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Immobilizing Lipase Catalyst for Biofuel Production – A Potential Path to a Greener Tomorrow

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Abstract

Clean renewable energy is one of the burning problems of this century.

Fossil fuels, global warming, carbon emissions threaten the survival and expansion of the human race as a whole. In this critical juncture, it is imperative that we shift to renewable, cleaner fuels and reduce our dependence on the conventional fossil fuels we've been accustomed to use.

Lipase is an enzyme which catalyzes the hydrolysis of lipids resulting in the formation of saturated as well as unsaturated fatty acid chains and glycerol. The saturated/unsaturated fatty acid chains being highly reduced releases a huge amount of energy on complete oxidation. This not only acts as a clean viable substitute to the fossil fuels we use but also reduces the pollution caused by the disposal of used waste vegetable oils which aren't fit for human consumption.

Lipase, being an enzyme can be reused and this technique is much more efficient and less polluting than the conventional base/acid catalyzed transesterification conventionally employed in biodiesel production nowadays. Immobilizing lipase offers some significant advantages over traditional means and is being adopted by more and more industries.

In this project we have attempted to produce and optimize enzymatic production of biodiesel using lipase obtained from lipid metabolizing microbial consortia. We have also tried to execute and optimize lipase immobilization to a substrate.Immobilizing lipase on a biopolymer substrate offers some significant advantages over traditional means and is being adopted more and more in industrial practices.

Keywords: lipase, biodiesel, transesterification, renewable energy

INTRODUCTION

The growing energy needs, depleting fuel sources and environmental pollution brought upon by conventional fossil fuels compel us to look for other renewable sources of energy. Liquid fuels are still the most energy dense source of fuel we know of and hence biodiesel is viewed as a viable alternative to conventional fossil fuels such as petroleum or diesel. Over the last several years, biodiesel has emerged as a renewable fuel which can serve as a viable alternative to the fossil fuels we use and has gained a lot of attention.

Some of the advantagesof biodiesel are as follows:

- Biodiesel is produced from agricultural crops that assimilate carbon dioxide from the atmosphere to grow and in turn produce vegetable oil. Thus, the carbon is being recycled.
- Biodiesel is the only alternative fuel that can run on a traditional unmodified or slightly modified diesel engine. Thus, if we are to shift to carbon neutral fuels, biodiesel will be the logical choice.
- Biodiesel can be used alone or be mixed in different ratios with traditional fossil fuels. The most popular blend is a 1:4 ratio of biodiesel:petroleum diesel.
- Biodiesel is non-toxic, biodegradable, has a safe flash point of around 150 degrees Celsius which makes it less prone to accidental ignitions/explosions.

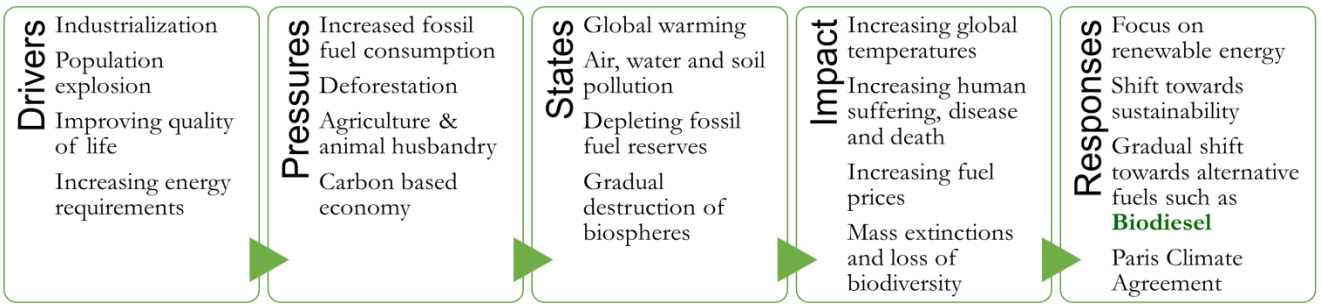
Chemically speaking, oils fall under a category of macromolecules called lipids. Lipids comprise a group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others.

Oils we consume and fossil fuels we use as our primary chemical source are fundamentally very similar. Salient features include:

They are used almost universally as stored forms of energy in living organisms in the form of fatty acids. Fatty acids are carboxylic acids with hydrocarbon chains ranging from 4 to 36 carbons long (C4 to C36).

- They are hydrocarbon derivatives with a low oxidation state i.e. highly reduced energy rich molecule.
- They may be saturated or unsaturated. The degree of saturation and unsaturation in turn dictates their physical properties in room temperature.
- They are insoluble in polar solvents, but soluble in organic solvents.
- As a renewable, biodegradable, and non-toxic fuel, biodiesel can be derived from vegetable oils, animal fats, or microbial oils through transesterification or esterification.Both reactions are with an alcohol (methanol or ethanol) in the presence of a catalyst which could a base (NaOH or KOH), an acid (HCl or H2SO4) or an enzyme (lipase).

The DPSIR analysis below illustrates how human activity and progress has necessitated the shift to cleaner and greener fuels to meet our energy requirements.



