2010

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Recommended Citation

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Abstract

A biosensor is an analytical device that uses biomaterials as elements of the sensing system and converts a biological response into an electrical signal. Biodiesel is a bio-based alternative, biodegradable, renewable, nontoxic diesel fuel made from a chemical reaction between alcohol (usually methanol or ethanol) and plant oil or animal fat. A need to provide accurate, real-time information for the quality sensing of biodiesel properties such as free and total glycerol has led to an ever-increasing demand for biosensor development. Being able to monitor specific physical and chemical properties is the prerequisite for developing a biosensor for quality sensing of the biodiesel. This article proposes a method for detection of the blend level of degraded biodiesel and lipase as a bioelement of biosensor systems. A design of an electrochemical potentiometric biosensor for quality sensing of biodiesel properties is proposed and discussed in detail. However, experimental trials, actual implementation and evaluations are necessary to understand the feasibility of the proposed biodiesel biosensor.

Keywords: Biosensor, biodiesel, lipase, enzymatic hydrolysis, immobilization, glycerol, quality sensing.

Introduction

A biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element that is in direct spatial contact with a transduction element (IUPAC 1996). Thus, a biosensor is a combination of two elements: the bioelement and the transducer or sensor element (Fig. 1).

Many biologically important biospecies such as enzymes, proteins and antibodies can be used as biological elements of recognition (bioelements) for
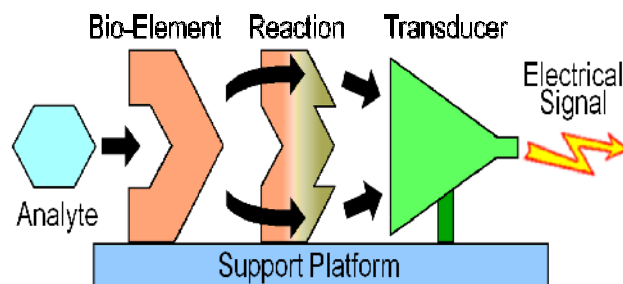

Figure 1. Model of a biosensor. The very specific bioelement (for example, a biocatalyst or enzyme) identifies a specific analyte, and the transducer or sensor element transduces the change in the biological molecules into a readable electrical signal.

biosensors (Zen et al. 1997, Lin and Shih 1999, Chang and Shih 2000, Chou et al. 2008). Enzymes are large, complex macromolecules, consisting largely of protein and usually contain a prosthetic group (one or more metal atoms). Enzymes hydrolyzing triglycerides have been studied for well over 300 years, and the ability of the lipases to catalyze the hydrolysis and synthesis of esters was recognized nearly 70 years ago (Hasan et al. 2006).

Lipase (EC 3.1.1.3) is a member of the broad classification of hydrolases, which transfer functional groups to water. Lipases are characterized by the ability to hydrolyze the long chain triglycerides or triacylglycerol (TAG) at an oil-water interface, resulting in the formation of fatty acids (Van Gerpen 2007, Hasan et al. 2006). The presence of lipases was observed as early as 1901 (Eijkman 1901) for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* (now named *Serratia marcescens*), which represent today's best-studied lipase-producing bacteria, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Lipases isolated from different sources have a wide range of properties with respect to positional specificity, fatty-acid specificity, thermostability and optimum pH (Huang 1984).

Biofuel is one of the most rapidly growing renewable energy sources. Bioenergy is stored during photosynthesis mainly in seed. Plants store oil as a dense form of energy that helps seed germination and early stage plants roots establishment. When the same oil is burned in a diesel engine, the oil releases its stored energy to power the vehicle. Biodiesel consists of the monoalkyl esters of long-chain fatty-acids produced by transesterification of triglycerides with primary alcohols in the presence of a catalyst (Fig. 2) (Knothe et al. 2005). The biodiesel production process can be summarized as a chemical reaction between primarily methanol or ethanol and an oil or fat. Biodiesel can be both cost and energy efficient to produce and can replace petroleum fuel in buses, trucks, tractors and cars with diesel engines (Tat and Van Gerpen 2003). Vegetable oils and animal fats are the only sources of triglycerides used in making biodiesel (Zawadzki et al. 2007). Completeness of the transesterification reaction is an important quality of biodiesel.
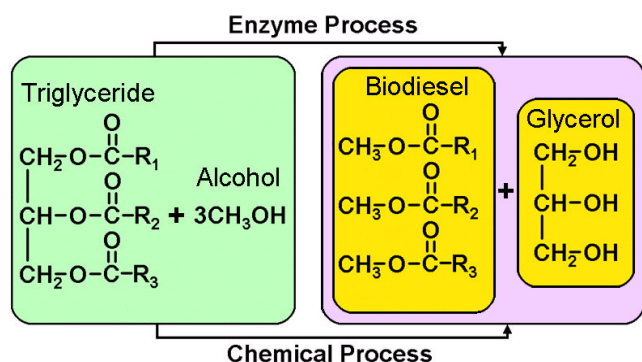

Figure 2. The transesterification reaction (Knothe et al. 2005). R_{1-3} represents various fatty acid chains. The alcohol used for producing biodiesel is usually methanol.

During biodiesel production triglycerides are converted to diglycerides, which in turn are converted to monoglycerides, and then to glycerol. Each step produces a molecule of a methyl ester of a fatty acid. If the reaction is incomplete, then there will be some triglycerides, diglycerides and monoglycerides left in the reaction mixture. ASTM D 6751-09 limits total glycerol to 0.24% and free glycerol 0.02% in biodiesel to be used in vehicle. Free and total glycerol is one of the most important indicators of the biodiesel quality (Van Gerpen 2007).

Generally, the quality of biodiesel depends on several factors. One of them is impurities. Biodiesel impurities include methanol, glycerides, unconverted or partly converted fat, bound glycerol, free glycerol

and catalyst (Van Gerpen 2007). Biodiesel can degrade in storage in two ways: oxidation and microbial contamination. Oxidation increases acidity and forms a gummy substance that then causes a concern for stability of the biodiesel. Microbial contamination can degrade the biodiesel since biodegradability is a property of biodiesel, and this requires water in storage tank, which causes housekeeping issues (McCormick 2006). Methanol can degrade some plastics and elastomers and is corrosive to metals. It can lower the flashpoint to unsafe levels, posing a fire hazard. Unconverted or partly converted fat is a form of bound glycerol, and may result in poor cold-flow properties, fuel-filter plugging, injector and in-cylinder deposits and potential engine failure. Free glycerol results in injector deposits, clogged fuel filters and can leave deposits at bottom of a fuel-storage tank. Therefore, in the biodiesel production process, glycerol comes as a co-product and thus, some free glycerol can be included in small amounts in the biodiesel (Oostdijk 2007). Several methods are known to determine it (Oostdijk 2007). The standard test method used for the quantitative determination of free and total glycerol contents in pure fatty-acid methyl esters (FAME) is American Society for Testing of Materials (ASTM) D 6584, ASTM D6584, 2000.

Literature review of biodiesel sensors

Armstrong (2007) described a gas-chromatographic method equipped with a flame ionization detector (FID). It is the technology recommended by both ASTM and European Union (EU) for the analysis of free and total glycerol. To determine free and total glycerol, the sample was first derivatized with a silylating agent and then injected into an open tubular GC column packed with a 5% phenylpolydimethylsiloxane. Calibration was achieved with two internal standards (butanetriol and tricaprin) and four reference materials. Mono-, di- and triglycerides were determined by comparison with mono-olein, di-olein and tri-olein, respectively. Hajek et al. (2006) introduced a new method that was based on the extraction of free glycerol into water and its subsequent determination in water solution by high-performance liquid chromatography (HPLC) with refractometric detection. They compared the GC and HPLC methods of free glycerol determination in biodiesel and obtained the same results, concluding that the new method was reliable and comparatively fast for free-glycerol determination in biodiesel.

Oostdijk (2007) demonstrated the suitability of an

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on-column injector and the Select Biodiesel for Glycerides UltiMetal column used with Varian's CP-3800 GC for the analysis of biodiesel. The calibration curves and repeatability data demonstrated excellent system integrity that makes the system ideally suited for the analysis of free, bound and total glycerol and mono-, di- and triglyceride content in biodiesel in accordance with the method ASTM D 6584. All samples analyzed were within standard specifications as stated in ASTM D 6751 with respect to the maximum level of free glycerol and total glycerol, except the in-house prepared biodiesel of low quality, which was as expected. However, because of the noted low levels of both glycerol and triglycerides, one sample (3S) was spiked with glycerol and triglycerides so an assessment could be made about the column's separation performance for those components. The new Select Biodiesel for Glycerides column was able to achieve a good resolution of the biodiesel sample and was a robust solution for the high-temperature application.

