malaysia, Indonesia, Argentina, and Brazil (Gao et al., 2011). The situation is further deteriorated by the competition for arable land for food and fiber plantation, high consumption of water and fertilizer, and poor agricultural practices. Thus, the first generation feedstock is claimed to be inefficient since it affects the food security and global food markets (Noraini et al., 2014). In order to overcome this problem, 2nd generation biodiesel produced from non-edible by-products and wastes has been proposed. However, the biggest challenge of the 2nd generation biodiesel production is the availability of cropland enough to produce these by-products for biodiesel production in a commercial scale. Hence, there is a need to

explore alternative feedstocks for biodiesel production that have less impact on the food industry (Cheng and Timilsina, 2011).

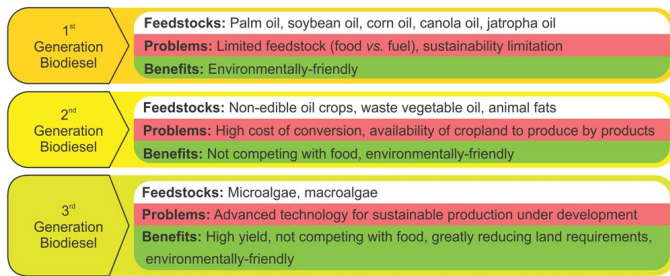


Fig.1. Development of biodiesel feedstocks.

1.2. Algae as a biodiesel feedstock

In the past decade, algae have emerged as a promising feedstock for biodiesel production. Numerous studies have indicated algae as a superior feedstock for biodiesel production compared to 1st and 2nd generation biodiesel feedstocks due to its high environmental index and high fuel efficiency (Brennan and Owende, 2010). The cultivation of microalgae can be performed on non-arable lands thus avoiding the increase of farmlands required for oleaginous plants. Oil yield per-acre-per-year of microalgae is approximately 150 – 200 times higher than that of plants (Chisti, 2007). Microalgae also have high photon conversion efficiency (as evidenced by the increased biomass yields per hectare) and the ability to utilize salt and waste water streams, thus significantly reducing freshwater consumption (Chisti, 2007; Li et al., 2008; Schenk et al., 2008). Under suitable conditions, algae can double its biomass in 24 h and produce up to 50% lipid to dry biomass ratio (Chisti, 2007). Algae do not compete with other feedstocks for natural resources and further; they do not impact food prices as is the case, for instance, with corn-based biodiesel. It is estimated that algae can produce up to 15,000 gal/acre/y compared to soybean oil (48 gal/acre/y), peanut oil (113 gal/acre/y), rapeseed oil (127 gal/acre/y), and palm oil (635 gal/acre/y) as reported by the U.S. National Renewable Energy Laboratory (NREL).

However, oil extracted from vegetables (plant or microalgae) introduces new problems if it is to be directly used in existing engines. Problems such as coking and trumpet formation on the injectors, carbon deposits, oil ring

sticking, thickening, and gelling of the lubricating oils have been encountered due to their high viscosity and tendency to polymerize during combustion (Ma and Hanna, 1999; Altun et al., 2001; Rakopoulos et al., 2006). Several methods have been introduced to solve these problems including microemulsions, pyrolysis, and transesterification with the sole purpose of reducing the viscosity of oil extracted from plants (Marchetti et al., 2007). Among all these alternatives, transesterification is likely the best choice as the process is simple, and the physical characteristics of the FAME (biodiesel) are very close to those of diesel fuel (Demirbas, 2009). Transesterification is the catalytic process of exchanging the alkoxy group of an ester by an alcohol (acyl acceptor) that converts the triglyceride in oils to FAME and glycerol. Chemical (acids or bases) and biological (enzymes) agents can be used effectively as catalysts (Noureddini and Zhu, 1997; Xu et al., 2003; Shah et al., 2004; Čerče et al., 2005; Leung et al., 2010).

2. Catalysts for lipid transesterification

As mentioned earlier, biodiesel is produced *via* transesterification reaction in the presence of a catalyst to convert raw lipid (triacylglycerol/FFAs) into renewable, environmentally friendly and biodegradable diesel known as mono-alkyl esters of long fatty acid (Baskar and Aiswarya, 2016). Besides feedstock, the type of catalyst is also important in biodiesel production. Various types of catalyst have been employed in transesterification process including chemical and biological catalysts, in homogeneous and heterogeneous forms. The type of catalyst and reaction condition used for lipid transesterification directly affects the yield and quality of the biodiesel produced (Table 1).

2.1. Chemical catalyst

During the conversion of triglyceride into FAME, the anion of alcohol attacks the carbonyl group of triglyceride molecules forming an intermediate tetrahedral, which rearranges to form diglyceride and fatty acid ester. The mechanism is repeated stepwise to liberate diglyceride, monoglyceride, and glycerol while the catalyst is later recovered through a proton transfer (Aransiola et al., 2014). The process is a reversible reaction although the reaction equilibrium favors the production of fatty acid and glycerol (Ma and Hanna, 1999).

2.1.1. Homogenous catalyst

Most industrial-scale biodiesel productions rely on homogenous catalysts that usually make use of either acid or base catalyst in liquid

Table 1. Reaction conditions and performance of biodiesel production using various types of catalyst (Lam et al., 2010; Leung et al., 2010; Math et al., 2010; Taher et al., 2011a; Sani et al., 2013).

Parameter	Homogenous catalyst	Heterogenous catalyst	Supercritical alcohol	Enzymatic catalyst
Reaction temperature	40 – 75 °C (base catalyst), 50–100 °C (acid catalyst)	60–65 °C (base catalyst), 30 – 460 °C (acid catalyst)	230–350 °C	35–60 °C
Reaction pressure	Atmospheric pressure	0.05 – 20 Mpa	19 – 35 Mpa	N/A*
Molar ratio Alcohol : oil	3:1 – 9:1 (base catalyst), 30:1 – 50:1 (acid catalyst)	6:1 – 18:1 (base catalyst), 6:1 – 70:1 (acid catalyst)	40:1 – 45:1	3:1–18:1
Reaction time for complete transesterification	1 – 4 h (base catalyst), more than 4 h (acid catalyst)	0.5–2 h (base catalyst), 3–20 h (acid catalyst)	3 min – 1 h	More than 24 h
Glycerol recovery	Difficult (base catalyst), moderate (acid catalyst)	Moderate (base catalyst), Easy (acid catalyst)	Easy	Easy
Product purification	Difficult (base catalyst), Moderate (acid catalyst)	Easy (base catalyst), Easy (acid catalyst)	Easy	Easy
Catalyst cost	Cheap	Moderate	Expensive	Expensive
Effect of water	Inhibit reaction (base catalyst), Tolerant to low water content (acid catalyst)	Inhibit reaction (base catalyst), Tolerant to low water content (acid catalyst)	Increase reaction rate	Negative effect if too much
Effect of FFA	Soap formation (base catalyst), No influence (acid catalyst)	Soap formation (base catalyst), No influence (acid catalyst)	No influence	No influence
Reusability	No	Yes	Yes	Yes

*N/A: not available

form. In the alkaline catalyst process, strong base such as potassium hydroxide (KOH) or sodium hydroxide (NaOH) at a concentration range of 0.5–1 wt.% is used as a catalyst along with short-chain alcohol such as methanol or ethanol at a temperature range between 45–80 °C. NaOH is the popular choice for industrial application because of the low price and high biodiesel yield (Kim et al., 2004). This process is the most efficient and least corrosive of all the processes (Ranganathan et al., 2008). Initially, reaction between catalyst and alcohol takes place forming alkoxy, the alkoxy is then reacted with any triacylglycerols forming biodiesel and glycerol. Product recovery is simple as glycerol being denser than biodiesel settles down at the bottom forming separated liquid phase allowing biodiesel to be decanted easily. Although the alkaline catalyst process produces biodiesel fuel of high quality, the formation of soap is likely to take place when high FFA or water content is present in the oil, which is the usual case for low cost biodiesel feedstock such as non-edible oil, used oil or animal fat, making this process unfavorable. Acid can be used to catalyze the conversion of lipid into biodiesel. Sulfuric acid or hydrochloric acid is commonly employed as a catalyst at concentrations in the range of 0.5–1 wt.% with temperatures of 60–100 °C. Although the biodiesel yield is high, acid-catalyzed process is often corrosive in nature causing damage to equipments and the reaction rate is low (Canakci and Gerpen, 1999). Moreover, the major drawback of a homogenous catalyst is the need to operate it at a relatively high temperature besides the release of undesired by-products. The requirement for separation and purification of final product also renders this technique challenging and costly.

