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Statistical Optimization of Enzymatic Transesterification Process to Produce Biodiesel from Honge Oil

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Abstract

The increasing environmental and economic concerns regarding the use of fossil fuels have led to the exploration of alternative energy sources that are renewable, eco-friendly, economically viable, and sustainable, to meet the growing energy demand. Biodiesel is a promising alternative fuel that has similar properties to diesel and can replace conventional diesel. Biodiesel is synthesized using oil sources and can be produced through transesterification processes, which can be catalyzed by chemical catalysts like NaOH/KOH or enzymes such as lipase. Enzymatic transesterification is a promising process for biodiesel production over chemical transesterification. In this study, Honge oil was converted to FAME (fatty acid methyl ester) using locally prepared immobilized beads of lipase extracted from an isolated strain of bacteria, *Lysinibacillus macroides* FS1. A statistical design was used to evaluate the impact of effective variables such as the amount of immobilized lipase (%), pH, and agitation speed (rpm) on biodiesel yield. The results showed that these factors had a positive effect on biodiesel production, indicating that the model is highly significant at a 95% confidence level. The optimum factors were found to be an amount of immobilized lipase of 12.5%, pH of 7, agitation speed of 125rpm, molar ratio of 1:3, temperature of 45°C, and reaction time of 24 hours, which yielded 96% yield. The model yields of 96% under these optimum conditions with one-time addition of methanol indicate that the prepared immobilized lipase is methanol tolerant, which has the potential for cost-effective large-scale production of biodiesel.

Keywords: Statistical optimization, Box Behnkem design, transesterification, immobilized lipase & biodiesel

Introduction

Energy requirement plays a vital role in the economic progress of the nation. Increasing demand for energy due to industrialization, urbanization and growing population builds much pressure on available energy resources. Our nation stood at 6th rank for growing energy consumers on a global scale and as the population is increasing, it is estimated that there is a rise in energy demand by 84% by the year 2050 (1). Environmental issues like pollution and global warming related to the burning of fossil fuels, intensive investigations are needed to find an alternative renewable source of energy like solar, thermal, hydropower, wind and bioenergy (2). Biofuels generated using biomass are considered as the best choice (3).

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Among the different alternative sources of energy, Biomass energy is vital due to the greater amount of biomass available. Biofuels are used for transportation and are derived from biomass such as plants, animals and microbial biomass (4, 5). The types of Biofuels are Bioethanol and Biodiesel which are alternatives to conventional petrol and diesel. Various types of such fuels can be produced but only ethanol and biodiesel production has gained commercial value (6).

Biodiesel defined as the fatty acid methyl (or ethyl) esters (FAMEs) produced from plant oils or animal fats, is a liquid fuel having similar composition like fossil/mineral diesel. It is one of the potential biofuels which reduces 85% of emissions of pollutants compared to petrodiesel (7,8). It is renewable, atoxic, perishable and environmentally compatible applied diesel engines with little or no modification due to its adjustable physical and chemical properties (9, 10).

Biodiesel production is carried out by different processes like microemulsions, thermochemical decomposition and transesterification (11). Transesterification is the most common and commercially well-developed method of biodiesel production due to the easy and efficient process to convert triglycerides into esters and glycerol and leads to a high yield of biodiesel compared to other methods. Therefore transesterification has become the more popular and best choice. The conventional method of transesterification is done by using an acid or base catalyst but in the last few years, the use of enzymes as a biocatalyst for biodiesel is inspired due to problems related to the acid or base-catalyzed transesterification process and that has shown significant progress. The enzymatic transesterification process is a more promising method in future days.

Most importantly, in enzymatic transesterification, glycerol can easily be recovered without requiring complicated processes and FFA present in oil can be converted to alkyl esters, thereby eliminating the need for subsequent wastewater treatment. The lipases which are nonstereospecific so that all types of glycerides are converted to alkyl esters and catalyze the esterification of FFA are preferred for the biodiesel industry. Instead of free lipase immobilized lipase are more stable and suitable to use in reaction mixture under harsher environmental conditions. The major type of plant oils used for biodiesel production are Jatropha, Pongamia and Polanga. *Pongamia pinnata* has great advantages for both the agriculture and transportation sector.

Process optimization is one of the important actions to achieve the maximum yield of biodiesel cost-effectively (12). Factors affecting the transesterification reaction involve the concentration of catalyst, type of alcohol, molar ratio, temperature, pH, agitation speed and time (13). Extensive research work is carried out on the use of inedible oils for transesterification, but there is no sufficient work is being performed on the optimization, oil characterization and fuel analysis. Hence there is much scope for optimization of the transesterification of non-edible oil sources. This can be performed either traditional way such as one factor at a time or in advanced methods like using statistical designs.

The statistical route is more advantageous than the conventional mode due to the benefits like the efficiency of the process, less number of experiments, reduced experimental time, consumables, human resources, economical, gives sufficient data for a statistically reasonable result and also it provides the details of the effective factors among various parameters and interactive effect of selected variables with few experiments (14, 15). Another advantage of the design of the experiment (DOE) is used to study multiple variables in the same experiment. There are various methods available among them the PB design is known for foundation studies to isolate effective factors which improve the yield of biofuels (16, 17).

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RSM is a statistical technique for designing experiments. It is widely used to evaluate effective factors, and interactions among chosen variables with fewer experiments, to find optimum conditions and quantify the relationships between one or more determined responses and the key input factors (18). It is the most frequently used for the optimization of the transesterification process. Box-Behnken Design is suitable for interaction study among factors and perfect to 3 factors (19).