Alhadeff et al. (2007) designed differential integrated systems with a bi-enzymatic biosensor; that works with two different methods of ethanol detection: flow injection analysis (FIA) and sequential injection analysis (SIA). The developed sensor was successfully applied to the detection of ethanol extracted from gasohol mixtures, and for samples of alcoholic beverages and fermentation medium. In a recent study, Zawadzki et al. (2007) proposed and evaluated a method for biodiesel blend-level detection using ultraviolet absorption spectra. The method was based on the absorbance of diluted samples with n-heptane in the UV absorption range and was found suitable for any biodiesel feedstock and independent of the diesel fuel.

The ASTM specification requires that the total glycerol be less than 0.24% of the final biodiesel product measured using a gas-chromatographic method as described in ASTM D 6584. Since the glycerol portion of the original oil is usually about 10.5%, this level of total glycerol, corresponds to 97.7% reaction completion (Van Gerpen 2007).

Compliance of ASTM D6584 requires a trained personnel and laboratory equipment. Proposed lipase-biosensor could be a means to meet ASTM standards in the quality sensing of the biodiesel. This paper proposes using lipase-biosensor that can be used to measure free and total glycerol quickly and easily. The proposed biosensor is capable of detecting and determining the quantity of glycerides (neutral fat) in the final biodiesel product and can be used as a mean of biodiesel-quality sensing.

Proposed design of a potentiometric electrochemical lipase biosensor for biodiesel-quality sensing

Potentiometric biosensors make use of ion-selective electrodes in order to transduce the biological reaction into an electrical signal. The simplest biosensor (Fig. 3) consists of an immobilized enzyme membrane surrounding the probe from a pH meter, where the catalyzed reaction generates or absorbs hydrogen ions. The reaction occurring next to the thin sensing glass membrane causes a change in pH that may be read directly from the pH meter's display. Typical of the use of such electrodes is that the electrical potential is determined at very high impedance, allowing effectively zero current flow and causing no interference with the reaction (Chaplin 2004).
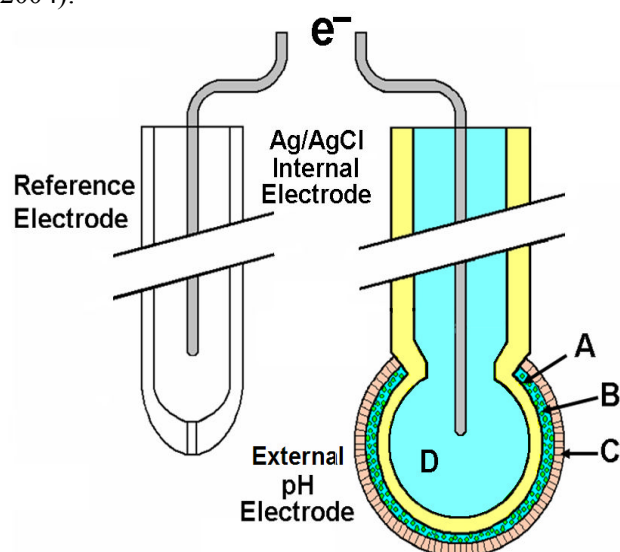

Figure 3. A generalized potentiometric biosensor. An active glass membrane (A) is surrounded by a biocatalyst (B), which is contained in place by a semi-permeable membrane (C). The electric potential (e⁻) is generated between the internal electrode, which is suspended in dilute HCl (D), and the external pH electrode.

The following equation describes the theory behind the proposed potentiometric biosensor principle. The fundamental of the proposed biosensor operation are simple. The response of an ion-selective electrode is given by Chaplin (2004);

$$E = E_0 + (RT / z F) \ln [i]$$

where E is the measured potential (in volts), E_0 is a characteristic constant for the ion-selective/external electrode system, R is the gas constant, T is the absolute temperature (°K), z is the signed ionic charge; either positive or negative , F is the Faraday constant and [i] is the concentration of the free uncomplexed ionic species. Strictly, [i] should be the activity of the ion but at the concentrations normally encountered in biosensors, this is effectively equal to the concentration.

Following is the lipase catalyzed reactions (Chaplin 2004) involving the release or absorption of H^+ ions that may be utilized by the proposed potentiometric lipase-biosensor.

<div align="center">Lipase</div>

Glycerides in biodiesel + H_2O → Glycerol + Fatty-acids + H^+
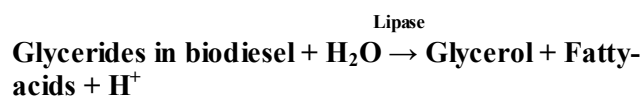
The proposed lipase-biosensor (Fig. 4) is a combination of two ideas: first, is a lipase electrode where lipase will act as a biological reporter (of any biological substance that can attach itself to a particular analyte) and second, an electrode to quantify the glycerides present as impurities in the final biodiesel products. The procedure for the construction of a lipase electrode and immobilization of lipase onto the glass electrode by means of a gelatin membrane for the glass-electrode-based lipase biosensor was described by Huang et al. (2001).
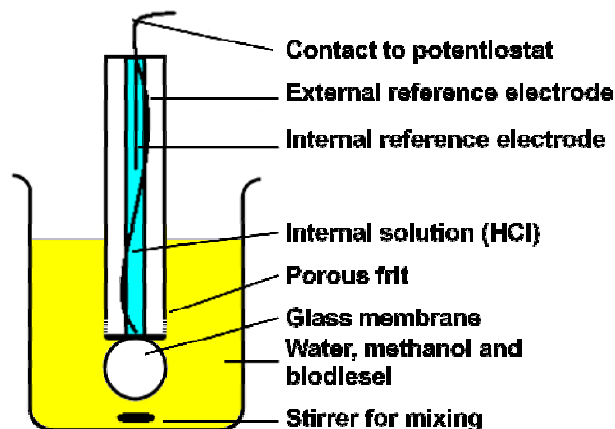

Figure 4. Diagram of the lipase biosensor for biodiesel-quality sensing.

The immobilization of lipase on the biosensor electrode is the key to the development of the biosensor. Immobilization of enzymes is necessary to ensure maximal contact and response, reusability of the enzyme electrode, guarantee of the enzyme stability, and less susceptibility to interference (Chaplin 2004). An important feature for the proposed biosensor is the employment of membrane technology in order to eliminate interference by other electro-active substances. This will be accomplished in the proposed biosensor with an immobilized enzyme layer in a gelatin membrane (Biosensors web book 2008). Once fabricated, the membranes have shelf lives exceeding six months (Huang et al. 2001) and are in operation two to three weeks depending upon the uses. The detector uses glass pH electrodes for H^+ cations (e.g. normal pH electrodes) in which the sensing element is a very thin hydrated glass membrane that generates a transverse electrical potential due to the concentration-dependent competition between the cations for specific binding sites. The selectivity of this membrane is determined via the composition of the glass (Huang et al. 2001).

The phenomenon of lipase-catalyzed hydrolysis reactions of glycerides causes a change in the pH of the solution (Reddy et al. 2001) and can be used to detect changes in pH during the hydrolysis as a shift in the capacitance-voltage (C–V) characteristics. The principle of the biosensor is based on the measurement of pH variation, which can be measured in millivolts, due to the enzymatic hydrolysis of the long-chain triglycerides (TAG) at the biodiesel-water interface, resulting in the formation of fatty acids. The procedure for the potential measurement of the lipase electrode was reported (Huang et al. 2001) as follows: potential measurements can be made at 37 ± 0.1°C on a pHS-3C pH / mV meter using the lipase electrode as an indicator (connected to the negative pole), a calomel electrode as reference (connected to the positive pole) and a Tris-HCl buffer (pH = 8.5) as medium (Huang et al. 2001). After the attainment of a steady-state potential response to the blank, biodiesel emulsion needs to be added quickly. Ten minutes later, the potential is recorded. Between measurements, the lipase electrode is rinsed with distilled water and kept in the buffer (Huang et al. 2001). The relationship between pH change and substrate biodiesel concentration is complex, including other such non-linear effects as pH-activity variation and protein buffering. However, there is a linear relationship between the apparent change in pH and the substrate concentration (Chaplin 2004, Kartal et al. 2007).

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Concluding remarks

Though there are many non-health application areas for biosensors, there are still gaps between research and commercial market of biosensors. These are barriers for wide non-health applications. If the non-health areas improve and flourish, the markets and real-life biosensors application areas will increase a thousand fold. Therefore, the study of non-health application areas such as biosensor applications in biological sciences and engineering is significant. Based on a review of the literature, we have detailed a potential design for a potentiometric electrochemical biosensor system for quality sensing of potential impurities of biodiesel. The benefits of the proposed biodiesel biosensor should be as follows: it is reagentless, and can detect the biodiesel impurities such as glycerol quickly with high accuracy, sensitivity and specificity. To develop a successful biodiesel biosensor, collective effort in both multidisciplinary areas and technologies is necessary. Over time, better-designed tools, improved and standardized biosensor technologies will allow numerous biodiesel biosensor products to be economically feasible.

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