2.1.2. Heterogeneous catalyst

Heterogeneous catalyst offers an economic solution to homogenous catalyst as they can be easily recovered and reused for successive cycles with ease of product separation and purification (Endalew et al., 2011). Heterogeneous catalyst is less hazardous and creates less environmental problems. Comprehensive reviews highlighting the various heterogeneous catalysts and their preparation have been reported in the literature (Helwani et al., 2009; Chouhan and Sarma, 2011; Borges and Diaz, 2012). Conventional heterogeneous base catalysts are derived from alkaline earth metal oxides and its derivatives (e.g., MgO, CaO, BaO). Meanwhile, acid catalysts are usually made from sulfate salts, zirconium oxide, titanium oxide, tin oxide, and heteropolyacid. The main advantage of heterogeneous base catalyst over acid catalyst is their ability to carry out transesterification at high reaction rates and lower reaction temperatures. Calcium oxide is among the popular choice of conventional heterogeneous base catalysts due to its long shelf-life, high basicity, moderate reaction conditions, and lower price in comparison with other heterogeneous base catalysts. (Zhu et al., 2006). Bai et al. (2009) reported that as high as 98% of FAME was obtained using CaO catalyst. Nevertheless, CaO has been reported causing leaching during transesterification, catalyst deterioration upon contact with air, and active site poisoning (Chouhan and Sarma, 2011). Moreover, the requirement for pretreatment of oil to overcome the problem of soap formation when high FFA content is present in the reaction has become a major hurdle for heterogeneous base catalyst in biodiesel processing.

Heterogeneous acid catalyst has the advantage when dealing with biodiesel feedstocks with high level of FFA contents. Besides being unsusceptible to high FFA contents, heterogeneous acid catalyst allows easy product separation and greater recyclability (Melero et al., 2009). Jitputti et al. (2006) reported over 90% FAME yield using heterogeneous acid catalyst for biodiesel production. Zirconia (Zr)-based acid catalyst is favorable due to the high number of Brönsted acid on its surface. However, in addition to longer reaction time and higher temperature requirement, Zr is a rare metal hence the catalyst fabrication is expensive. The constant efforts made in addressing the issues associated with heterogeneous base and acid catalysts have led to the development of advanced heterogeneous catalysts that offer simultaneous esterification and transesterification benefits.

Generally, the building block of modern heterogeneous catalysts is a spinel mix of two (non-noble) metals (Helwani et al., 2009) which determine the basic and acidic characteristics of the catalysts. These features allow users to fabricate catalysts with preferred functionalities by adding certain metals that favor the desired catalytic properties. Yan et al. (2009) reported the application of a modern heterogeneous catalyst for the conversion of unrefined waste cooking oil into biodiesel. A conversion efficiency of 96%

was observed in a reaction performed at 200 °C for 3 h using zinc/lanthanum ratio of 3:1. The mixture of zinc and lanthanum was found to exhibit higher catalytic activities than pure metal oxides with higher tolerance against FFA and water content.

Kondamudi et al. (2011) employed quintinite-based heterogeneous catalyst for biodiesel synthesis from various waste vegetable oils. The reaction was carried out at a temperature of 70 °C for 2 h using 2:1 Al/Mg ratio, giving a conversion efficiency of ~ 98% and a reasonable catalytic stability after 5 runs. Other successful applications of modern heterogeneous catalysts have also been reported elsewhere (Soldi et al., 2009; Cannilla et al., 2010; Macario et al., 2010; Salinas et al., 2010; Wen et al., 2010). Despite the high conversion efficiency, such catalysts however, have several disadvantages including harsh reaction conditions, energy intensive process, difficulty in by-product removal, and feedstock pretreatment requirement.

2.2. Supercritical fluid technology

Although heterogeneous catalysts have been explored to overcome problems related to homogenous catalysts, the same inherent drawbacks are still found on both technologies, the requirement of pretreatment of FFA and separation of water and glycerol. If the mixture is left untreated, soap formation may occur and consequently the catalysts are deactivated. Thus, non-catalytic supercritical transesterification of lipid has been proposed to overcome the above problem. As a result of the catalyst absence, product separation is much simpler and environmental friendly. Moreover, studies have shown that the presence of water in the reaction further promotes supercritical transesterification (Minami and Saka, 2006; Tan et al., 2010a; Niza et al., 2013). The principle of supercritical fluid (SCF) in biodiesel processing is where the transesterification reaction is carried out at temperatures and pressures above the critical value of the fluid. In this state, the fluid properties are the intermediate of its liquid and gas phases. SCFs have higher solvent properties than gases and better transport properties than liquids. Consequently, mass transfer rate is improved due to the relatively low viscosity, high diffusivity, and low surface tension (Kondoh and Fukuda, 2008). Under such conditions, the mixture of feedstock (triglyceride) and acyl acceptor (alcohol) becomes homogenous where esterification and transesterification can be carried out simultaneously at a high rate. Homogenous reaction in supercritical fluid can be explained by the increase in solubility of non-polar triglyceride as temperature and pressure increase, while lowering the dielectric constant of alcohol resulting in a hydrophobic nature of alcohol. As a result, non-polar triglycerides dissolve easily forming a single phase of oil/alcohol/SCF mixture (Lee and Saka, 2010). As opposed to the previously mentioned catalyst reaction, supercritical transesterification is suitable for any types of biodiesel feedstocks especially low cost feedstocks containing a high level of FFA. Additionally, the reaction rate of supercritical transesterification is proven to be superior to catalyst-assisted reaction (Demirbas, 2005; Minami and Saka, 2006; Endalew et al., 2011).

2.2.1. Supercritical alcohol

The most studied SCF-based biodiesel production method deals with supercritical methanol and also known as the Saka method (Saka and Kusdiana, 2001). The supercritical methanol method has been proven to produce a high yield of biodiesel at a very fast reaction rate compared to other methods aforementioned. In this reaction, hydrogen bonding of methanol is significantly reduced allowing methanol to become a free monomer. It is suggested that, depending on pressure and temperature, methanol molecule can directly attack the carbonyl group of triglyceride. The transesterification is then completed *via* a methoxide transfer. The process mechanism is almost similar to that of chemical transesterification through stepwise reaction of converting triglyceride, diglyceride, monoglyceride, methyl ester, and lastly glycerol. Parameters affecting transesterification reaction *via* supercritical-methanol process such as temperature, pressure, and molar ratio of alcohol to triglyceride have been discussed previously (Lee and Saka, 2010). It is also noted that the optimum temperature needed in order for the transesterification to occur lies within the range of 270 °C to 300 °C with a supercritical pressure of

8.09 Mpa and higher. Although higher temperature has been proven to increase the transesterification rate (Saka and Kusdiana, 2001), isomerization of polyunsaturated methyl ester from cis-type to trans-type isomers as well as thermal decomposition has been observed (Imahara et al., 2008). In this situation, the reaction should be conducted below 300 °C. Tan et al. (2009) reported the comparison between conventional catalyst (homogeneous and heterogeneous) and supercritical methanol using the same oil (purified palm oil). The result revealed that supercritical methanol reaction rate was 3-folds higher compared to the conventional catalyst. Alternative alcohol such as ethanol, 1-propanol, 1-butanol, and 1-octanol have also been studied under supercritical condition (Warabi et al., 2004). However, due to the requirement of high temperature and pressure when only alcohol was employed, addition of co-solvent such as carbon dioxide, calcium oxide, propane, and hexane has been proposed to address this problem (Yin et al., 2008a). The addition of a small amount of catalyst and co-solvent in the reaction significantly reduced the amount of methanol and temperature required (Yin et al., 2008b). The reaction produced 98% yield of methyl esters within 10 min reaction time. Nevertheless, the reaction condition is still considered as energy intensive, and the addition of co-solvents resulting in increased reaction volume is less practical at large-scale.