Box Behnken Design (BBD) is applicable for optimising a few numbers of variables at a few levels. Thus, BBD is extensively applied in various applications such as microwave-assisted transesterification, optimizing the biodiesel production process and optimization of media components (20). The yield and conversion efficiency of the enzymatic method is affected by a number of factors the nature and properties of the enzyme catalyst, immobilization techniques, pretreatment, use of a wide variety of substrates, acyl acceptors, their step-wise addition and use of solvents, operating conditions like water content, temperature, pH and bioreactor design. The success of enzymatic transesterification is depends on optimizing these factors with respect to raw material and catalyst (21). It is crucial to optimize these parameters to make the enzymatic process applicable for large-scale production.

Materials and Methods

Collection & characterization of honge oil

For the present study *Pongamia pinnata* (Honge) is selected as the source of oil as it is one of non-edible oil and its greater availability in the North Karnataka region as well as its fatty acid composition is more suitable as feedstock for biodiesel production (22, 23). Honge oil is collected from BRIDC BEC-STEP Bagalkote in purified form. The collected honge oil is analyzed for physical properties like color, odour, density, viscosity, acid value and FFA% which are important parameters for feedstock. Further fatty acid profiling of *Pongamia* oil is performed by GCMS analysis (24, 25).

Production of lipase

Lysinibacillus macroides FS1 (MW940857) novel bacteria is used for the present study which was isolated in previous studies. The production of lipase is done in optimized media which is formulated in previous studies (26). Media is prepared using the optimized ingredients mentioned in Table 1. pH is adjusted to 7 using buffer solution and media is incubated at 37℃ at 120rpm for 48 hours. After incubation time lipase is extracted by centrifugation at 10000rpm at 4℃ for 30min, as lipase is produced extracellularly, the supernatant is considered a crude lipase source (27).

Table 1: Optimized media for lipase production

Sl. No.	Ingredients	Quantity (g/L)
1	Beef extract	30
2	Galactose	30
3	Ammonium chloride	30
4	$CaSO_4$	0.1
5	KH_2PO_4	0.5
6	$MgSO_4.7H_2O$	0.1
7	Honge oil	10

Partial purification of lipase by ammonium sulphate precipitation

The produced lipase is partially purified by ammonium sulphate precipitation (0-30%). The purification is done by mixing solid ammonium sulphate in collected supernatant with constant stirring at 4℃. 3gm ammonium sulphate is uniformly dissolved and the flask is kept for incubation at 4℃ for 30min. The mixture was centrifuged and partially pure lipase is collected at 10000rpm at 4℃ and dissolved in phosphate buffer of pH7. Further, it is tested for lipase activity by titrimetric method and quantitative estimation of protein content is carried out to find the specific activity of an enzyme (28).

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Lipase activity assay

The lipase activity assay is performed by Titration. The titrimetric assay of lipase is one of the simple and most commonly used methods. The assay is performed by preparing the reaction mixture in 50 ml conical flask by adding 1ml of the supernatant along with 2ml of phosphate buffer (pH 7) and 1ml of olive oil. The flask containing the reaction mixture is kept for incubation at 37℃ for 60min. After an incubation period of 60min, 1ml of acetone:ethanol solution in (1:1) ratio is added to the reaction mixture to stop the reaction and further titration is carried out using 0.05M NaOH with a few drops of phenolphthalein as an indicator until the reaction mixture pH reached to 10.5. The amount of NaOH consumed is recorded. Lipase activity is measured in a unit of U/ml. The amount of lipase produced is correlated with lipase activity. Lipase activity is calculated by using Eq.1

$$\text{Lipase Activity}\left(\frac{U}{ml}\right) = \frac{\text{Volume of alkali consumed x Strength of alkali x 1000}}{\text{Volume of sample x Time in min}}$$

Eq.1

Immobilization of lipase

Immobilization was performed by entrapment using sodium alginate (4%) and calcium chloride (200 mM). Sodium alginate is prepared in 0.05M Tris HCl buffer (pH 7). The supernatant is mixed with sodium alginate in equal proportion (1:1) by continuous stirring. The enzyme alginate mixture is added dropwise to cold $CaCl_2$ solution (200 mM) using a hypodermic needleless syringe of 5ml. The produced beads are preserved for curing at 4°C for 1 hour (29). After curing, the beads (approx. 3mm diameter) are collected from the solution by filtration through Whatmann filter paper 1. The beads were stored in distilled water at 4°C till further use.

Determination of immobilization efficiency

Immobilization efficiency is calculated using the below Eq.2(30). The activity assay of immobilized lipase is measured by titrimetric method and activity is calculated using the Eq.1. Calculated activity of immobilized lipase is used for calculating the immobilization efficiency.

$$\text{Immobilization efficiency \%} = \frac{\text{Activity of immobilized lipase}}{\text{Activity of free lipase} - \text{Wash water activity}} \times 100$$

Transesterification reaction

Honge biodiesel is produced using locally synthesized immobilized lipase. The reaction mixture is prepared in 100 ml conical flask by adding 25gm of honge oil, 3.4ml of methanol (1:3), 5% immobilized lipase and placed in a shaking incubator at 37℃ with an agitation speed of 120rpm (**Fig. 1**). After 24 hours collected filtrate is added to separating funnel and left for separation (**Fig. 1**). Clean glycerol and biodiesel were separated. Produced biodiesel is used for quantification to calculate the yield using the **Eq.3** [2].

$$\text{Biodiesel yield Y (\%)} = \frac{\text{Weight of the biodiesel produced}}{\text{Weight of crude oil used}} X100$$

Experimental design

Optimization studies are helpful to achieve a higher yield of biodiesel. It is necessary to optimize the process for all types of feedstocks and catalysts as yield varies. Any optimized condition of the transesterification process may not be suitable for all types of feedstocks because each type of feedstock has different composition of fatty acids and properties therefore they need different reaction conditions. Hence in this study biodiesel production from honge oil is optimized to identify the reaction conditions suitable for honge oil by using response surface methodology.

Factors affecting enzymatic transesterification [31,24,32]

Molar ratio: 1:3 to 1:15

Amount of immobilized lipase: 5 to 20% to the wt. of oil

Temperature: 25 to 45℃.

Reaction time: 2 to 24 hours

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pH: pH 6 to 8

Agitation speed: 50 to 200rpm

Water content: 1 to 20%

Response surface methodology (rsm)

RSM is effective for screening more variables at a time with minimizing experimental trials and resources (14,15). The experiment is divided into two phases, phase 1 Plackett-Burman and phase 2 Box Behnken.

Box behnken design

Effective factors are identified by PB design in previous studies (26). Among the seven variables studied, three factors such as the amount of immobilized lipase (%), pH and agitation speed (rpm) have shown significant effects on biodiesel yield hence using these three factors further optimization is performed using Box Behnken design which is specifically used for three factors. The BBD is employed for this study, which includes 15 trials. Variables chosen for BBD are based on PB design analysis. Factors selected were in three levels low (-1), central (0) and high (+1) as shown in Table 2.

In the design, there are 3 factors, 15 base runs with three center points, 1 replicate, 1 block and 15 total runs. The amount of immobilized lipase high value is 20%, central value is 12.5% and 5% is low value. pH high value is 8, central value 7 and low value is 6. Agitation speed high value 200rpm, central value 125rpm and low value 50rpm. The rest of the factors and their level is selected based on the PB responses. Temperature set to 45℃ as immobilized lipase has shown its maximum activity at 45℃ and molar ratio (1:3) is selected for transesterification 3moles of methanol is required for 1mole of triglyceride as per stoichiometric ratio. The reaction mixture is prepared using 25gm of honge oil, 3.4ml methanol (1:3 ratio- which is calculated based on the average molecular weight of oil) and varying amounts of immobilized lipase, pH and agitation speed as per experimental layout (Table 3). Transesterification is carried out in a shaking incubator at 45℃ for 24 hour. After completion of the reaction, immobilized lipase beads are collected from the reaction mixture by filtration. The collected filtrate is kept for the separation of biodiesel and glycerol in a separating funnel overnight. The upper layer of Biodiesel is collected and quantified by using Eq.3.

Table 2: Process variables for Box-Behnken (BB) Design

Process Variables	Levels		
	-1	0	+1
Amount of Immobilized Lipase (%)	5	12.5	20
pH	6	7	8
Agitation speed (rpm)	50	125	200

Table 3: Experimental layout for BB design

Standard order	Run	Block	Amount of Immobilized lipase (%)	pH	Agitation speed (rpm)
12	1	1	12.5	8	200
14	2	1	12.5	7	125
3	3	1	5.0	8	125
1	4	1	5.0	6	125
7	5	1	5.0	7	200
15	6	1	12.5	7	125
10	7	1	12.5	8	50
11	8	1	12.5	6	200
2	9	1	20.0	6	125
13	10	1	12.5	7	125
9	11	1	12.5	6	50
4	12	1	20.0	8	125
8	13	1	20.0	7	200
5	14	1	5.0	7	50
6	15	1	20.0	7	50

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Biodiesel production using immobilized lipase under optimum conditions

Biodiesel is produced under optimum conditions which are revealed by BBD design such as molar ratio 1:3, pH 7, amount of immobilized lipase 12.5% for 25gm of *Pongamia* seed oil at 45℃, 125rpm for 24 hour. The reaction mixture is prepared in 100 ml conical flask by adding 25gm of oil, 3.125gm of immobilized lipase, 3.4 ml methanol, pH is set to 7 using phosphate buffer, flask was kept in shaking incubator at a speed of 125rpm at 45℃. After 24 hour of reaction mixture is collected and filtered through filter paper to collect immobilized beads. The collected filtrate is kept for settling in the separating funnel. Biodiesel is collected and quantified.

GC-MS analysis

The produced biodiesel is subjected to GC-MS analysis to analyze the fatty acid content. For the analysis GC-MS Shimadzu (QP2020) instrument with a column SH-Rtx, wax having dimensions of 0.50µm T, 30.0m L & 0.25µm diameter is used. Analysis is performed using the standard method EN 14103. The detection of FAMEs is ascertained using Mass Spectrometry (MS) by comparing the relative retention time of each discrete FAMEs with reliable standards of fatty acid methyl esters [33, 34, 31].

Fuel properties of biodiesel

Characterization need to be performed to evaluate whether the produced biodiesel will obey the fuel properties as per ASTM standards. Fuel properties like viscosity, density, flash point, fire point, pour point, cetane number, calorific value, carbon residue etc. were tested by using standard procedures. These are compared with ASTM standard limits (American Standard of Testing material, ASTM 6751–3**)** [23, 2].

Results and Discussion

Statistical optimization of biodiesel production to identify the reaction conditions suitable for transesterification to achieve maximum yield of biodiesel. These optimized conditions for the transesterification of *Pongamia* oil could be promising for biodiesel production in a large scale for future scope.

Physicochemical properties of honge oil

The properties of the biodiesel mainly depend on fatty acid content and physicochemical properties of the feedstock used. Hence, it is important to know the relationship between these to synthesize good-quality biodiesel [35]. Physicochemical analysis of honge oil is performed using standard methods (Table 4).

GC-MS analysis using GCMS Shimadzu (QP2020) instrument, reported the fatty acid composition of honge oil based on peak position and peak area. The fatty acid composition of honge oil is presented in Table 5 the major FA composed in honge oil is oleic acid (54.09%), palmitic acid, stearic acid and linoleic acid among others. The results are in agreement with studies for honge oil showing that it is suitable as feedstock for biodiesel synthesis [36].

Table 4: Physicochemical properties of Pongamia pinnata oil

Sl.No.	Test Property	Test results	Units
1	Color	Yellowish orange color	-
2	Odor	Undesirable	-
3	Density	932.8	kg/m^3
4	Viscosity	45.27	cSt
5	Acid value	9.96	mg KOH/g of oil
6	Saponification value	199.99	mg/g of oil
7	FFA	4.98	%

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Table 5: Fatty acid composition of Pongamia pinnata oil

Fatty acid	Systematic name	Structure	Retention time	Composition %
Palmitic acid	Hexadecanoic acid	C16:0	15.235	13.52
Stearic acid	Octadecanoic acid	C18:0	19.321	7.59
Oleic acid	**9-Octadecenoic acid**	**C18:1**	**19.811**	**54.09**
Linoleic acid	9,12-Octadecadienoic acid	C18:2	20.875	17.02
α-Linolenic acid	9,12,15-Octadecatrienoic acid	C18:3	22.380	2.29
Arachidic acid	Eicosanoic acid	C20:0	23.887	1.29
Gondoic acid	11-Eicosenoic acid	C20:1	24.436	0.69
Heneicosanoic acid	Henicosanoic acid	C21:0	27.854	3.50
Saturated fatty scid	-	-		25.9
Monounsaturated fatty acid	-	-		54.78
Polyunsaturated fatty acid	-	-		19.31
Degree of unsaturation	-	-		93.4

Lipase production and immobilization

Lipase produced in optimized media has an activity of 16.75 U/ml. The partial purification of lipase yields 50.74% with 15.28 fold purification having a specific activity of 21.25U/mg. The efficiency of immobilization is around 75% is achieved.

Transesterification using biological catalyst

Transesterification of honge oil is carried out using biocatalyst (Locally prepared immobilized lipase) under the reaction conditions mentioned in the material and methods. As shown in Fig.1 (e), biodiesel stands at upper layer and glycerol at the bottom. Glycerol is removed from the bottom and biodiesel is collected and recorded a yield of 58%. Biodiesel is produced successfully using immobilized lipase (Fig.1 (f**)**) but lower yield is recovered hence to enhance the yield further optimization studies are performed. Biodiesel yield from honge oil is observed by many authors to be around 75-92% (24,31).

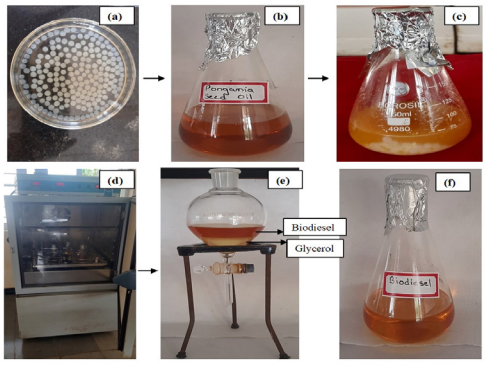

Fig.1: Enzymatic Transesterification a: Immobilized lipase beads b: *Pongamia* oil c: Reaction mixture d: Reaction mixture in shaking incubator e: Separation of biodiesel and glycerol f: Produced Biodiesel

Optimization Studies

Optimization studies are performed to enhance the yield of FAME (biodiesel) which is

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key aspect for an economically viable process. Generally, optimization is performed to identify the optimum conditions which will give higher conversion rates and yield. Biodiesel yield is depending on type of feedstock and reaction conditions used. Yield may vary with different reaction conditions and type of feedstocks and its characteristics. Hence to achieve greater yield it is mandate to optimize the reaction conditions as per the feedstock. In the present study, optimization is performed by using statistical designs (PB & BBD). None of the studies reported on optimization studies using enzymes through statistical designs.

Conventional optimization of multiple parameters is a difficult as greater number of trials were involved in the process. Therefore, the present study is employing Response surface methodology **(**RSM) for optimization of biodiesel production as this method is more advantageous than conventional method. RSM is novel an effective statistical tool for evaluating the relationships between independent variables and response. RSM determines the optimized reaction conditions of the process. RSM is widely used for optimization of biodiesel synthesis by several scientists throughout the world; very few reports are available on enzymatic biodiesel production from *Pongamia* seed oil (15).

MINITAB-14 software is used to analyse the experimental design. *p-value* is used to test the significance of the hypothesis. If the *p-value* is less than 0.05, indicates that selected variables are significant and non significance if it is more than 0.05. The factors which have p>0.05 are excluded and factors have p<0.05 are chosen for further studies. Box-Behnken Design (BBD) is used in the present study as it was found to be superior to central composite designs (CCD) and most suitable for optimization including three variables [19]. An effort has been made in the current study to know the effect of three factors such as concentration of biocatalyst, pH and agitation speed on biodiesel yield. These effective factors are identified by Plackett-Burman experiment. In Box-Behnken design the interactions of the three variables are studied. When one variable is on hold as high, central and low the interactions and effects of other two variables are studied. The experimental layout of BBD is designed using three variables at three levels (+1, 0, -1) which gives out 15 experimental trials which are performed and biodiesel yield is recorded as a response.

The experimental layout and corresponding response of Box-Behnken Design are presented in Table 6. It is observed that there is an appreciable variance in the yield of biodiesel under diverse conditions.

The required quadratic equation for biodiesel:

$$\text{Biodiesel Yield} = -464.519+5.304X_1+139.833X_2+0.839X_3-0.148X_1^2-9.333X_2^2-0.002X_3^2-0.400X_1X_2+0.005X_1X_3-0.053X_2X_3$$

Note: X_1=Concentration of Biocatalyst

X_2=pH

X_3=Agitation speed

The ANOVA for regression models of optimization shows the model is well fitted with regard to the relationship between the biodiesel yield and the process variables. *p-value* defines the significance of each term in ANOVA (Table 8). The results of ANOVA summarized in Table 8 show that the *p-value* of the model is found to be 0.007 and F-value is 12.24, indicating that the model is highly satisfactory and significantly approves the model for validation. The lack of fit is insignificant as the *p value* is 0.218 confirming the validity of the model indicates data perfectly fit into the model. The high value of R^2 (95.7%) and adj-R^2 (87.8%) describes that the model represented the actual relationship is well correlated between the response and independent variables. In Fig.2 obtained values and predicted values are on par with each other in all the runs. As shown in Fig.2 the results show that the experiment is in agreement with predicted values.

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The shape of the surface plot illustrates the different interactions between the variables [37]. The graphs represent the effect of variables on biodiesel yield by using two continuous variables and the other one is fixed at three levels such as (+1, 0, -1). The analysis reveals pH is the most significant factor to affect yield followed by concentration of biocatalyst and agitation speed.

Table 6: Box-Behnken experimental design matrix

Runs	Concentration of biocatalyst (%)	pH	Agitation speed (rpm)	Yield of Biodiesel (%)	Predicted Yield (%)
1	12.5	8	200	64	61
2	12.5	7	125	92	94.6
3	5.0	8	125	80	81.5
4	5.0	6	125	84	80.5
5	5.0	7	200	68	69.5
6	12.5	7	125	96	94.6
7	12.5	8	50	80	78
8	12.5	6	200	72	74
9	20.0	6	125	80	78.5
10	12.5	7	125	96	94.6
11	12.5	6	50	72	75
12	20.0	8	125	64	67.5
13	20.0	7	200	68	67.5
14	5.0	7	50	84	84.5
15	20.0	7	50	72	70.5

Table 7: Estimated regression coefficient for biodiesel yield verses concentration of biocatalyst, pH and agitation speed

Term	Coefficient	SE Coefficient	T value	P value
Constant	-464.519	101.230	-4.589	0.006
Concentration of Biocatalyst %	5.304	2.010	2.639	0.046
pH	139.833	27.778	5.034	0.004
Agitation speed rpm	0.839	0.201	4.176	0.009
Concentration of Biocatalyst %* Concentration of Biocatalyst %	-0.148	0.035	-4.259	0.008
pH*pH	-9.333	1.956	-4.770	0.005
Agitation speed rpm*Agitation speed rpm	-0.002	0.000	-6.815	0.001
Concentration of Biocatalyst %*pH	-0.400	0.251	-1.596	0.171
Concentration of Biocatalyst %* Agitation speed rpm	0.005	0.003	1.596	0.171
pH*Agitation speed rpm	-0.053	0.025	-2.128	0.087

S = 3.759 R-Sq = 95.7% R-Sq(adj) = 87.8

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Table 8: Analysis of Variance for Biodiesel Yield (%) verses concentration of biocatalyst, pH and agitation speed

Source	DF	Seq. SS	Adj. SS	Adj.MS	F value	P value
Regression	9	1557.07	1557.07	173.007	12.24	0.007
Linear	3	340.00	584.23	194.745	13.78	0.007
Square	3	1081.07	1081.07	360.356	25.50	0.002
Interaction	3	136.00	136.00	45.333	3.21	0.121
Residual Error	5	70.67	70.67	14.133		
Lack-of-Fit	3	60.00	60.00	20.000	3.75	0.218
Pure Error	2	10.67	10.67	5.333		
Total	14	1627.73				

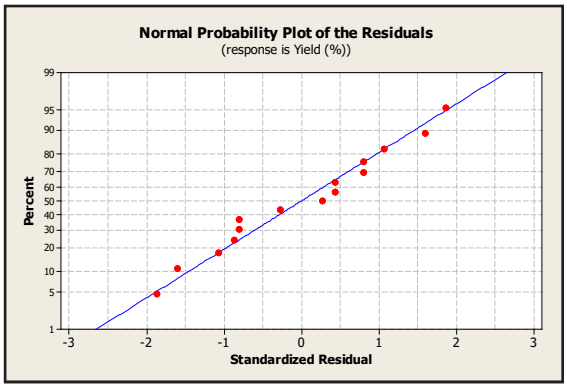

Fig. 2: Predicted vs Actual Yield of Pongamia Biodiesel

Effect of concentration of biocatalyst and pH

As shown in Fig. 3 when the concentration of biocatalyst is varied with pH and agitation speed being constant shows the effect on yield as at the high and low level of concentration of biocatalyst, yield is low rather at central level (12.5%) the yield is maximum it may be due to saturation of substrates after central level hence even though the increase in the concentration of biocatalyst doesn't have an impact on yield therefore yield decreases. Many authors reported the same phenomenon as enzyme content increases yield and also increases upto its saturation level (25% to 35%).

An optimum lipase concentration of 10% to the weight of oil is considered in studies carried out by Kumar et al. (24). This concludes that yield depends on the type of enzyme as well as the type of feedstocks.

Similarly, for pH (pH 7) central value has shown greater yield indicating the suitability of pH 7 for lipase as its optimum pH that shows its maximum activity. At low pH 6 and high pH 8 activity is reduced hence yield also decreases it may be due to conformational changes in the active site of lipase (25). Similarly reported in many studies (30).

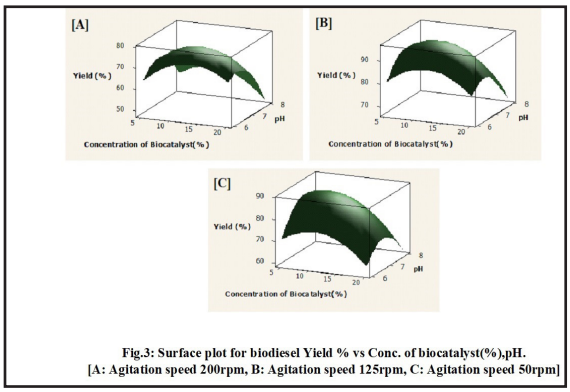

Fig. 3. Surface plot for biodiesel yield % vs Conc. of biocatalyst (%), pH. A. Agitation speed 200 rpm, B. Agitation speed 125 rpm, C. Agitation speed 50 rpm.

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Effect of Concentration of Biocatalyst and agitation speed

When the concentration of biocatalyst is varied with agitation speed and pH being constant the effect on yield is observed. As shown in Fig. 4 yield is higher at the central level for both concentration of biocatalyst (12.5%) and agitation speed (125 rpm) indicating that increasing agitation speed enhances the yield as mixing provides greater surface area for the interaction of the enzyme with its substrate, but at higher speed it may damage the immobilized beads hence the yield may decrease. As even when the concentration of biocatalyst increases yield is low due to higher agitation speed that may damage the beads and due to high pH (8) and low pH (6) values it may distort the active site of an enzyme that could not carry out reaction hence even at high concentration yield is low even at pH 7 which is optimum pH value, high concentration of biocatalyst and low concentration of biocatalyst both gives lower yields may be due to low concentration not sufficient to hold all the substrates and high concentration get saturated with all substrates.

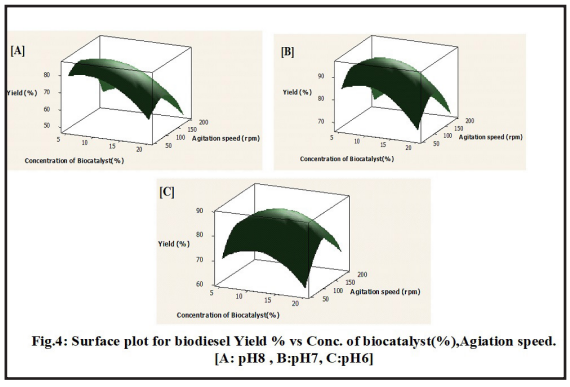

Fig.4: Surface plot for biodiesel Yield % vs Conc. of biocatalyst(%),Agiation speed. [A: pH8 , B:pH7, C:pH6]

Fig. 4. Surface plot for biodiesel yield % vs Conc. of biocatalyst (%), Agitation speed. A. pH 8, B. pH 7, C. pH 6.

Effect of pH and Agitation speed on yield

When pH is varied with agitation speed and concentration of biocatalyst being constant, yield is more at the central level for both pH (7) and Agitation speed (125 rpm) when the concentration of biocatalyst is at three levels (Fig. 5). As pH and agitation speed increases for all levels of concentration of biocatalyst shows a similar pattern, indicates that ionic concentration is one of the important factors for enzymes activity and agitation speed influences exposure of substrates to enzymes but higher speed may damage the enzymes at any concentration of biocatalyst.

Effective factors such as concentration of biocatalyst, pH and agitation speed were identified in previous studies by PB design and further optimization of these factors with the use of RSM BBD revealed the optimum conditions for biodiesel production from honge oil using locally prepared immobilized lipase.

The optimum parameters obtained as follows: concentration of biocatalyst (X1) = 12.5%, pH (X2) =7, agitation speed (X3) =125rpm and remaining reaction conditions viz. temperature and molar ratio are maintained at 45℃ and 1:3 respectively yields 96%. To validate the model, experimental studies are performed with optimized conditions that lead to the average conversion yield of 94.8% which is close to the predicted response demonstrating that statistical designs can effectively estimate the optimal conditions for the biodiesel production process.

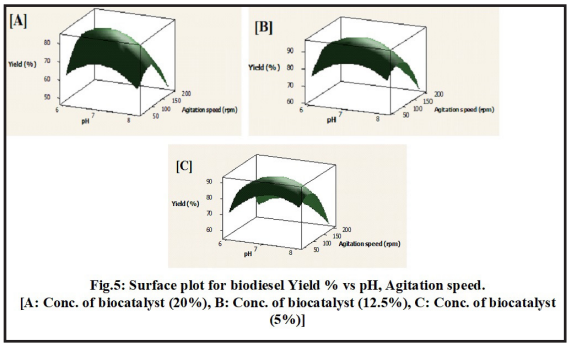

Fig.5: Surface plot for biodiesel Yield % vs pH, Agitation speed. [A: Conc. of biocatalyst (20%), B: Conc. of biocatalyst (12.5%), C: Conc. of biocatalyst (5%)]

Fig. 5. Surface plot for biodiesel yield % vs pH, Agitation speed A. Conc. of biocatalyst (20%), B. Conc. of biocatalyst (12.5%), C. Conc. of biocatalyst (5%)

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Biodiesel production under optimal conditions

Using the optimized conditions 94.8% biodiesel yield is obtained, which is higher than previously reported yield from honge oil using immobilized lipase. One of the studies reported a biodiesel yield of 91.3% using 10% Novozyme 435. The optimum biodiesel yield of 72% is recorded by using *Pseudomonas fluorescens* entrapped in the sodium alginate and 92% yield at 5% enzyme, 1:6 molar ratio and 600rpm for 6 hours. Many authors have reported BBD for optimization of transesterification of different feedstocks apart from honge oil and major studies have been carried out for chemical transesterification than enzymatic hence there is wide scope for optimization of enzymatic process for different feedstocks as each type of feedstock yields biodiesel differently (25, 35, 38). Based on the literature, previous studies are not available on statistical optimization of biodiesel synthesis from *Pongamia* oil using locally prepared immobilized lipase from local isolated strain. From the results, it is revealed that the applied statistical tools for optimization studies are successful.

GC analysis of pongamia biodiesel

The produced biodiesel is subjected to GC-MS analysis using GCMS Shimadzu (QP2020) instrument to evaluate the chemical components of the biodiesel (FAME) produced by enzymatic transesterification process under optimized conditions. As per Fig. 6 it is found that the highest peak is observed for palmitic acid methyl ester and oleic acid methyl ester indicating higher conversion of these two fatty acids during transesterification (34, 2). The fuel properties of biodiesel play a vital role that will affect engine performance and emission characteristics hence to evaluate manufactured biodiesel for its technical compatibility with conventional diesel and to ensure that it meets international standards. For commercial applications of biodiesel, fuel property analysis is mandatory. Standard analytical methods are applied to the final biodiesel product to determine the fuel characteristics and the results are compared with the diesel standards of EN 14214 and ASTM for biodiesel and are listed in Table 9. The result revealed that the properties of produced biodiesel are in accordance with ASTM (American Standards for Testing and Materials) standards. The mentioned fuel-quality parameters are found comparable with those from previous studies (24,36,23).

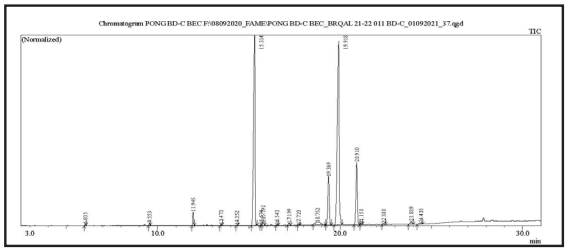

Fig. 6: GC Chromatogram of Pongamia Biodiesel

Table 9: Properties of Pongamia biodiesel and method used

Fuel Properties	ASTM Used	Pongamia Biodiesel	Limits	Standard Biodiesel ASTM D 6751-18	Petro diesel
Density (kg/m3)	ASTM D 4052	890.0	850-900kg/m3	880 kg/m3	860-900kg/m3
Viscosity @ 40°C (cSt)	ASTM D445	5.8233	1.9-6.0mm2/s or	1.3-4.1cSt	1.9-6.0cSt
Cloud point (°C)	ASTM D2500	18.4°C	-3 to 12	-3 to 12	-15-7
Flash point	ASTM D93C	143°C	93 °C min	100-170	60-80
Fire point	-	165 °C	-	120-190	80-100
CFPP	ASTM D6371	21 °C	Max 19	Location & season dependent	Location & season dependent
Oxidation stability	EN 14112	3.11	6hrs(B6-20) 3HRS(BIOO)	3hrs	6hrs
Calorific Value	ASTM D240	38,082 J/g	J/g	29850	29307– 62801 J/g

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Conclusion

This study focuses on the optimization of biodiesel production using immobilized lipase through Response Surface Methodology. Previous studies have identified variables such as the amount of immobilized lipase, temperature, reaction time, pH, agitation speed, and water content to be effective factors for biodiesel yield. Among these variables, the amount of immobilized lipase, pH, and agitation speed were found to have significant effects on yield. The Box-Behnken Design (BBD) was used to optimize these effective variables, and the central value results showed a greater response for all variables. The optimized conditions resulted in a yield of 94.8%. These findings demonstrate that statistical optimization is an effective approach to improve the efficiency of the transesterification process and enhance the yield of biodiesel. Additionally, the use of enzymatic conversion with immobilized lipase represents a promising process for the conversion of honge oil to biodiesel.

References

1. Dilip, K., Das, T., Balendu, S. G. and Verma, B. (2020) Preparation and characterization of novel hybrid bio-support material immobilized from *Pseudomonas cepacia* lipase and its application to enhance biodiesel production. Renew Energ, 147(1), 11-24.

2. Chourasia, V.R., Gawas, A. S., Menon, A.S. and Shinde, P.M. (2015) Production of biodiesel by enzymatic transesterification using immobilized lipase. Int J Eng Res, 3, 1238-1246.

3. Patel, R. and Patel, S. (2017) Renewable hydrogen production from butanol: a review, Clean Energ, 1(1), 90–101.

4. Casimir, C. A., Shu-Wei, C., Guan-Chiun, L. and Jei-Fu, S. (2007) Enzymatic approach to biodiesel production. J Agric Food Chem, 55(22), 8995-9005.

5. Antczak, M.S., Kubiak, A., Antczak, T. and Bielecki, S. (2009) Enzymatic biodiesel synthesis-Key factors affecting efficiency of the process. Renew Energ, 34(5), 1185-1194.

6. Uthoff, S., Bröker, D. and Steinbüchel, A. (2009) Current state and perspectives of producing biodiesel like compounds by biotechnology. Microb Biotechnol, 2(5), 551–565.

7. Christopher, L.P., Kumar, H. and Zambare, V.P. (2014) Enzymatic biodiesel: Challenges and opportunities. Appl Energ, 119, 497-520.

8. Yucel, S., Terzioglu, P. and Ozqmen, D. (2012) Lipase Applications in biodiesel Production. InTech Open, Chapter 8, 209-234.

9. Yamane, K., Ueta, A. and Shimamoto, Y. (2001) Influence of physical and chemical properties of biodiesel fuels on injection, combustion and exhaust emission characteristics in a direct injection compression ignition engine. Int J Engine Res, 2(4), 249-261.

10. Thangaraj, B., Solomon, P.R., Muniyandi, B., Ranganathan, S. and Lin Lin. (2018) Catalysis in biodiesel production-a review. Clean Energ, 3(1), 2-23.

11. Sankaran, R., Show, P.L. and Chang, J.S. (2016) Biodiesel production using immobilized lipase: feasibility and challenges. Biofuels Bioprod Bioref, 10(6), 896–916.

12. Ong, H.C., Milano, J., Silitonga, A.S., Hassan, M.H., Shamsuddin, A.H., Chin-Tsan, W., Mahlia, T.M.I., Siswantoro, J., Kusumo, F. and Sutrisno, J. (2019)

Vol. 17(Supplementary issue - 3B) 1208-1222,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.57

Biodiesel production from *Calophyllum inophyllum* - *Ceiba pentandra* oil mixture: Optimization and characterization. J Clean Prod, 219, 183-198.

13. Marchetti, J. M., Miguel, V. U. and Errazu, A. F. (2007) Possible methods for biodiesel production. Renew Sust Energ Rev, 11(6), 1300–1311.

14. Gwi-Taek, J. & Don-Hee, P. (2009). Optimization of Biodiesel Production from Castor Oil Using Response Surface Methodology. Appl Biochem Biotechnol, 156, 431–441.

15. Manojkumar, N., Muthukumaran, C. and Sharmila, G. (2022) A comprehensive review on the application of response surface methodology for optimization of biodiesel production using different oil sources. J King Saud Univ Eng Sci, 34(3),198-208.

16. Laureano, C., David, E. L.C., Cubero-Atienza, A.J., Ruiz, J.J. and Pilar Dorado, M. (2018) Optimization of the Transesterification of Waste Cooking Oil with Mg-Al Hydrotalcite Using Response Surface Methodology. Energies, 11(2), 302.

17. Mehta, A., Sharma, R. and Gupta, R. (2019) Statistical Optimization by Response Surface Methodology to Enhance Lipase Production *Aspergillus fumigatus,* Open Microbiol J, 13(1), 86-93.

18. Kenthorai, R. J., Sariah, A., Denis, P., Eng, S. C. and Pogaku, R. (2008) Production of Biodiesel Using Immobilized Lipase - A Critical Review. Crit Rev Biotechnol, 28(4), 253-264.

19. Ferreira, S. L., Bruns, R. E., Ferreira, H. S., et al. (2007) Box-Behnken design: An alternative for the optimization of analytical methods. Anal Chim Acta, 597(2), 179-186.

20. Supardan, M.D., Fahrizal., Moulana, R., Safrida, D., Satriana. and Wan, A. M. (2017) Optimisation of Process Parameter Conditions for Biodiesel Production by Reactive Extraction of Jatropha Seeds. J Eng Sci Technol, 2(3), 847 – 859.

21. Al-Zuhair, S. (2007) Production of biodiesel: possibilities and challenges. Biofuels Bioprod Bioref, 1(1), 57–66.

22. Islam, A.K.M.L., Primandari, P. and Yaakob, Z. (2018) Non-Edible Vegetable Oils as Renewable Resources for Biodiesel Production: South-East Asia Perspective. Adv Biofuels Bioenerg, 3, 201-215.

23. Mamilla,V.R., Mallikarjun, M. V. and Rao, L. M. (2011) Preparation of Biodiesel from Karanja Oil. Int J Energ Eng, 1, 94-100.

24. Kumar, D., Das, T., Giri, B. S. and Verma, B. (2018) Characterization and compositional analysis of highly acidic Karanja oil and its potential feedstock for enzymatic synthesis of biodiesel. New J Chem, 42(19), 15593-15602.

25. Katerine, S. M., Lourembergue, S. M., Rodolpho, R. C. M., et al. (2020) Optimization of the Production of Enzymatic Biodiesel from Residual Babassu Oil (Orbignya sp.) via RSM. Catalysts, 10(4), 1-20.

26. Shilpa, K. J. and Bharati, S. M. (2021) Isolation, Optimization and Molecular Characterization of Novel Lipase Producing *Lysinibacillus macroides* FS1 from Oil Rich Sample for Biodiesel Production. Curr Trends Biotechnol Pharm, 15(5), 115-123.

27. Mobarak-Qamsari, E., Kasra-Kermanshahi, R. and Moosavi-nejad, Z.

Vol. 17(Supplementary issue - 3B) 1208-1222,September 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.3s.57

(2011) Isolation and identification of a novel lipase producing bacterium, *Pseudomonas aeruginosa* KM110. Iran J Microbiol, 3(2), 92–98.

28. Bharathi, D., Rajalakshmi, G. and Komathi, S. (2018) Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil. J King Saud Univ Sci, 31(4), 898–901.

29. Ertan, F., Hulya, Y. & Bilal, B. (2007) Optimization of α-Amylase Immobilization in Calcium Alginate Beads. Prep Biochem Biotechnol, 37(3), 195-204.

30. Kareem, S. O., Falokun, E. I., