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Effect Of Different Amount Of Enzyme And Acyl Acceptor On Lipase-Catalyzed Transestrification Reaction For High Yield Of Biodiesel From Microalgal Oil

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Abstract: Enormous use of petroleum fuels is now widely recognized as unsustainable because of depleting supplies and these fuels contributes to the accumulation of CO_2 in environment. Renewable, carbon neutral, transport fuels are necessary for environmental and economical consideration. Biodiesel is monoalkyl ester of long chain fatty acids produced from the trans-esterification reaction of vegetable oil with alcohol in the presence of catalyst & can be used as fuel. Biodiesel is derived from oil crops, waste cooking oil and animal fat or micro algae. Micro algae have emerged as one of the most promising sources for biodiesel production. It can be inferred that micro algae grown in CO_2-enriched air can be converted to oily substance. Such an approach can contribute to solve major problems of air pollution resulting from CO_2 evolution and future crisis due to a shortage of energy sources. Oil productivity of many micro algae greatly exceeds the oil productivity of the best producing oil crops. Approaches are taken for making microbio-diesel economically competitive with petrodiesel. The production of biodiesel from microalgae oil by transesterification chemically and enzymatically. In the present work *Aspergillus niger* lipase were screened for a transesterification reaction of algal oil using different acyl acceptor (methanol, ethanol, propanol-2 and n-butanol) in reaction mixture to produce biodiesel; only methanol was found to give appreciable yield. The application of crude lipase was determined to catalyze the transesterification process, and a conversion of 44% was achieved under selected conditions (reaction temperature 40 °C, algal oil/methanol molar ratio 1:4, 5 ml of lipase based on the oil weight, and reaction time of 24h). It was seen that algal oil and optimization of transesterification conditions resulted in adequate yield of biodiesel in the case of the enzyme-based process.
Keyword: Biodiesel, Transestrification, Microalgae, Alkyl Ester.

Introduction

Majority of the worlds energy needs are supplied through petrochemical sources, natural gases and coal, with the nuclear energy and exception of hydroelectricity, of all, these sources are finite and at current usage rates will be consumed shortly[1]. Conventional diesel fuels have an essential function in the industrial economy of a

developing country and used for transport of industrial and agricultural goods and operation of diesel tractor and pump sets in agricultural area. Economic growth is always accompanied by commensurate increase in the transport. Usually high energy demand in the industrialized world as well as in the domestic sector and pollution problems caused due to the widespread use of fossil fuels make it increasingly necessary to develop the renewable energy sources of limitless duration and smaller environmental impact than the traditional one. These have stimulated recent interest in alternative sources for petroleum-based fuels. Another fuel must be technically economically competitive, feasible, readily, and environmentally acceptable. Solitary possible alternative to fossil fuel is the use of oils of plant origin like vegetable oils and tree borne oil seed. This alternative diesel fuel can be termed as biodiesel. This alternative fuel is biodegradable and non-toxic and has low emission profiles as compared to petroleum diesel. Practice of biodiesel will allow a balance to be sought between economic development, agriculture and the environment.

Biodiesel is a clean-burning fuel currently being produced from grease, animal fats or vegetable oils. Though, recent advance in bio-resource proved that some microorganism species including microalgae, fungi, and yeast[2-4] which have a large amount of fatty acids, be able to also be used as potential sources for biodiesel production. In the direction of distinguish from the biodiesel derived from conventional sources (vegetable oils or animal fats), we use a novel word "**microbio-diesel**" to describe fatty-acid methyl esters (FAMEs) transesterified from microorganism oils. This word might be a suitable extension for traditional concept of biodiesel and its chemical structure is that of fatty acid alkyl esters. Biodiesel be produced by transesterification of oils with short-chain alcohols or by the esterification of fatty acids. A transesterification reaction consists of transforming triglycerides into fatty acid alkyl ester, in the occurrence of an alcohol such as methanol or ethanol and a catalyst such as an alkali or acid, by glycerol as a by-product[5]. Two approaches for transesterification of vegetable oils for production of biodiesel are suggested[6]. The early is a chemical one in which alcoholysis of oil by methyl or ethyl alcohol in the presence of a strong acid or base produces biodiesel and glycerol[7]. The base-catalyzed transesterification is much faster and less corrosive than the acid-catalyzed reaction. Thus alkali hydroxides are the most commonly used catalysts. However, if the feedstock has a high free fatty acid (FFA) content (as is common with rendered fats and spent restaurant oils), excess of alkali causes loss of the free fatty acids as their insoluble soaps. That decreases the final yield of ester and consumes alkali. Seeing that an alternative, in these cases, one can conduct an acid-catalyzed reaction that requires higher reaction temperatures (100 °C) and longer reaction times than alkali-catalyzed transesterification[8].

The second approach is the enzymatic one, inside which lipase-catalyzed transesterification is carried out in nonaqueous environments[9]. Chemical transesterification is efficient in terms of reaction time; though, the chemical approach to synthesize biodiesel from triglyceride has drawback, such as difficulty in the recovery of glycerol and the energy-intensive nature of the method. In contrast, biocatalysts allow synthesis of specific alkyl esters, simple recovery of glycerol and transesterification of glycerides with high free fatty acid content[10].

Many starting materials such as Jatropha oil[11], soybean oil[12,13], sunflower oil[14,15], cotton seed oil[16,17] , rapeseed oil[18], palm oil[19,20] and restaurant kitchen wastes[21] have been evaluated for preparation of biodiesel by the enzymatic route. Microalgae can provide several different types o renewable biofuels. These comprise methane produced by anaerobic digestion of the algal biomass[22] biodiesel resulting from microalgal oil[23-25] and photobiologically produced biohydrogen[26,27]. Chemically and enzymatically both methods are applicable for produce biodiesel from Microalgal oil.

The need for alternative energy sources that combine environmental friendliness with biodegradability, less dependence, renewability, and low toxicity on petroleum products has never been enhanced. One such energy source is referred to as micro-biodiesel which is produced by microalga. Chemically they are known as monoalkyl esters of fatty acids. The straight method for producing biodiesel involves acid and base catalysts to form fatty acid alkyl esters. Downstream giving out costs and environmental problems associated with biodiesel production and byproducts recovery have led to the search for alternative substrates and alternative production methods. Enzymatic reactions involving lipases can be an excellent alternative to produce biodiesel through a process commonly referred to alcoholysis a form of transesterification reaction or through an interesterification (ester interchange) reaction.

Microalgae appear to be the only source of biodiesel that has the potential to completely displace fossil diesel. Distinct other oil crops, microalgae produce extremely rapidly and many are exceedingly rich in oil content. Microalgae commonly double their biomass within 24 h. Biomass doubling-up times during exponential growth are commonly as short as 3.5 h. Oil component in microalgae can exceed 80% by weight of dry biomass. Oil content levels of 20–50% are quite frequent. Photosynthetic growth requires light, water, inorganic salts and

carbon dioxide. Biocatalysts are naturally occurring lipases which have been identified as having the ability to perform the transesterification reactions that are essential to biodiesel production. Advantages of by means of lipase include ease of product recovery, temperature requirements and low energy, ease of enzyme recovery, mild reaction conditions of pH and pressure and reuse of the enzyme several times, give of accepting various substrates and alcohols and reaction in solvent and solvent-free systems lipases allow reactions in systems that contain acceptable levels of water and can esterify free fatty acids therefore making the overall process economically viable.

Material And Methods

Microalgal species were collected from the Upper Lake Bhopal Madhya Pradesh India. Media was prepared with lake water came from upper lake, a lake/sand mix soil and CHU 13 media (Table: 1) mixed them and autoclaved for 20 min at 121º C, 103.35 kPa, and cooled overnight.

Table 1: Composition of CHU 13 Medium (Modified)

S.No.	Composition	Stock Media 20X	Working Media 1X mg/L
1.	KNO_3	8 gm/L	400
2.	K_2HPO_4	1.6 gm/L	80
3.	$MgSO_4$ heptahydrate	4 gm/L	200
4.	$CaCl_2$ dihydrate	2.14 gm/L	107
5.	Ferric Citrate	0.4 gm/L	20
6.	citric acid	2 gm/L	100
7.	$CoCl_2$ dihydrate	2.14 gm/L	107
8.	H_3BO_3	114.4 mg/L	5.72
9.	$MnCl_2$ tetrahydrate	73.4 mg/L	3.67
10.	$ZnSO_4$ heptahydrate	8.8 mg/L	0.44
11.	$CuSO_4$ pentahydrate	3.2 mg/L	0.16
12.	Na_2MoO_4	1.68 mg/L	0.084
13.	0.072 N H_2SO_4		1 drop

Growth Parameter

Temperature	26ºC
pH	7.8
Day/Night period	14:10
Areation	Continuous
Total Algal Culture	27.6 L

Harvest Algal Biomass

Algae can be harvested from 5-gallon carboy culture by the addition of 0.2% ferric chloride algal culture and after the addition of the chemical flocculants algae settled down in the bottom of the 5-gallon carboy. Settled algal biomass removes out from the container and then dry in the oven for 2days at 55ºC. Dry algal biomass used further oil extraction

Oil Extraction

The simplest method is mechanical crushing. Dried algae it retains its oil content, which then can be pressed out with an oil press.

Production of Lipase

Fungal lipase was produced using *Aspegillus niger* MTCC 3390 taken from IMTECH, Chandigarh. Seed culture was grown in 200-mL Erlenmeyer flasks containing 20 mL of mineral medium. The production media were sterilized by autoclaving at 121 °C for 15 min. After cooling, the sterilized medium was inoculated with 4-day-old seed culture.

Table 2: The Composition of Culture Medium for Submerged Fermentation.

Composition	Quantity
MgSO4·7H2O	0.5 g/l
KH2PO4	1 g/l
NaNO3	3 g/l
Peptone	30 g/l
Glucose	2%
Olive Oil	2%
pH	6.0

After cultivation, the culture broth of *A. niger* was centrifuged at 4,000 rpm for 15 min and the supernatant was prepared as a crude lipase solution. Ammonium sulphate was then added to this crude lipase solution (to give 60% saturation) and the resulting suspension was centrifuged at 12,000 rpm for 15 min to obtain the supernatant. The precipitate was then suspended in 20 mM sodium phosphate buffer (pH 7.8) and this solution was concentrated using an ultra filtration membrane and stored at 4°C. Lipase activity was determined by spectroscopy using 1ml olive oil, 5 ml of 50 mM sodium phosphate buffer of pH 7.8 and 1 ml enzyme solution. One unit of lipase activity was defined as the amount of enzyme liberating 1μ mol of fatty acid per min under the experimental conditions (pH=7 and 37 °C).

Lipase-Catalyzed Transesterification

Algal oil (0.25 g) and acyl acceptor (n-butanol, propanol-2, ethanol, and methanol) were taken in the ratio of 1:4 (mol mol-1) in a screw-capped vial. To this mixture, 5 ml of enzyme preparation (crude) was added and incubated at 40 °C with constant shaking at 200 rpm. The progress of the reaction was monitored by removing aliquots (20 μL) at various time intervals (4, 8..., 24 hrs). The aliquots were appropriately diluted (with hexane) and analysis by thin-layer chromatography.

Effect of Different Amount of Enzyme on Transesterification

Algal oil (0.25 g) and acyl acceptor (n-butanol, propanol-2, ethanol, and methanol) were taken in the ratio of 1:4 (mol mol-1) in a screw-capped vial. The crude enzymes were prepared by adding of 20 mM sodium phosphate buffer, pH 7.8 and take this crude lipase solution in varying amount viz., 1, 2, 3, 4, and 5 ml. To the reaction mixture, these enzyme preparations were added and incubated at 40°C with constant shaking at 200 rpm for 24h. The aliquots were appropriately diluted (with hexane) and analysis by thin-layer chromatography.

Effect of Different Amount of Acyl Acceptor on Transesterification

Algal oil (0.25 g) and acyl acceptor (n-butanol, propanol-2, ethanol, and methanol) were taken indifferent ratio such as 1:1, 1:2, 1:3, 1:4, 1:5 (mol mol-1) in a screw capped vial and in this mixture 5ml of prepared crude lipase enzyme preparation was added. The reaction mixture was incubated at 40 °C with constant shaking at 200 rpm for 24 h. The aliquots were appropriately diluted (with hexane) and analysis by gas thin-layer chromatography.

Quantitative Analysis of Esters by Titration (Remaining fatty acid in reaction mixture)

Prepare solvent mixture (95% ethanol/diethyl ether, 1/1, v/v), 0.1 M KOH in ethanol and 1 % phenolphthalein in 95% ethanol. Total reaction mixture in conical flask and dissolve in at least 50ml of the solvent mixture. Titrate, with shaking, with the KOH solution (in a 25 ml burette graduated in 0.1 ml) to the end point of the indicator (5 drops of indicator), the pink color persisting for at least 10s. The acid value is calculated by the formula: 56.1xNxV/M where V is the number of ml of KOH solution used and N his exact normality, M is the mass in g of the sample.

Result And Discussion

Large Scale Production

Total Algal Culture	27.6 L
Total Dry Biomass	132.06 gm
Total Oil Extract	24.3 ml

Lipase-Catalyzed Transesterification

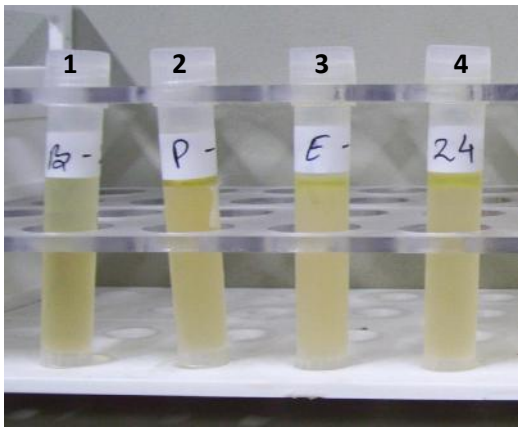

Figure 1: The lipase-catalyzed transesterification reaction. Tube 1, 2, 3 and 4: reaction mixtures of, n-butanol, propanol-2 ethanol and methanol, respectively, after 24h at 40ºC.

Table 3: yield of biodiesel production (%) by different acyl acceptor at different time interval

Time Interval hrs	Yield of Biodiesel Production (%)			
	n-Butanol	Propanol-2	Ethanol	Methanol
4	0	0	0	6
8	0	5	0	14
12	0	8	4	33
16	0	19	10	40
20	0	22	12	43
24	0	22	12	44

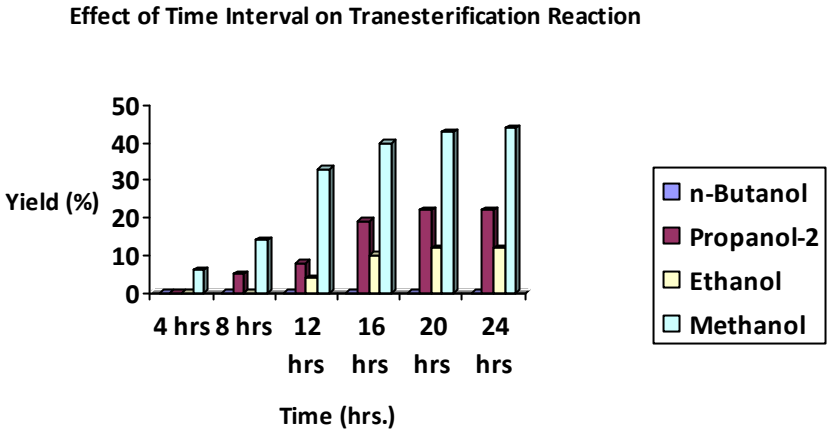

Figure.2 : Graph shows the effect of incubation time on biodiesel production at 40°C using 5ml *A. niger* lipase.

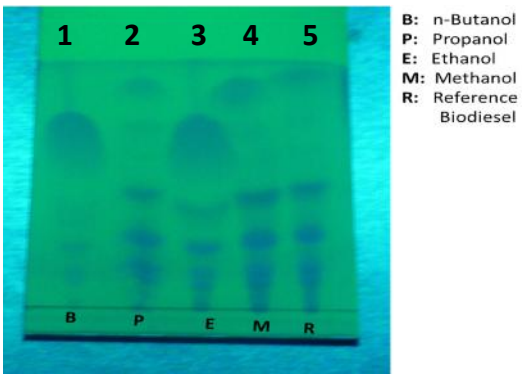

Figure 3: TLC analysis of the different esters. Lanes 1, 2, 3,4 and 5: reaction mixtures of n-butanol, propanol-2, ethanol, methanol and biodiesel respectively.

The lipase from *Aspergillus niger* sources (which were prepared crude in laboratory) were screened for transesterification. Figure 1 shows that lipase from *A. niger* is capable of transesterification. With this enzyme, a yield of 44%, 24%, 22% and0% respectively for the methyl ester, ethyl ester, propanyl ester and butanyl ester could be obtained after 24h (figure 2). The reaction could be qualitatively followed by TLC (figure 3) and quantitatively by titration. The formation of biodiesel did not increase beyond 24 h, which is likely to be due to inactivation of the lipase by substrate methanol. The inactivation of lipase during transesterification reactions has also been observed by others.

Effect of Different Amount of Enzyme on Transesterification

Table 4: Yield of Biodiesel production (%) by different acyl acceptor with different amount of enzyme

Crude Lipase Enzyme (ml)	Yield of Biodiesel Production (%)			
	n-Butanol	Propanol-2	Ethanol	Methanol
1	0	0	0	15
2	0	3	0	16
3	0	7	4	21
4	0	18	9	43
5	0	22	12	44

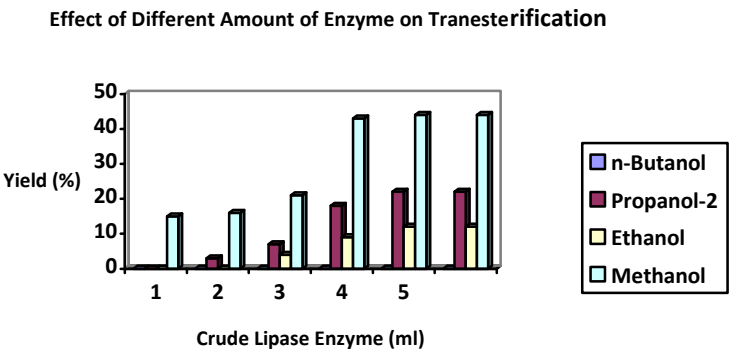

Figure 4: Graph shows effect of amount of enzyme addition on biodiesel production at 40°C using 1:4 ratios of algal oil and acyl acceptor.

The effect of varying amount of *A. niger* lipase enzyme (1, 2, 3, 4, 5 ml) shows in Figure 4, under these optimized conditions. The separately crude enzymes were added to reaction media containing algal oil and different acyl acceptor in the molar ratio of 1:4. The best results were obtained with 4 ml or 5 ml of enzyme. The higher amount of enzyme (i.e., 5 ml) in fact equal the product yield, this was presumably due to increase in the viscosity which reduced the reaction rate to the extent that an additional amount of enzyme did not help[28].

Effect of Different Amount of Acyl Acceptor on Transesterification

Table 5: Yield of Biodiesel production (%) by different acyl acceptor with different molar ratio of algal oil:acyl acceptor

Molar Ratio (Oil:Alcohol)	Yield of Biodiesel Production (%)			
	n-Butanol	Propanol-2	Ethanol	Methanol
1	0	5	0	12
2	0	9	3	18
3	0	15	7	37
4	0	22	12	44
5	0	21	10	44

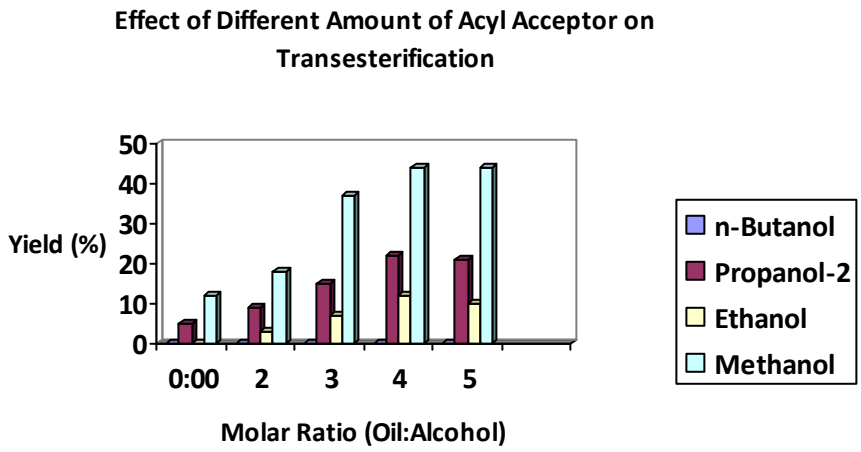

Figure 5: Graph shows the effect of varying amount of acyl acceptor addition on biodiesel production at 40°C using 5ml *A. niger* lipase.

The effect of varying amount of acyl acceptor under these optimized conditions. Varied amounts of acyl acceptor (1, 2, 3, 4, 5 ml) were ratio with algal oil such as 1:1, 1:2, 1:3, 1:4 and 1:5. The separately acyl acceptor were added to reaction media containing 5ml *A. niger* lipase. The best results were obtained with 1:4 ml or 1:5 ml of enzyme (figure 5) [28].

Conclusion

As demonstrated at this point, microalgal biodiesel is technically possible. It is the only renewable biodiesel that can potentially completely displace liquid fuels derived from petroleum. Financial side of producing microalgal biodiesel need to improve substantially to make it competitive with diesel, but the level of improvement necessary appears to be within reach. Producing low-cost microalgal biodiesel requires primarily improvements to algal biology through genetic and metabolic engineering. Employ of the biorefinery concept and advances in photobioreactors engineering will further lower the cost of making. In view of their much greater productivity than raceways and tubular photobioreactors are likely to be used in producing much of the microalgal biomass required for making biodiesel. Photobioreactors give a controlled environment that can be tailored to the specific demands of highly productive microalgae to attain a consistently good annual yield of oil content.

Micro-biodiesel production can be done through the use of Chemical (acid or base) or Enzyme (lipase-catalyzed) reactions. Readily available is a current interest in using immobilized lipases or immobilized whole cells as green alternatives to chemical catalysts to produce biodiesel industrially. Though, the cost of the final enzymatic product remains a hurdle compared to the cheaper alternative of using chemical catalysis. With the tools of recombinant DNA technology and it is possible to increase the supply of suitable lipases for biodiesel production. Site-directed mutagenesis and protein engineering may be used to alter the enzyme–substrate specificity, thermostability and stereospecificity or to increase their catalytic efficiency, which resolve benefit biodiesel production and lower the cost of the overall procedure. Indeed a thermo stable and short-chain alcohol-tolerant lipase suitable for biodiesel production was recently cloned and more research efforts should be focused in modifying and cloning more lipases. Lipases enzyme can be immobilized to increase their operational stability, creation reuse several times possible and the alcoholysis process economically feasible and competitive with conventional processes.

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