2.2.2. Supercritical carbon dioxide

SC-CO₂ is a potentially promising alternative to supercritical methanol. A distinctive advantage of SC-CO₂ is that supercritical condition can be achieved at mild temperature and pressure below the denaturation conditions of the enzyme. Final CO₂ removal can easily be achieved by reducing the pressure level since the product and enzyme are not soluble in CO₂. Thus, SC-CO₂ has the potential to be integrated into a continuous industrial enzymatic reactor for biodiesel production. The utilization of SC-CO₂ in enzymatic processes is further discussed in Section 5.

2.3. Enzymatic catalyst

Recent studies show that biocatalytic (enzymatic) process is more favorable than conventional catalyst (Aransiola et al., 2014). Lipases from different sources have been explored for their ability to convert lipid into biodiesel by means of enzyme immobilization (Christopher and Zambare, 2014). Figure 2 shows the general transesterification reaction using lipase catalyst. Enzymatic transesterification comprises four steps: i) the formation of enzyme-substrate complex by nucleophilic addition; ii) the proton transfer from conjugate acid of amine to alkyl oxygen atom of substrate; iii) the addition of oxygen atom from alcohol to the acyl-enzyme intermediate forming acylated enzyme-alcohol complex; and iv) the elimination of oxygen atom from the complex followed by the formation of FAME by proton transfer from conjugate acid of amine (Al-Zuhair et al., 2007). During the transesterification process, breakdown of triglyceride is performed *via* hydrolysis in the presence of water. However, if alcohol is in excess, lipase will accept alcohol as substrate and start converting lipid into biodiesel by following the bi-bi ping pong reaction (Fjerbaek et al., 2009). This can be explained by the fact that the catalytic site of lipase is hidden within the structure of its monomeric protein which is only exposed at the lipid-water interface (Nigam et al., 2014).

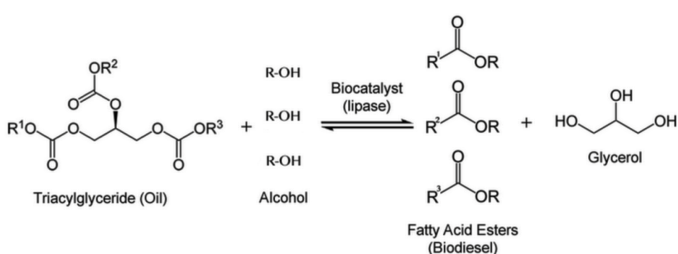


Fig.2. General transesterification reaction using lipase catalyst.

Enzymatic reaction offers significant benefits in terms of its low energy consumption and high compatibility with high FFA content of feedstock. It

also provides ease of product separation and catalyst recovery as well as good regenerability (Fukuda et al., 2001). Lipase requires water molecules in order to be activated for biodiesel production. Optimal water activity needed for lipase activation relies on the lipase origin, solvent used, and enzyme condition (suspended or immobilized) (Noureddini et al., 2005; Oda et al., 2005).

The enzymatic transesterification process is normally carried out in the presence of an organic solvent to obtain a homogenous reaction mixture (Lu et al., 2008; Park et al., 2008). Lipases can be deactivated by a short chain alcohol (e.g., methanol and ethanol) as reported by several researchers (Watanabe et al., 2002; Du et al., 2004; Modi et al., 2006). It has been reported that tert-butanol is more suitable as an acyl acceptor (Li et al., 2006, 2007; Raita et al., 2011). Besides reducing the inhibition factor, tert-butanol stabilizes and promotes enzyme activity mainly due to the moderate polar properties of tert-butanol that allow complete solubilization of methanol and glycerol (Li et al., 2006). However, tert-butanol is least preferred to short chain alcohol in large scale processes due to their relatively expensive price. A solvent-free system has been employed to synthesize biodiesel at a comparable productivity (Bélafi-Bakó et al., 2002; Shah and Gupta, 2007; Ognjanovic et al., 2009), however, a lower reaction rate due to poor mass transfer efficiency between the oil/methanol and the enzyme was observed. To enhance the mass transfer rate in a solvent-free system, an interconnected porous reactor that offers convective mass transfer property has been proposed. Such a reactor is further highlighted in Section 4. New technologies and strategies to address the issues of high enzyme cost and slow reaction rate are reviewed in Section 5.

3. Strategies for lipase immobilization

Enzyme immobilization on support matrix for biodiesel processing has been rapidly gaining its pace. Immobilization technology has been known to shelter and/or stabilize enzymes against harsh environments (e.g., pH, temperature, chemicals, and agitation), reduce contamination of products (Mateo et al., 2003), and more importantly promote the recovery and reuse of enzyme in a continuous process (Husain et al., 2011; Misson et al., 2015a). There are three strategies of enzyme immobilization: (i) direct immobilization on modified surfaces; (ii) immobilization on beads; and (iii) immobilization of enzymes on monoliths (He et al., 2009). Some disadvantages were reported for both direct immobilization and immobilization on beads namely; (1) long diffusion times; (2) enzyme denaturation; (3) and the need of high enzyme load during immobilization (Park and Clark, 2002; Peterson et al., 2002; Křenková and Foret, 2004). The key component in producing high quality immobilized enzyme is the appropriate selection of immobilization techniques and support material to be used that permit high enzyme activity whilst reducing inhibition effects, promotes wide ranges of pH and temperature tolerance, and high stability under the constraints imposed by the substrate medium (Noureddini et al., 2005; Shao et al., 2008). Enzyme has been employed in various types of support materials including natural/synthetic polymers or inorganic materials as reported by Datta et al. (2012) or advanced nanostructured materials as reviewed by Misson et al. (2015b). Understanding the strategies of enzyme immobilization is important in order to preserve the native functionality of a biocatalyst. Enzyme immobilization can be done by physical adsorption, covalent binding, cross linking enzyme aggregate (CLEA), and entrapment methods (Tan et al., 2010b).

3.1. Physical adsorption

The adsorption of enzymes onto insoluble supports occurs through weak forces like Van der Waals or hydrogen bonding under suitable conditions such as optimal pH and ionic strength (Jegannathan et al., 2008). Adsorption has been widely employed for lipase immobilization due to the simple and inexpensive process as well as the potential reuse of carrier. The standard procedure for adsorption onto solid carriers includes dissolving lipase in a buffer solution with gentle mechanical mixing. Salis et al. (2003) revealed that lipase adsorption took a longer time by displacing other proteins with lower affinity for the support. Among the supports used for lipase adsorption are silica (Blanco et al., 2004),

styrene-divinylbenzene copolymer (Dizge et al., 2009b), chitosan based support (Badgujar et al., 2013; Nwagu et al., 2013), carbon monolith (Luangon et al., 2012), activated carbon (Ramani et al., 2012), polysulfone membrane (Yujun et al., 2008), aldehyde-resin (Mendes et al., 2011), and polypropylene (Gitlesen et al., 1997). The support properties such as hydrophobicity, particle size, and porosity significantly affect the enzyme binding capacity and activity upon immobilization (Palomo et al., 2002). Low immobilized lipase activity is usually attributed to the alteration in structural conformation of the enzyme, low enzyme immobilization yield, and reduction in the ability of hydrophobic substrates to reach the active site of lipase (Xavier Malcata et al., 1990). Hydrophobic interaction between lipase and the support may also occur resulting in lipase adsorption as reported by Gitlesen et al. (1997).

The pore diameter of mesoporous and macroporous supports affects the lipase immobilization yield, where the degree of immobilization yield increases with increasing pore diameter to a certain point (Lauren et al., 2013). Adsorption isotherm illustrating the trend of support affinity for lipase can be used to study lipase-support interaction and to screen for a suitable support for lipase immobilization (Blanco et al., 2004). Despite the advantages of simple process and mild reaction conditions (Jesionowski et al., 2014), lipase attachment through adsorption suffers a major drawback that is leakage due to the weak binding between lipase and carrier thus reducing the enzyme recyclability.

3.2. Covalent binding

Enzyme immobilization *via* covalent binding provides a strong attachment of enzymes on the support matrix. As a result, covalent binding can potentially hinder enzyme leakage upon immobilization. Binding can occur through the interaction between the functional groups available on the surface of the enzyme and carrier. Functional groups such as amino, carboxylate, thiol, and hydroxyl are present on enzyme surfaces located in lysine, arginine, glutamic, and aspartic acid residues (Sakai et al., 2010). The functional groups on the carriers are introduced *via* surface functionalization (Misson et al., 2016) or activation using coupling agent (Xie and Ma, 2009). Coupling agents determine the type of functional group deposited on the support surface. The commonly used coupling agent glutaraldehyde introduces aldehyde groups that will interact with lipase amino group (Barbosa et al., 2012). Epichlorohydrin, on the other hand, will bind to amino, hydroxyl, and sulfhydryl groups of lipase (Rodrigues et al., 2008; Nigam et al., 2014). Many other functional groups of lipase have also been explored for covalent immobilization. Knezevic et al. (2006) reported lipase immobilization on Eupergit C support *via* carbohydrate moiety demonstrating nearly 2-fold higher enzyme stability than conventional method and 18-folds higher than free lipase. Successful implementations of this technique have also been reported for other types of enzyme such as invertase (Prodanović et al., 2001) and glucoamylase (Milosavić et al., 2007). Support surface modifications using a spacer arm have also shown positive impacts on the enzyme stability (Bayramoglu et al., 2005; Ozyilmaz, 2009). Modifications of the length of the spacer arm can be achieved through a series of chemical reactions as demonstrated by Arica et al. (2009) and (2000). Despite providing strong binding and enhancing enzyme stability against leakage (Rodrigues et al., 2013), covalent binding requires rigorous conditions during immobilization that may potentially reduce the lipase activity thus rendering the technique less economical.

3.3. Cross linking enzyme aggregates (CLEA)

CLEA is a carrier-free enzyme immobilization technique first discovered in 1960s (Sheldon, 2007). The enzyme acts as a carrier and precipitates forming a high concentration of enzymes (Hanefeld et al., 2009). The immobilization approach includes enzyme precipitation by the addition of salts/water-miscible organic solvents/non-ionic polymer (e.g., acetone, ammonium sulphate, ethanol or polyethylene glycol - PEG) followed by the addition of a cross-linker (e.g., glutaraldehyde) (Jegannathan et al., 2008; Hanefeld et al., 2009). It has been known that the addition of additives such as surfactants and amines enhances the activity of the immobilized enzyme (Sheldon, 2007; Nigam et al., 2014). The purification and immobilization processes are combined into a single step, thus simplifying the process and lowering the production cost. The successful applications of CLEA in biodiesel production

have been demonstrated previously (Kumari et al., 2007; Raita et al., 2011; Sheldon, 2011; Lai et al., 2012). Hilal et al. (2004) reported a new approach by embedding CLEA onto a microporous polymeric membrane producing an effective lipase-embedded biocatalytic membranes. However, CLEA displays some drawbacks when it is used in a heterogeneous reaction. As the size of CLEA is usually below 10 μm (Jegannathan et al., 2008), the enzyme activity declines drastically, and separating CLEA from the product can be problematic (Cao et al., 2003).

3.4. Entrapment (physical immobilization)

Entrapment, as in the case of a sol gel technique, involves mixing of enzymes in a monomeric solution before the polymerization occurs. Enzymes are protected against environmental exposure within the polymeric matrix. The porosity of an enzyme-immobilized polymer can be varied based on the polymerization conditions, for instance, the drying conditions (Sheldon, 2007). The gel formed can be classified into three categories in the order of decreasing densities namely; xerogels, ambigels, and aerogels, respectively. Lipase has also been entrapped in methylenebisacrylamide, chitosan, cellulose, agarose, calcium alginate, and kappa carrageenan (Ghaly et al., 2010; Nigam et al., 2014). It is important to note that hydrophilic carriers may reduce the activity of an enzyme hence is not recommended for lipase entrapment (Reetz et al., 1996). As the enzyme is entrapped during the polymerization process, it is also important that the reaction conditions do not denature or damage its structural conformation. Additionally, additives such as surfactants and crown ethers have proven to increase lipase loading capacity and activity (Sheldon, 2007). Soares et al. (2004) demonstrated the activity of entrapped lipase in the presence of PEG of three times higher than without additives. Although the entrapment method is cheap, fast, and involves mild conditions (Lalonde and Margolin, 2008), mass transfer resistance and enzyme leakage have hindered its successful application. Table 2 compares the different techniques involved in lipase immobilization.

Although enzyme immobilization offers various advantages, the conversion of lipid into biodiesel involves large molecules such as triglyceride thus diffusion of such molecules can be problematic. On the other hand, large pore size can lead to internal mass transfer limitations as the effective contact between the enzyme and substrate is reduced. The formation of a glycerol film in a continuous porous biodiesel reactor has been reported previously (Fjerbaek et al., 2009). Thus, appropriate selection of support matrices and methods for enzyme immobilization is vital in order to produce highly efficient biodiesel pathways. Selection of support matrices is dependent upon a number of factors such as mechanical strength, microbial resistance, thermal stability, chemical durability, hydrophobicity, ease of regeneration, loading capacity, and production cost (Jegannathan et al., 2008).

4. Monolithic bioreactor for biodiesel production

Various types of support matrix for enzyme immobilization have been used for the biocatalytic conversion of lipid into biodiesel. STR in which the immobilized enzyme is suspended in a liquid medium is a simple bioreactor design consisting of a reactor vessel and a propeller. STR offers high heat removal and excellent mass transfer characteristics due to the very small particle size, hence the increase in surface area. However, STR needs multiple purification steps which are inconvenient in a large scale application. PBR can be operated in either batch or continuous mode by recirculating the reaction mixture. In PBR, substrate flows concurrently upward or downward over the immobilized enzyme. However, a downward substrate flow is not always preferable due to poor oxygen transfer and high pressure drop caused by the compressed enzymatic bed support (Fjerbaek et al., 2009). PBR is often limited by large biomolecular assemblies, highly concentrated feedstocks, reduced liquid-solid contacting efficiency, and bed instability (Stickel and Fotopoulos, 2001). Figure 3 shows the schematic diagram of biodiesel production route *via* STR and PBR.

In the continuous search for suitable processes of fabricating enzymatic bioreactors, scientists have discovered the idea of using structured packing instead of randomly packed bed (Strancar et al., 2002). To date, various types of structured packing material are available such as organic

Table 2.
Various lipase immobilization techniques.

Source of enzyme	Type of support	Method of immobilization	Acyl acceptor	Oil feedstock	Reaction condition	FAME (%)	Recyclability	Reference
<i>Candida</i> sp. 99-125	Textile	Adsorption	Methanol	Lard	8 ml n-hexane as solvent, 20% water based on the fat weight, temperature 40 °C, three-step addition of methanol, solvent free	87.4%	Stable when used repeatedly for 180 h	Lu et al. (2007)
<i>Thermomyces lanuginosa</i>	Immobead 150	Adsorption	Methanol	Soybean oil	temperature 40 °C, and three-step addition of methanol, solvent free, 9.65 g soybean oil	90%	N/A*	Du et al. (2005)
<i>Candida antarctica</i>	Acrylic resin	Adsorption	Methanol	Cotton seed oil	24h at 50 °C with a reaction mixture containing 32.5% tert-butanol as solvent, 13.5% methanol, 54% oil	95%	Stable when used repeatedly for 500 h	Royon et al. (2007)
<i>Thermomyces lanuginosa</i>	Immobead 150	Adsorption	Methanol	Soybean oil	methanol/oil molar ratio 3.6:1, temperature 40 °C, 150 r/min. tert-butanol as solvent	97%	Stable when used repeatedly for 120 cycle	Wang et al. (2006)
<i>Thermomyces lanuginosa</i>	Toyopearl AF-amino-650M	Covalent	Ethanol	Palm oil	Ethanol/oil ratio 18:1, 48h at 45°C, agitation speed 180 rpm	100%	N/A	Mendes et al. (2011)
<i>Thermomyces lanuginosus</i>	(STY-DVB) copolymer	Covalent	Methanol	Canola oil	50°C in 24h reaction, 3 step addition of methanol, solvent free, 6:1 methanol/oil	97%	No changes after 10 repeated batch reactions	Dizge et al. (2009b)
<i>Thermomyces lanuginosus</i>	Aldehyde Lewatit beads	Covalent	Ethanol	Soybean oil	7.5:1 ethanol/oil, 4 % water, 10h at 30 °C in n-hexane	100%	Similar activity after 3 batches	Rodrigues et al. (2010)
<i>Thermomyces lanuginosus</i>	Olive pomace	Covalent	Methanol	Pomace oil	25 °C in 24 h reaction. 3 step addition, solvent free. 1:6 methanol/oil,	93%	Similar activity after 10 batches	Yucel (2011)
<i>Penicillium expansum</i>	N/A	Cross-linked	Methanol	Microalgae oil	0.5 g algal oil was mixed with 1.0 ml IL with addition of distilled water (15µL), 3:1 methanol/oil, at 50 °C at a speed of 220 rpm for 48 h	85.7%	N/A	Lai et al. (2012)
<i>Pseudomonas cepacia</i>	N/A	Cross-linked	Ethanol	Madhuca indica oil	4:1 ethanol/oil, 40 °C for 2.5 h with a constant shaking at 200 rpm	92%	N/A	Kumari et al. (2007)
<i>Candida</i> sp. 99-125	Macroporous silica	Cross-linked	Ethanol	Jatropha oil	40 °C for 24 h, 0.21g oil/0.175ml ethanol, 1.5 ml cyclohexane	72%	Maintain more than 85% of initial activity after 16 days	Jiang et al. (2014)
<i>Pseudomonas cepacia</i>	Sol-gel support	Entrapment	Methanol	Soybean oil	35°C, 1:7.5 oil/methanol molar ratio, 0.5g water in 1 h reaction	67%	Decrease to below 50% after 12 cycles	Noureddini et al. (2005)

*N/A: not available

and inorganic supports called “monolith” (Ameur et al., 2014), “sandwich packing” (Kashani et al., 2005), “open cross flow structures” (OCFS), and “closed cross flow structures” (CCFS) (Pangarkar et al., 2009). These materials show several advantages in many applications such as biochromatography, membrane separation, effective reaction column, and potentially as support matrices for enzyme immobilization. Enzyme immobilization employing monolith as the support matrix has been shown to possess a number of advantages over PBR and STR (Huang et al., 2008; Dizge et al., 2009a). Monolithic reactor provides several predominating features, including lower bed pressure drop, better mass transfer performance, higher specific surface area, lower axial dispersion, and back mixing as well as lower investment cost and reliability to scale-up (Santos and Costa, 2009).

Moreover, monolithic reactor allows higher substrate flow rate compared to conventional PBR thus resulting in increased productivity. The monolith concept was first mentioned in the early 1950s but did not gain popularity due to the low mechanical stability limitation at that time (Svec, 2010). The orthodox monolith was a structure made of metal or ceramic with a large number of straight, identical, and parallel channels. Monoliths fabricated from polymers termed ‘monolithic polymers’ were first introduced by Hjertén et al. (1989). Later, Svec and Fréchet (1992) published their first paper on ‘continuous porous rods’ revealing the potential of monolithic technology in the field of bimolecular separation. Since then, a vast number of studies have been conducted to test the applicability of monolithic polymers in biocatalytic separation (Gusev et al., 1999; Tanaka et al., 2002; Cabrera, 2004; Guillarme et al., 2010; Lv et

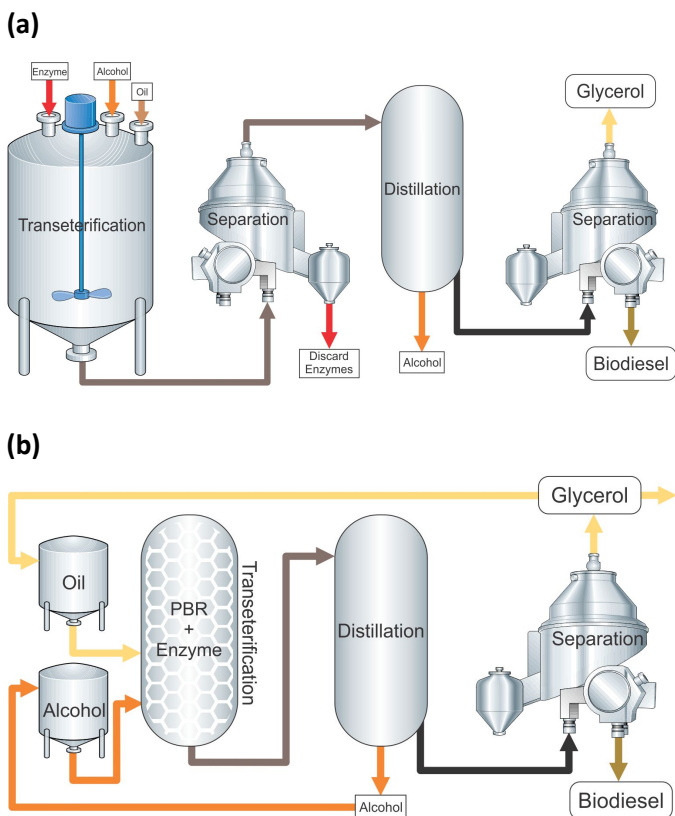


Fig.3. Biodiesel production route via (a) Stirred tank reactor (STR) and (b) Packed bed reactor (PBR).

al., 2014). Countless methods for the preparation of monolithic polymers have been reported in the literature. In 2010, a summary of different polymerization techniques developed to fabricate monolithic polymer was published (Svec, 2010). Regardless of the methods used, the fundamental concept of monolith preparation is a reaction that takes place within a mold containing monomers, porogenic solvents, and a radical initiator, and initiated by heat. The selection of monomer/s used in the polymerization reaction is important as it reflects the final functionality on the monolith surface.

4.1. Hydrodynamics of monolith

The morphology of a monolithic polymer is basically a single interconnected porous structure. The geometry of the monolith channels is usually rectangular; however, more complex geometries also exist (Williams, 2001). **Figure 4** shows the differences in internal structure of a packed column and a monolithic column. Alternatively, the monolith functional group, shape, and size of pore can be readily altered by modifying the type and ratio of monomer/s and porogenic solvent according to the user's preferences hence the versatility of the monolith. Understanding the hydrodynamics of a monolithic reactor is crucial in achieving high lipid to biodiesel conversion rate (Krishna and Sie, 1994). The physical properties and superficial velocity of liquid as well as the type of structured packing used are the main hurdles that affect the hydrodynamic parameters such as liquid-holdup volume, flow rate, and pressure drop (Roy et al., 2004). Besides the high conversion rate, other factors such as low pressure drop, high substrate flow rate, lower axial dispersion, and back mixing, higher specific surface area, and high mass transfer rate are also desirable in designing a monolithic biodiesel reactor. The main difference between a conventional reactor and a monolithic reactor is the internal structure of the monolith. Monolith is a piece of porous material with a significant amount of interconnected pores thus allowing the mobile phase to flow through the column by convection at a relatively very low pressure.

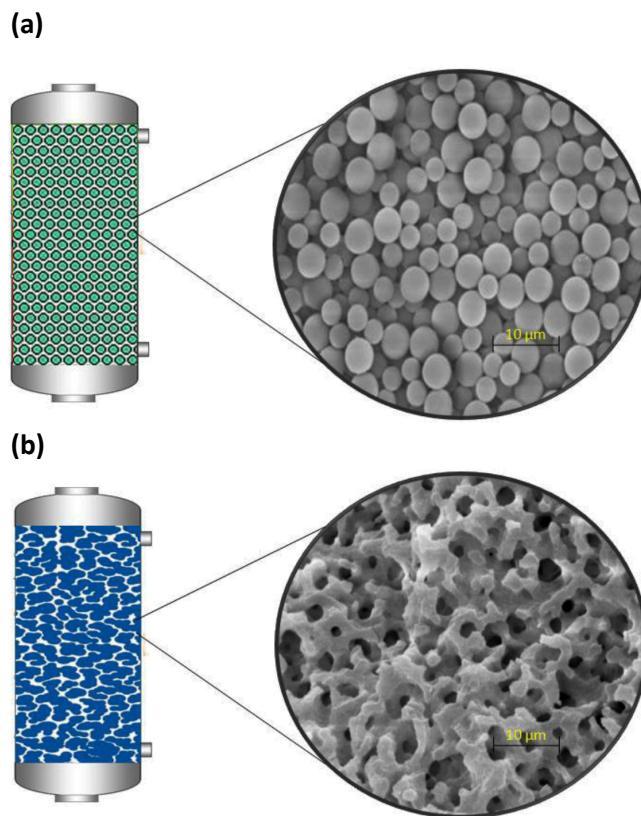


Fig.4. The difference in internal structure of (a) packed column (b) monolith column.

4.2. Organic monolith

In 1992, Svec and Fréchet pioneered the *in situ* preparation of monolithic polymer within the confine of a standard stainless steel column. Discs cut from this monolithic polymer showed high permeability allowing fast separations of proteins (Tennikova et al., 1990). Since then, a multitude of studies have been carried out, and have made significant impacts on the separation technology. The diverse and relatively simple methodologies involved in organic monolith preparation have led to rapid advancement of the technology. Organic monoliths are commonly composed of methacrylate, acrylamide, and styrene monomers. To date, various methods for the preparation of monoliths can be found extensively in the literature such as free radical processes (thermally/photoinitiated/radiation), high internal phase emulsions, cryogels, living polymerizations (nitroxide mediated/organotellerium initiators/atom transfer/ring-opening metathesis), polycondensation, and preparation of monoliths from soluble polymers (Svec, 2010). Thermal and photoinitiated radical polymerizations are the most commonly used methods and widely reported in the literature purportedly due to their simple processes.

In the last two decades, separation of large molecules such as protein in reversed-phase (Liu et al., 2009) and in ion-exchange (Chen et al., 2009) chromatography has been driven towards the utilization of organic polymer monoliths focusing on biocompatibility, morphology, and surface area enhancements. Recent approaches including the utilization of epoxy functional groups (Meador et al., 2005), incorporation of nanoparticles of varied functionalities (Liu et al., 2011), and the use of supramolecular templates (Wan et al., 2008) have been tested to enhance the performance of organic polymer monoliths. Organic polymer monoliths offer higher permeability, better biocompatibility, higher pH tolerance, and faster mass transfer rate in comparison with random packed polymer beads. However, some limitations such as poor heat dissipation, uneven pore size distribution, "wall channel" effect, and relatively low mechanical strength

particularly during scale-up have also been reported (Ongkudon et al., 2014). The use of organic polymers as support matrices specifically for lipase immobilization has been demonstrated, however, its real application in biodiesel production is yet to be discovered. Dizge et al. (2009a) published their work on the immobilization of *Thermomyces lanuginosus* lipase onto a microporous polymeric matrix (MPPM) in different forms (powder, bead, and monolith) through the aldehyde functional group for the synthesis of biodiesel. This was an improvement made over their previous research findings (Dizge and Keskinler, 2008) where they developed a novel method utilizing polyglutaraldehyde and polyglutaraldehyde-activated poly(styrene-divinylbenzene) to immobilize lipase within hydrophilic polyurethane foams (Dizge et al., 2008). The MPPM was synthesized via poly-high internal phase emulsion (polyHIPE) technique using styrene, divinylbenzene, and polyglutaraldehyde. The highest immobilization efficiencies up to 89% were achieved by immobilizing *T. lanuginosus* lipase onto MPPM in monolithic forms. The optimal biodiesel yields were obtained from sunflower oil at 97% conversion, and the immobilized lipase retained its activity after 10 repeated batch reactions at 25 °C, each lasting 24 h.

Urban et al. (2012) demonstrated the application of enzyme-immobilized monolithic supports in both organic solvents and their biphasic mixtures with water (Urban et al., 2012). The poly (alkylmethacrylate-co-ethylene dimethacrylate) monolith was prepared based on their previous work (Eeltink et al., 2007). The monolith was functionalized with azlactone in a two-step grafting technique (Stachowiak et al., 2007) prior to the attachment of porcine lyophilized lipase. The optimized monolithic reactor was then used for the transesterification of soybean oil in pure organic solvent. The mixture of methanol and 2-methyl-2-propanol was used as acyl acceptor. The results obtained under the optimum operating conditions (37 °C with 10 min of substrate residence time) were superior compared to those obtained using mobile lipases, and the enzyme activity was retained even after pumping through a solution of substrate equaling 1,000 reactor volumes.

4.3. Inorganic monolith: silica

Successful preparation of silica-based monolith including the synthesis of monolith with desired porous properties was reported by Nakanishi and Soga (1991) and (1992). Later on, a modification was made where they successfully eliminated the need for post treatment of monolith by substituting ammonium hydroxide with urea as the source of ammonia (Nakanishi et al., 2000). Fields (1996) was the first person to prepare a silica-based capillary column via *in situ* solidification of formamide solution of potassium silicate. Sol-gel approach is favorable in preparing silica-based monolithic columns in which the hydrolytic polycondensation of alkoxysilane forms the pores inside the silica rod. Sol-gel process allows users to fabricate silica monoliths according to their desired properties by regulating the rate of hydrolysis and condensation via the selection of suitable precursors, temperature, pH, reactant concentration, incubation time, and type of catalyst. Tetramethoxysilane (TMOS), tetraethoxysilane (TEOS), and polyethoxydisiloxane (PEDS) are the most commonly used precursors. TMOS has been found to be superior to TEOS and PEDS in terms of pore distribution and surface area (Wagh et al., 1999). The problem that commonly occurs in sol-gel process is the insolubility of alkoxide precursors in water forming two separate phases when constituted together. This bottleneck can be overcome by using alcohol (Šefčík and McCormick, 1997) or PEG (Nakanishi et al., 2000) as the solubilizing agents. Although this process allows the silica-based monoliths to have excellent mechanical stability, their preparation is quite difficult.

Other methods have also been introduced including embedding particles inside the pores (Dulay et al., 1998), encapsulating silica particles within a polymeric backbone (Bakry et al., 2006), and using mixed alkoxysilanes within the confine of a capillary (Motokawa et al., 2006). Subsequent developments in phase separation techniques in addition to using hydrolysis-controlling additives have made it possible to synthesize commercial silica-based monoliths. Like organic polymer monoliths, silica-based monoliths offer numerous advantages such as high mass transfer rate, excellent permeability, and low back pressure drop (Cabrera, 2004; Kato et al., 2005). A common property shared by all silica-based supports is that they can only operate in a certain range of pH (2-8) for a given time and degrade rapidly in certain mobile phases, such as in phosphate buffers (Shi et al., 2004). However, silica-based monolith is suitable for the separation of biomolecules

of varied molecular sizes as it possesses a defined macro/meso-pores network apart from the ability to comprehend extreme temperatures of over 750 °C (Bacskey et al., 2014).

Recently, silica-based monoliths have been adopted as support matrices for enzyme immobilization in biodiesel production (Yu et al., 2013). The utilization of silica-based monoliths in biodiesel production can be found widely in the literature. Anuar et al. (2013) immobilized *Candida antarctica* lipase on a silica monolith with 15 cm in length and 320 µm in internal diameter using glutaraldehyde as a cross-linking agent. The optimized microreactors were used for the transesterification of lipid with atmospheric pressure photoionization (APPI) thus becoming the first demonstration of an online microreaction. The results showed that the reactor was reusable without a noticeable loss of activity up to 15 runs when operated at a room temperature and flow rates of <1 µL/min. Kawakami et al. (2011) demonstrated the use of n-butyl-substituted hydrophobic silica monolith as support matrices for the immobilization of *Burkholderia cepacia* lipase to convert crude jatropha oil into biodiesel. A biodiesel yield of 90% was obtained in a 12-h batch reaction while in continuous production; a biodiesel yield of 95% was attained at a low flow rate of 0.6 mL/h (2.79 g-lipase h/mL). Approximately 80% of the initial yield was retained even after 50 d of continuous operation. A number of papers published on silica-based lipase immobilization for the production of biodiesel can be found elsewhere (Kawakami et al., 2007, 2009, 2012; Shakeri and Kawakami, 2008).

5. Supercritical carbon dioxide-based enzymatic bioreactor for biodiesel production

The development of a sustainable production technology for biodiesel is significantly influenced by the cost of raw materials, pre-treatment processes, and catalysts used which can reach up to 60% of the overall cost (Baskar and Aiswarya, 2016). Numerous alternatives have been explored to overcome the high cost-related factors in biodiesel production including synergistically fusing SC-CO₂ technology and enzymatic bioreactor (Taher et al., 2011b and 2014).

5.1. Inorganic enzymatic reactor with SC-CO₂

Among the available catalytic transesterification processes for biodiesel production, enzyme-catalyzed process is more attractive due to the simplicity, being non-toxic, and low energy consumption. However, the slow reaction kinetics has hindered the application of this technology at a commercial scale. Moreover, the use of organic solvents in the reaction raises concerns due to its volatility, toxicity, and flammability issues (Wen et al., 2009). The integration of enzymatic catalytic processes with SC-CO₂ technology offers, in addition to the non-flammable and non-toxic features, a cheaper alternative to the conventional method due to the abundant natural carbon dioxide gases. SC-CO₂ diffuses easily into solid matrices and drastically enhances the diffusivity of the dissolved mixture. The solvation properties of SC-CO₂ can easily be manipulated by varying the temperature and pressure of the reaction. These features allow simple separation of product and unreacted reagents by decreasing the temperature and pressure to create two distinct liquid phases at atmospheric conditions (Reverchon and De Marco, 2006), hence allowing CO₂ to be recycled back into subsequent reactions. Moreover, SC-CO₂ integration with enzymatic reactor has been proven to address the issues related to high temperature and high pressure requirements in non-catalytic supercritical techniques and the slow reaction rate associated with enzymatic production route while retaining the advantages of both techniques. The mass transfer rate is extensively increased due the high solvation properties of SC-CO₂ allowing homogenous mixture of oil/immobilized enzyme/methanol, increased interaction with the active sites of an enzyme, hence the increase in reaction rate. Figure 5 shows the visual observation of the mixture under different pressures.

5.2. Biodiesel production in supercritical enzymatic reactor

Rodrigues et al. (2011) reported the continuous biodiesel production using virgin and waste sunflower cooking oil as feedstocks catalyzed by Lipozyme TL-IM in SC-CO₂ condition. A FAME yield of > 98 % was

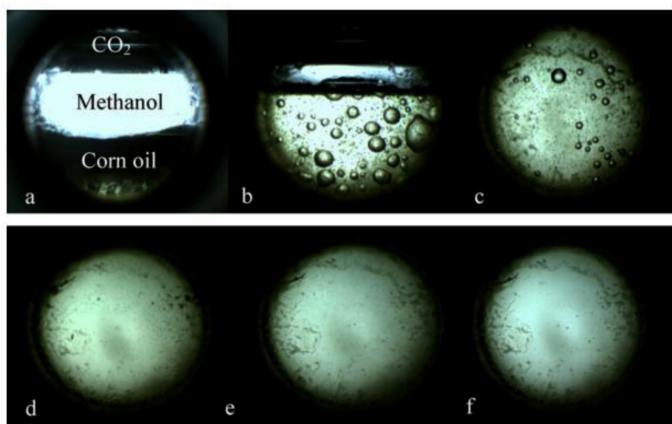


Fig.5. Visual observation of corn oil, methanol and CO₂ mixture at different pressures at 55°C. (a) 0.102 MPa, (b) 3M Pa, (c) 7 MPa, (d) 11 MPa, (e) 20 MPa, (f) 35 MPa. (Adopted from Ciftci and Temelli (2011)).

obtained at 20 MPa, 40 °C temperature, 20s residence time, and oil to methanol molar ratio of 1:24. The efficiency of Lipozyme TL-IM, however, decreased when waste sunflower cooking oil was used. Ciftci and Temelli (2011) optimized the biodiesel production from corn oil in a SC-CO₂ bioreactor using immobilized lipase (Novozym 435) by varying the operating conditions using response surface methodology (RSM) yielding $93.3 \pm 1.1\%$ FAME at 19.4 Mpa, 62.9 °C, 7.03 substrate mole ratio, and 0.72 L/min CO₂ flow rate. In their recent study using a batch reactor, the highest FAME content of 81.3% was obtained at 15% enzyme loading, 60 °C temperature, 1:6 substrate mole ratio, 10 Mpa, and 4 h reaction time. Lozano et al. (2011) demonstrated that continuous biodiesel production from triolein with immobilized *C. antarctica* lipase B (Novozym 435) coated with an ionic liquid (IL) in SC-CO₂ system was better than batch process with the highest biodiesel yield of 98.6% obtained after 6h. They also reported that coating immobilized lipases with IL improved its operational stability. A yield of up to 85% was observed even after 12 cycles of 4h reaction at 60 °C and 180 bar. Lozano et al. (2007) reported the use of immobilized *C. antarctica* lipase B (CALB) on monolith-supported IL phases (M-SILPs) for the continuous synthesis of citronellyl propionate in SC-CO₂ condition by transesterification. The catalytic activity of the mini-flow-bioreactors remained practically unchanged for 7 operational cycles of 5 h each in different supercritical conditions. The best results were obtained when the most hydrophobic monolith, M-SILP-8-CALB, was used at 80 °C and 10 MPa, reaching a total of 35.8×10^4 mol product/mol enzyme. The results significantly exceeded those obtained using PBRs with supported silica-CALB-Si-4 catalyst under the same experimental conditions.

These results prove that monolithic reactors, as discussed in Section 4, offer more advantages over the conventional supports. Therefore, it is of great interest to further optimize monolithic supports for lipase immobilization in a SC-CO₂ system. Generally, to ensure successful conversion of lipid into biodiesel using this system, the required conditions include temperature (40-60 °C), pressure (10-20 Mpa), methanol to oil ratio (more than 1:6 ratio, although excess methanol is problematic), and flow rate of CO₂ (<0.1 L/min). In addition to the aforementioned conditions, selection of enzyme also plays an important role as some enzymes may not be compatible with this process. It has been suggested that the interaction between CO₂ and the enzyme (free amine groups) at high pressure and temperature will lead to a gradual change in enantioselectivity of the enzyme (Matsuda et al., 2004). The primary advantage of utilizing enzymatic SC-CO₂ reactors over conventional techniques (chemical solvent, organic solvent, and free-solvent systems) is improved reaction rate; less than 8 h (SC-CO₂ system) and more than 24 h (conventional system) for a complete reaction. Studies have also been conducted on the effect of reaction conditions towards lipase activity. Lanza et al. (2004) reported that the increase in temperature and density of CO₂ resulted in loss of enzyme activity while the CO₂ decompression rate had no significant effect. It has been suggested that the magnitudes of pressure,

temperature, decompression rate of CO₂, and exposure time needed to inactivate the enzyme strongly depend on the nature and source of enzyme, as well as whether the enzyme is in its native or immobilized form. Figures 6 and 7 show the schematic diagrams of batch and continuous processes in SC-CO₂, respectively.

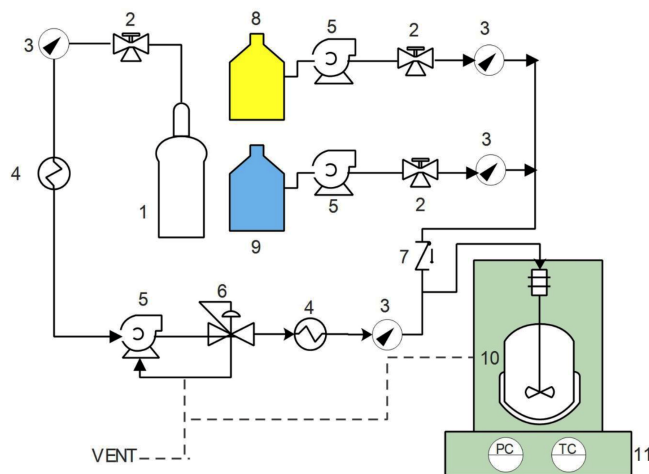


Fig.6. Schematic diagram of a batch SC-CO₂ bioreactor. (1) CO₂ cylinder; (2) control valve; (3) pressure gauge; (4) heat exchanger; (5) high pressured pump; (6) back pressure regulator; (7) check valve; (8) oil tank; (9) methanol tank; (10) stainless steel vessel equipped with stirrer; (11) oven equipped with temperature and pressure controller and indicator.

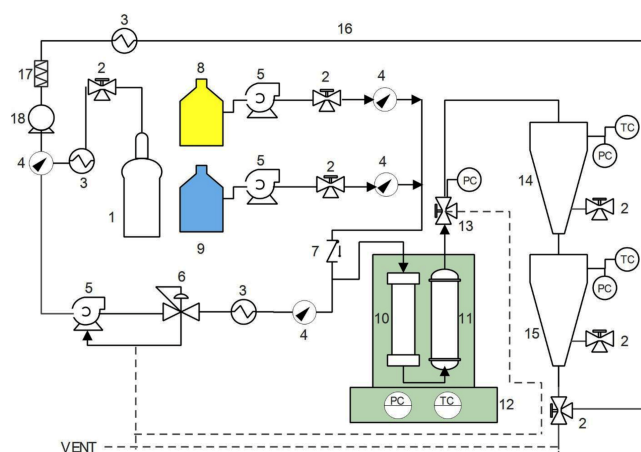


Fig.7. Continuous high pressure pilot scale biodiesel production system. (1) CO₂ cylinder; (2) control valve; (3) heat exchanger; (4) pressure gauge; (5) high pressured pump; (6) back pressure regulator; (7) check valve; (8) oil tank; (9) methanol tank; (10) static mixer; (11) monolithic reactor; (12) oven equipped with temperature and pressure controller and indicator; (13) control valve with pressure indicator and controller; (14) static mixer; (15) separator (cyclone); (16) carbon dioxide recycle loop; (17) filter; (18) compressor.

5.3. Oil extraction in supercritical enzymatic reactor

Although enzymatic SC-CO₂ reaction has been demonstrated successfully, the uneconomical price of equipments has hindered the application of this technology at a commercial scale. A technology combining the process of biodiesel production and the extraction of oil from the same reaction system has been proposed. Chen et al. (2010b) investigated the extraction of triglyceride from powdered *Jatropha curcas* kernels by comparing SC-CO₂ technique with conventional soxhlet extraction method. The oil recovery was lower when using SC-CO₂ approach, however, the purity of triglycerides was better than that of

Soxhlet extraction method. In a different study, [Chen et al. \(2010b\)](#) revealed an optimum oil yield of 43.51% extracted from *J. curcas* L. seeds using SC-CO₂ technology at 350 bar, 333 °K, and a solvent to solid ratio value of 125:1. The concentration and extraction efficiency of triglycerides reached 657.1 mg/g and 97.62%, respectively. A comparison between conventional extraction (static solvent extraction) and SC-CO₂ extraction has been made by investigating the effect of biomass pretreatment prior to microalgae extraction ([Taher et al., 2014](#)), and the results showed that SC-CO₂ extraction was the most efficient method. Similar findings have also been reported by other researchers ([Santana et al., 2012](#); [Aresta et al., 2005](#); [Soh and Zimmerman, 2011](#)). Such an advancement in the SC-CO₂ technology has led to many new strategies combining oil extraction and enzymatic reaction to produce biodiesel in a single SC-CO₂ bioreactor system. This technique effectively allows SC-CO₂ to be recycled back into the extraction unit without having to change to a different system, and substantially reduces the overall operating cost. Currently, only a limited number of reports on the integrated extraction/enzymatic SC-CO₂ system are available ([Al-Zuhair et al., 2012](#)), highlighting the need for further optimization and improvement of the technology. [Figure 8](#) shows an example of integrated extraction/enzymatic SC-CO₂ system.

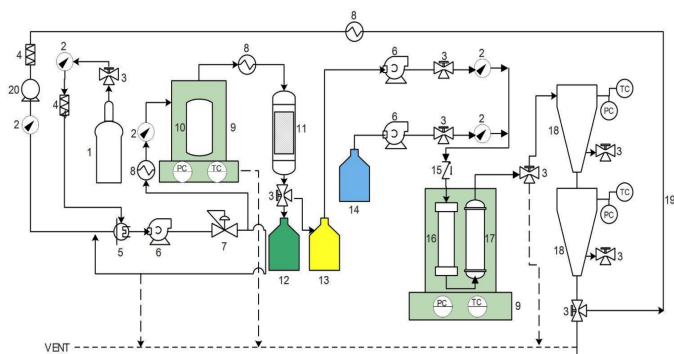


Fig.8. Schematic diagram of the integrated oil extraction–enzymatic reactor in SC-CO₂. (1) CO₂ cylinder; (2) pressure gauge; (3) control valve; (4) filter; (5) cooler; (6) high pressure pump; (7) back pressure regulator; (8) heat exchanger; (9) oven equipped with temperature and pressure controller and indicator; (10) extractor; (11) separator; (12) biomass collector; (13) oil and CO₂ collector; (14) methanol tank; (15) check valve; (16) static mixer; (17) monolithic reactor; (18) separator (cyclone); (19) carbon dioxide recycle loop; (20) compressor.

6. Future prospects

Integrated biodiesel production system, which incorporates an enzymatic reactor and SC-CO₂, has rapidly gaining pace in recent years. The combination of an oil extraction unit with the enzymatic reactor is a promising platform to reduce energy consumption and operating cost in biodiesel production. This exciting yet challenging technology endeavor however seeks further affirmation before it can finally be realized at a commercial scale.

Monolithic reactor has shown a great potential for enzymatic-based reaction in SC-CO₂ system due to its durability in high temperature and pressure applications. The pore size can easily be tailored for large and viscous biomolecules such as triglycerides thus eliminating the issues of pressure build-up especially during a high-throughput process. The pressure drop in monoliths is significantly lower than conventional supports at a given flow rate due to its largely interconnected pore and convective mass transport properties. These features allow monoliths to be applied as an industrial-scale biodiesel reactor where time and volume are two major factors contributing to its economic feasibility.

Co-immobilization of multiple enzymes on a monolith surface is another promising approach that may facilitate an enhanced reaction in a single reactor. Transesterification of lipids involves several steps where acyl migration is the rate-determining factor. [Lee et al. \(2011\)](#) have shown that the combination of *Rhizopus oryzae* lipase (ROL) and *C. rugosa* lipase (CRL) in

a SC-CO₂ process could eliminate the acyl-migration step. Although co-immobilization of enzymes on monoliths shows promising results, the feasibility of the technique at a commercial scale remains questionable.

Surface modification such as the addition of chemicals to generate functional groups may have detrimental effects on the native structure of the enzyme. As the enzyme conformation may change upon immobilization, strategies on how to improve immobilization efficiency still remain as a research endeavor. [Hanefeld et al. \(2009\)](#) demonstrated an oriented immobilization of *T. lanuginosus* lipase through the removal of lysine residues near the active site thus promoting greater substrate-enzyme interfacial reactions as depicted in [Figure 9](#). [Lozano et al. \(2004\)](#) revealed an optimum microenvironment for the synthesis of short chain alkyl esters in SC-CO₂ system by coating *C. antarctica* lipase B with ILs and obtained an increased free energy of deactivation to 25kJ/mol and improved half-time by 2000-folds.

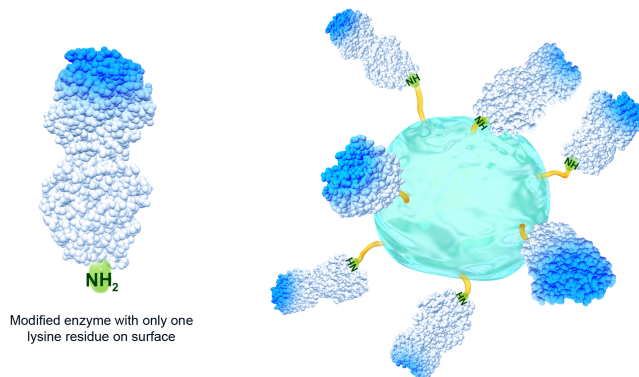


Fig.9. Modified enzyme with just one amino group on its surface, ideally oriented when covalently immobilized (adopted from [Hanefeld et al., 2009](#)).

The inhibition effect of alcohol in a reaction medium can be minimized using a solvent-free transesterification system or a stepwise addition of alcohol ([Shimada et al., 1999, 2002](#); [Fernando and Vasudevan, 2006](#); [Kawakami et al., 2011](#)). The stepwise addition of alcohol has achieved a complete lipid-to-biodiesel conversion within 2 h as compared to 3 h by bulk addition of alcohol with an increased reusability of up to 20 cycles ([Lee et al., 2011](#)).

As a conclusion, the search for alternative feedstocks for green biodiesel production is still ongoing. Microalgal biomass has been proposed as the next-generation feedstocks for biodiesel mainly due to its high lipid content and high yield per acre compared to other plants with zero impact on food security. Apparently, the incorporation of monolithic enzymatic reactors, particularly in a microalgal-based biodiesel production platform, shows a highly promising economic potential. An integrated system that combines the extraction of oil from feedstock and the direct bioconversion of lipid into biodiesel using an enzymatic reactor in SC-CO₂ has been discussed. Although still in the infancy stage, the aforementioned technology has been demonstrated successfully, at least, in bench-top studies.

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