Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Spl Issue [3] 2022: 245-252

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Production of Biodiesel & Qualitative Screening on Thin Layer Chromatography

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ABSTRACT
Lyophilized lipase (0.6g) prepared from of Bacillus tequilensis lipase enzyme was used as a catalyst in the first method, wherein soybean oil (16ml) was used as a substrate for biodiesel Production using methanol as the source of alcohol (4ml.) In the second method, immobilized Candida antarctica lipase (0.064g) (Sigma Aldrich) was used as a catalyst for biodiesel Production from soybean oil (10.4ml) and methanol (2.3ml). In this condition, the lipase efficiently trans-esterified soybean oil with methanol to produce biodiesel, giving an overall conversion of 98%. In another method B .tequilensis lipase (1.5ml) was used to hydrolyze the soybean oil (7.5ml) into free fatty acid. The free fatty acid mixture was then subjected to acid catalyzed esterification reaction using methanol (0.2ml) as the alcohol source to produce biodiesel. This was carried out on thin layer chromatography with the aid of Reference Spotted (Methyl ester).
Keywords:-*Biodiesel, methyl ester, soybean oil, thin layer chromatography.*

Received 12.08.2022 Revised 21.09.2022 Accepted 20.10.2022

INTRODUCTION

Biodiesel a mono-alkyl ester is currently an accepted and dependable substitute for diesel. Esterification, trans-esterification, inter-esterification, hydrolysis, and other renowned reactions of lipase are only a few of many other biotransformation potentials of them which are industrially exploited As a biocatalyst and environmentally sociable procedure, lipase is used in many industrial biocatalyst conversions .Making biodiesel with lipase enzyme is a good and well-known environmentally friendly progression because it does not contain chemicals and the adaptation of biodiesel is good, which increases the calorific value and also reduces viscosity, compared to the chemical method, so that biodiesel is a good option for the future. Currently vegetable oils or animal fats and grease are used to create biodiesel, a fuel with a hygienic burn. It has an alkyl fatty acid ester chemical configuration. Using short-chain alcohols to trans-esterify oils or fatty acids to form esterified fatty acids is how biodiesel is Produced. An alcohol, such as methanol or ethanol, a catalyst, such as an alkali or acid, and triglycerides are transformed into fatty acid alkyl ester during the trans-esterification reaction, which also produces glycerol as a derivative [1]. An assortment of oils is used to make biodiesel, such as the oil from cotton seed oil, Karanja oil, jojoba oil, soybean oil, Jatropha Curcas, linseed oil, castor oil, soybean oil, and leftover cooking oil. FAME or fatty acid methyl esters are also referred to as biodiesel and biodiesel has a lot of impending as a substitute to biodiesel fuel. Fundamentally, triglycerides (oil or fat) are trans-esterified using a chemical catalyst or a lipase biocatalyst to make biodiesel.

Due to the exhaustion of fossil fuels and the issues, the world's natural surroundings are facing as a result of the current use of fossil fuels, the development of green energy that is clean and renewable has gained ever-increasing consideration [2,3,4] Enzymatic trans-esterification has some recompense larger than chemical trans-esterification, as well as the capability to recuperate glycerol voluntarily and a lower energy requirement in addition to the ability to trans-esterify glycerides with high free fatty acid stuffing [5,6,7].

Due to the potential, it receives from the increase in petroleum costs in addition to its environmental benefits; biodiesel has become a more accepted option for future use. Biodiesel production is a forthcoming and enormously interesting vital topic of research [8]. One of the incredible uses of lipase is the creation of biodiesel. Mittelbach was the first to report on lipase-catalyzed biodiesel synthesis [9].The ester link is hydrolyzed in step one of the lipase-catalyzed trans-esterification before the second substrate is esterified [10]. A ping-ping bi bi mechanism that is characteristically employed for kinetic studies of

trans-esterification that is mediated by enzymes. Due to their rapid generation times, microbes are normally selected for commercial enzyme synthesis .The high yield of substrate to product conversion, considerable adaptability to environmental conditions, ease of genetic manipulation, and simplicity of growth settings are further remuneration of microorganisms [11]. The most popular bacterium for producing lipase is *Candida rugosa*, which is derived from yeast [12]. *Streptomyces sp*. has recently received attention as a powerful lipase-producing bacterium for the generation of biodiesel and has been proven to be useful [13]. The capability of lipases to discriminate between different acyl chains' structural characteristics is a key component of their substrate specificity, which influences the selection of the best enzyme based on the symphony of the raw materials [14,15].

Candida rugosa, Pseudomonas fluorescens, Pseudomonas cepacia, Candida antarctica, and lipases have extensive substrate specificity and regio-specificity, making them appropriate for trans-esterification reactions [16]. Additionally, the ability of lipases to be reused may be a way to reduce the high cost of the enzymes and make them feasible for use on an industrial scale. Table 1 provides an evaluation of immobilized and free enzymes. Adsorption, covalent bonding, trapping, and cross-linking are the several types of enzyme immobilization techniques. For the purpose of producing an effectual lipase, technique and support material selection are important.

Comparative studies' findings showed that, after immobilization onto various supports, the same lipase molecule might exhibit substantially varied catalytic activity [17]. An important and well-known biocatalyst for the trans-esterification process is biodiesel. Microbial lipases can convert triglycerides (oil) into fatty acid methyl esters under a variety of conditions and with less waste than chemical-based catalytic processes, which lowers processing costs.

Table 1:- The evaluation of immobilized and free enzymes [18].

Characteristics	Free Enzyme	Immobilized Enzyme
Price	High	Low
Efficiency	Low	High
Activity	Unstable	Stable
Reusability and recovery	Not possible	Possible
Tolerance to temperature, pH, etc.	Low	High
To separate from the substrate	Difficult	Easy
To separate from the product	Difficult	Easy

Table 2:- Comparison of the chemical and elemental composition of biodiesel and diesel [19].

	Biodiesel Content (%)	Diesel Content (%)
Carbon	79.6	86.4
Hydrogen	10.5	13.6
Oxygen	8.6	-
Nitrogen	1.3	-
C/H	7.6	6.5
n-Aliphatics	15.2	67.4
Olephenics	84.7	3.4
Aromatics	-	20.1
Naphtens	-	9.1

However, the lipase-catalyzed biodiesel production method is still a developing process that necessitates thorough comprehension of the process at different levels. [20,21].

The following are some benefits of using lipase biodiesel:

1. Lipase enzyme serves as a catalyst that completely converts oil (triglycerides) into biodiesel (Fatty acid methyl ester).
2. Receive a high caloric value (energy)
3. Reusing catalytic lipase that has been immobilized
4. Obtain a high yield from the trans- and esterification processes.

Lipase biodiesel's drawbacks:

1. These processes are more expensive than chemical ones; the biodiesel in this research is generated from soybean oil.
2. Because this type of action is reversible and can change the product into a reactive agent, many parameters must be taken into consideration when producing biodiesel using the lipase method, including temperature, incubation time, and Rpm.

MATERIAL AND METHODS

1. Lipase lyophilized enzyme was prepared from freeze drying of cell free supernatant of fermented broth having 55U/ml of *B. tequilensis* lipase:-In this research, lyophilized lipase was utilized as a bio-catalyst in the trans-esterification reaction because lipase has hydrolysis, esterification, and trans-esterification capabilities [22, 23].Methanol was purchased from hi-media (India) and edible-grade soybean oil was brought from a nearby food store. The standards of mono-lein,diolein and oleic acid were purchased from Sigma-Aldrich. All other chemicals used of AR grade purchased from Hi-media (India).

Three different methods were used for producing biodiesel using the *B. tequilensis* lipase. In the first approach, soybean oil was used as a substrate or source of fatty acid to be transesterified with methanol and lyophilized lipase enzyme was used as the bio-catalyst In the presence of lyophilized lipase enzyme the reaction combination of soybean oil and methanol produces both biodiesel and glycerol (0.6 g of lyophilized lipase enzyme, 4 ml methanol, 16 ml soybean oil, 24 hours of incubation, Temperature 50°C, and 150 rpm [24].

2. Immobilized lipase enzyme (*Candida antarctica)* (as a biocatalyst) with soybean oil:- Candida Antarctica [26,27] was bought from Sigma Aldrich. Methanol was purchased from hi-media (India) and edible-grade soybean oil was brought from a nearby food store. The standards of mono-lein,diolein and oleic acid were purchased from Sigma-Aldrich. All other chemicals used of AR grade purchased from Hi-media (India).

In the second method, immobilized *Candida antarctica* lipase (0.064g) (Sigma Aldrich) was used as a catalyst for biodiesel Production from soybean oil (10.4ml) and methanol (2.3ml). In this condition, the lipase efficiently trans-esterified soybean oil with methanol to produce biodiesel, giving an overall conversion of 98%. Temperature 50^0C, incubation duration 24 hours, and rpm 120 and tert-butyl alcohol (6.5ml) [28].

3. Soybean oil hydrolyze with *B .tequilensis*:-In third method *B .tequilensis* lipase (1.5ml) was used to hydrolyze the soybean oil (7.5ml) into free fatty acid. The free fatty acid mixture was then subjected to acid catalyzed (Sulfuric acid) esterification reaction using methanol (0.2ml) as the alcohol source to produce biodiesel. [30, 31, 32]. Incubation time 48 hrs.150 rpm and heated to 40°C and then centrifuged 10000 rpm for 10 minutes. In the first approach, soybean oil was used as a substrate or source of fatty acid to be hydrolyzed with *B .tequilensis* lipase enzyme was used.

Hydrolysis: - Triglycerides (soybean oil) + $3H_20$ lipase enzyme Glycerol + free fatty acid

4. Lipase Activity Measurement:- The p-NPP (*p*-Nitrophenol palmitate) assay was used to evaluate the lipase activity using a colorimetric technique [33]. When combined with 90 ml of Tris- buffer, 30 mg of p-NPP was dissolved in 10 ml of Iso-propanol (0.05 M, pH 8.5). 0.1 millilitre of culture supernatant was added to 2.4 millilitres of freshly produced p-NPP solution. The reaction was carried out by keeping the mixture in the reaction vessel at 50^0C for 5 minutes by adding 0.1 ml of fused 100 mM $CaCl_2$, the reaction was stopped, and the tubes were kept on ice. To clarify the solution, the reaction mixture was then centrifuged for 10000 rpm for 10 minutes. The yellow color's absorbance was measured at 410 nm.

Enzyme unit: One micromole(s) of *p*-Nitrophenol released per minute by the hydrolysis of p-NPP by one ml of soluble enzyme is considered one unit (IU) of lipase activity.

RESULT AND DISCUSSION

1. Lipase lyophilized Powder with soybean oil (First method):- 10µl samples were taken from this reaction mixture the different intervals and spotted on thin layer chromatography Furthermore [34] and observed the result.

- Sampling point for TLC: - Take 10µl samples at different intervals (1, 3,6,24 hrs) for TLC observations.
- TLC analysis for biodiesel production: - 10 µl of samples spotted on TLC plates and then plates were dried in air and kept in TLC jar.
- Analysis: - solvent system contents:- Petroleum ether: diethyl ether: acetic acid (85:15:1). The plates were developed in an iodine chamber and observed for the spot of biodiesel.
- TLC result: - 10 µl samples spotted on TLC plates and observed for the spot of biodiesel. After 1, 3,6,24 hrs. FAME (fatty acid methyl ester) biodiesel spot did not appear on TLC plates (from Method 1) Methyl stearate was spotted as reference biodiesel.
- Control: - In control enzyme was not added and other conditions were the same as for the test reaction.

2. Immobilized lipase enzyme (*Candida antarctica*) with soybean oil (Second method):-10µl samples were taken from this reaction mixture at different intervals and spotted on thin layer chromatography with the help of capillary and observed the result.

- Sampling point for TLC:- Take 10µl samples at different intervals (1,3,6,24 hrs) for TLC observations (batch process) [35,36,37]
- TLC analysis for biodiesel production: - 10 µl of samples spotted on TLC plates and then plates were dried in air and kept in a TLC jar.
- Analysis:- Solvent system contents:- Petroleum ether: diethyl ether : acetic acid (85:15:1). The plates were developed in an iodine chamber and observed for the spot of biodiesel.
- TLC result: - 10 µl samples spotted on TLC plates and observed for the spot of biodiesel. FAME (fatty acid methyl ester) biodiesel spot appeared on TLC plates. Methyl stearate was spotted as reference biodiesel. After 6 and 24 hrs. FAME spot appeared on the TLC plate from Method 2).
- Control: - In control enzyme was not added and other conditions were the same as for the test reaction.

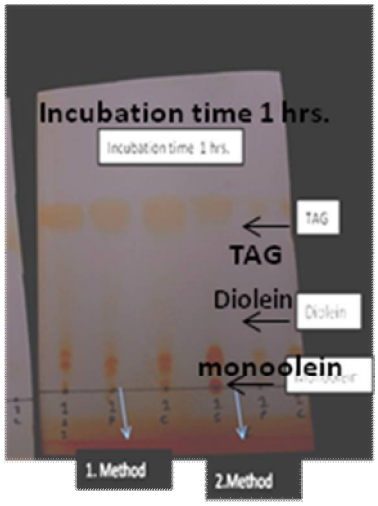

Fig 1:- Determination of biodiesel spot used method (Inubation time 1 hrs.)
S :- Standard immobilized enzyme *Candida antarctica*
P:- lyophilized powder, C:- control

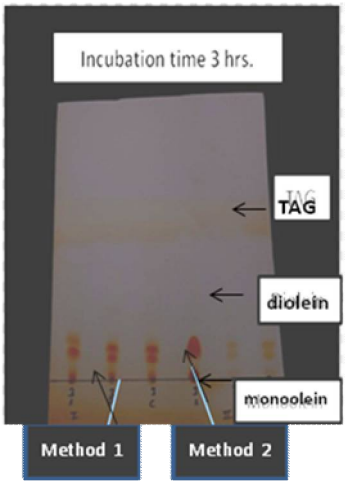

Fig 2:- Determination of biodiesel withTLC spot (Incuabtion time 3hrs.)
S:- Standard immobilized enzyme *Candida antarctica*
P:-lyophilized powder, C:- control

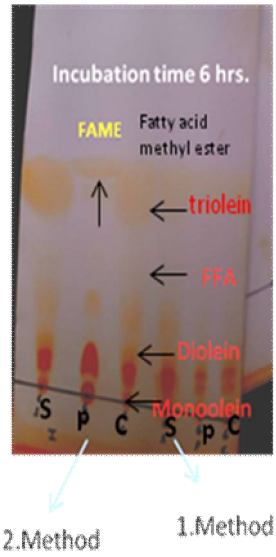

Fig 3:- Determination of biodiesel spot
After 6 hrs FAME spot appeared on TLC plate

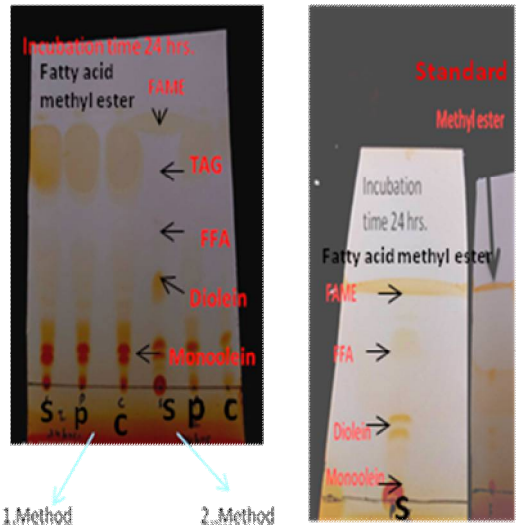

Fig 4:- Determination of biodiesel spot
After 24 hrs.FAME spot appeared on TLC plate
with Standard Methyl ester

S:- Standard immobilized enzyme *Candida antarctica*
P:- lyophilized lipase powder, C:- control

Table 3:- Biodiesel spot determination with different incubation times (batch process) and Methods

Oil/raw material	Methods	Incubation time	Rpm	Temperature	FAME(Biodiesel) Spot
Soyabean oil	From method 1.	1 hrs	150	50^0 C	--------
Soybean oil	From method 2.	1 hrs	150	50^0 C	--------
Soybean oil	From method 1.	3 hrs	150	50^0 C	--------
Soybean oil	From method 2.	3 hrs	150	50^0 C	--------
Soybean oil	From method 1.	6 hrs	150	50^0 C	--------
Soybean oil	From method 2.	6 hrs	150	50^0 C	+++++
Soybean oil	From method 1.	24 hrs	150	50^0 C	----------
Soybean oil	From method 2.	24 hrs	150	50^0 C	++++++

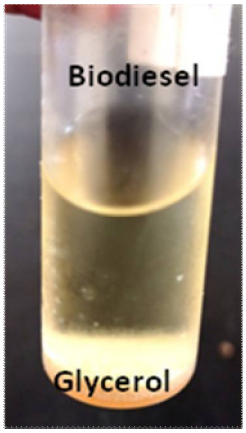

Fig 5:- Biodiesel made from standard immobilized lipase enzyme from (Method 2)

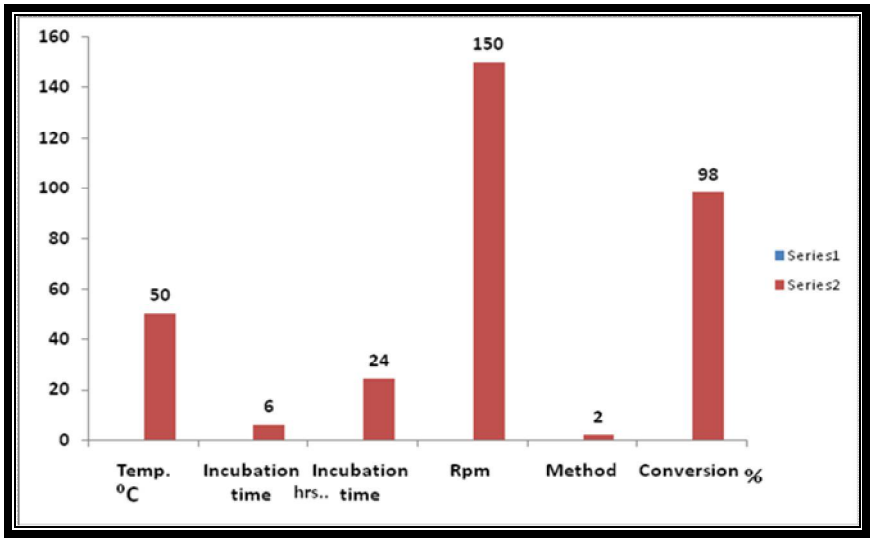

Fig. 6: Determination of various biodiesel production parameters (method 2).

3. Soybean oil hydrolyze with *B .tequilensis* (Third method):- The esterification reaction occurred in the presence of sulfuric acid catalyst from this fatty aid reaction mixture and water was produced. 10 µl samples spotted on TLC plates and observed for the spot of biodiesel. FAME (fatty acid methyl ester) biodiesel spot appeared on TLC plates. Methyl stearate was spotted as reference biodiesel spot.

Oil/raw material	Method	Incubation time	Rpm	Temperature	FAME (Biodiesel) spot
Soybean oil	From Method 3.	48 hrs.	150	40^0C	++++++

Table 4:- Biodiesel spot determination with incubation time &temp. (From Method no.3)

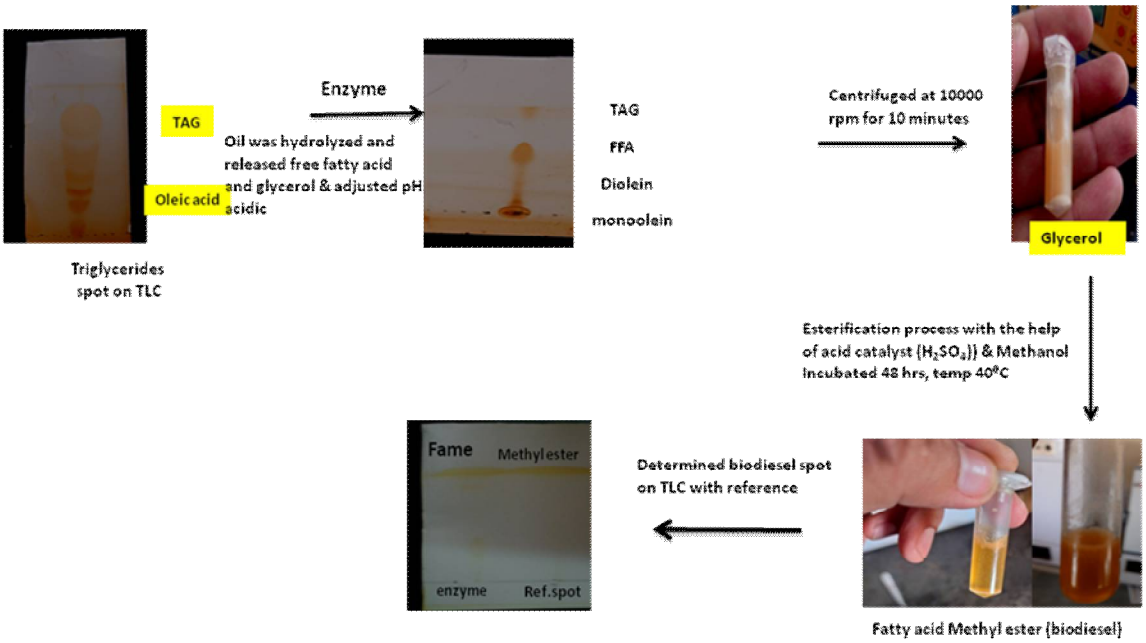

Fig 6:- Determined biodiesel spot used with different steps (esterification process) from (Method 3.)

CONCLUSION

In the future, replacing diesel with biodiesel is a good alternative. In this study, the lipase enzyme serves as a good biocatalyst that demonstrates the actions of lipid hydrolysis, esterification, and trans-esterification. Immobilized lipase enzyme can reduce the cost of biodiesel by taking in different types of spots like monoolein, di-olein, and free fatty acids and completely converting them into FAME. Good biodiesel is only considered good when its calorific value (energy value) is high and its viscosity is helpful (fatty acid methyl ester). Using acid catalyst to speed up the reaction of free fatty acid and methanol to

produce methyl ester and water, which was determined using thin layer chromatography; biodiesel is a good option and is used as a good parameter to the product.

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CITATION OF THIS ARTICLE

Ajaj Ahmed, Gautam Kumar Meghwanshi. Production of Biodiesel & Qualitative Screening on Thin Layer Chromatography. Bull. Env. Pharmacol. Life Sci., Vol Spl Issue [3] 2022: 245-252