The advantages of using Biodiesel over Petroleum Diesel are due to the following differences:

Biodiesel	***Petroleum Diesel***
Biodegradable, nontoxic.	Non-degradable, polluting.
Lubricity benefits, maintainsengine health.	No engine maintaining functions.
Releases carbon dioxidesequestered from the atmosphere during combustion.	Releases new carbon dioxide payloads into the atmosphereduring combustion.
Relatively cheap and not subject to inflation.	Increasingly costly and pricesubject to external factors.
Renewable energy resource,practically limitless.	Non-renewable energy resource, depleting rapidly.

Enzymatic Transesterification

The first report on the application of lipase to produce methyl esters (biodiesel) dates back to 1986.

As compared to other catalyst types, biocatalysts have several advantages over traditional means.

Some of them are:

- Milder, more energy efficient reaction conditions.
- No side reactions. Hence the glycerol produced as the by-product doesn't need to be subjected to additional steps of purification to remove the soaps and hence no alkaline wastewater is produced.
- Both the transesterification of triglycerides and the esterification of free fatty acids occur in one process step. As a consequence, highly acidic fatty materials, such as palm oil or waste oils, can be used without any form of pre-treatment.
- Phase separation is easier. Hence the glycerol can be sold in the market as a pure product.
- Lipases show considerable activity in catalyzing transesterification with long or branched chain alcohols, which is not achievable in the presence of conventional alkaline catalysts.

Enzymatic Transesterification: Lipase

Lipases, or triacylglycerol acyl ester hydrolases (EC3.1.1.3), are enzymes possessing an intrinsic capacity to catalyze cleavage of carboxyl ester bonds in tri-, di-, and monoacylglycerols (the major constituents of animal, plant, and microbial fats and oils). Lipases are one of the most important enzymes found in nature. They have critical roles in lipid metabolism, transport and processing in almost all living organisms. Carboxylic acids and alcohols with a lower number of ester bonds (and eventually glycerol) are released as a result of lipase metabolism. More than 50 lipases have been identified, purified and characterized to date, which originate in natural sources as plants, animals, and (native or genetically engineered) micro-organisms. Other features of lipase which spark further interest include:

- Non-toxic, environment friendly
- Stereo-selective, substrate specific, allows manufacture of high value industrial products
- Catalytic efficiency, high rates of substrate conversion
- Mild pH and temperature requirements

The advantages of using lipase as biocatalyst are:

- Milder reaction conditions, faster, more energy efficient.
- No yield reducing side reactions and toxic waste effluent streams.

- Wider substrate range.
- Product separation easier.
- By-products can be sold as a pure product.

The majority of enzymes are fairly unstable and industrial application is often hampered by a lack of long-term operational stability and the technically challenging recovery process and reuse of the enzyme. Also, a protein's sequence and interactions between residues in the protein core are naturally not fully optimized and only achieve the minimum requirements for proper functioning. This situation leaves plenty of room for improvement.

Moreover, one of the main hurdles in the widespread application of lipases in industrial processes is their high price and lack of reusability if they are used in homogenous enzyme preparations. Downstream processing also becomes an important consideration in homogeneous enzyme preparations since the presence of protein in the final product may not be desired.

In view of the above, it is important to increase the reusability of the enzyme in the bioprocess.

In order to make enzyme utilization in biotechnological processes more favorable, different methods for cost reduction have been put into practice and, immobilization is one of them. The term 'immobilized enzymes' refers to 'enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously.

Immobilization of lipase (or any enzyme in general) on a solid substrate has the potential to be more cost effective and efficient.

Some of the advantages it offers are:

- The enzyme can be recovered at the end of the reaction and hence can be reused.
- Multienzyme catalyzed complex reactions are a possibility.
- Much more economic.
- Downstream processing is much less time and resource intensive.
- Efficiency of the enzymes has been observed to increase in several cases.
- Increase in enzyme stability.
- Better process control.
- Lesser downtime.
- Suitable with both batch-fed, fed-batch and continuous flow reactors.

There are four principal techniques for immobilization of enzymes namely. They are:

1) Covalent attachment: Enzymes are covalently bound to particular groups in the substrate or carrier.
2) Adsorption: Enzyme is adsorbed on the external surface to the support.
3) Entrapment: Enzymes are physically trapped inside a porous matrix.
4) Cross-linking: Enzymes are covalently bound to each other forming an aggregate.

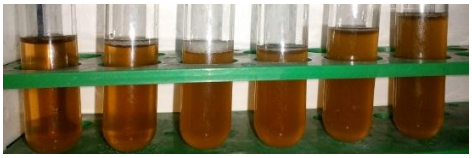
MATERIALS & METHODS

Environmental Sample Collection

Environmental samples from three distinct sources having very different microbial populations and pollutants were obtained. The samples were obtained from highly contaminated drain water and subjected to tenfold dilution.

Microbial Culturing

The diluted samples were cultured overnight at room temperature in modified nutrient media optimised to favour the growth of lipid metabolizing microbes.



Cell Lysate Extraction

The overnight cultures were centrifuged and the supernatant was discarded. The pellet was resuspended in phosphate buffer in a clean eppendorf tube. The pellet was then subjected to multiple heat treatment cycles (40ºC, 30 seconds) followed by centrifugation at 12000rpm for 15 minutes.

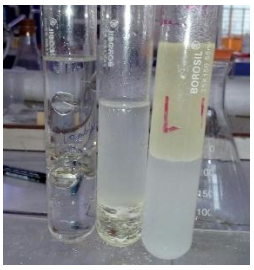
Following the heat treatment cycles, the proteins are separated from the mixture using the alcohol-acetone treatment method.

Protein Quantification

The extracted protein and the crude cell lysate were both subjected to protein quantification using Lowry's method. Both demonstrated the presence of protein. The cell lysate (**left**) had nearly 3x more protein content than the alcohol-acetone method extracted protein (**right**).

Ethanol:Water Ratio

Different ethanol:water ratios were used to detect the optimum solvent polarity. A water:ethanol ratio of nearly 1.2:1 demonstrated the highest deposition of glycerol at the bottom of the test tubes. Quantitative measurements were however, not carried out.



(**L** to **R**) Soybean oil in a) a 1:1 solution of ethanolic lipase and aq. lipase b) a 100% ethanolic lipase solution c) a 100% aq. lipase solution mixed with a 100% aq. lipase solution.

Addition to Used Soybean Oil

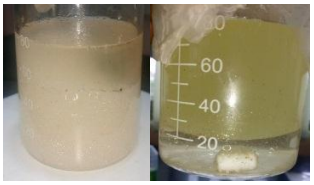
Regular used cooking oils were used as the substrate to produce biodiesel. The reason for choosing cooking oils are as

follows:

1) Cooking oils are rendered unusable after a few cycles of use and are consequently discarded. Reutilization of cooking oil is harmful for human health as it contains harmful polyaromatic compounds. Sustained exposure to these polyaromatic compounds can cause cancer as well as a variety of other medical conditions. Using the same oil to cook different types of proteins may also cause an overall degradation of flavour. Recycling these cooking oils to produce fuel would help reduce wastage significantly.

2) Cooking oils are broken up into fatty acid chains by lipase. These fatty acid chains chemically resemble fossil fuels which can easily be used to produce energy. Among cooking oils, soybean oil was chosen primarily due to ease of availability. The used soybean oil was sourced from several domestic kitchens on volunteering basis. The oil was used an average of 3 times before disposal.

Both the protein extracts were added to a methanolic solution of used soybean cooking oil. The setup was kept at 35ºC and stirred overnight (**left**). A clear layer separation was observed the next day. The upper layer burnt readily with bright yellowish-orange flame when exposed to fire. This indicates the activity of lipase present in the cell protein extracts added to the solution (**right**).



Bile Salts

Varying concentrations of sodium taurocholate (bile salt) was added to the reaction mixture to enhance fat emulsification. However, no significant benefits were visually observed.

(**L** to **R**) Soybean oil and sodium taurocholate in a) a 100% ethanolic lipase solution b) a 100% aq. lipase solution c) a 1:1 solution of ethanolic lipase and aq. Lipase.

Attempts at Immobilization: Agarose Encapsulation

The cell lysate was added to a 1% solution of agarose to entrap the enzyme. The agarose particles were then cut into fine pieces after solidification. The particles were added to the solvent oil-mixture. However, no enzymatic activity was observed indicating significant protein leakage and hence the need for more optimized protocols. Sodium alginate beads were also used to entrap the enzyme. However, no significant improvements were observed.

DISCUSSIONS AND FUTURE DIRECTIONS

Contaminated waste water contains a variety of chemicals including various types of fats and lipids. Thus, a notable fraction of the microbes in the water sample would be positive for lipase expression.

Using the cell lysate from waste water had several advantages. It was not only an easy and cheap source of lipase but it also allowed us to obtain different variants of lipase in the same mixture. This allowed the system to be more robust and tolerant to fluctuating conditions.

Used soybean oil was primarily used due to its ease of availability. Quantification of the glycerol deposition found at the bottom of the test tube would provide better insights regarding the efficiency of lipase action.

The use of bile salts as emulsification agents produced no significant results. However, use of other emulsification agents such as Tween 80 remains to be investigated.

Although the attempts of lipase agarose immobilization produced negative results, spectrophotometric analysis of the buffer used to suspend the agarose particles revealed the presence of proteins. Therefore protein (and hence lipase) leakage may be the predominating issue with the current protocol.

Deeper quantitative studies involving theenzyme and immobilization kinetics would be the next step in this project. Optimizing protocols for better immobilization would also be a priority. Quantitatively studying the enzyme kinetics after successful immobilization of the enzymes on a substrate would also help provide a better understanding of the system as a whole.

Finally, quantitatively measuring the chemical properties of the biodiesel obtained would help validate its use as a fossil fuel alternative.

This method, when further optimized can be used to produce biodiesel in a cheap and sustainable manner. Since environmental samples were used to develop a lipid expressing microbial consortia, a wide range of substrates can be used to produce biodiesel. Moreover, on successful execution of the lipase immobilization, one batch of immobilized lipase can be used multiple times thus increasing efficiency and lowering the price of biodiesel.

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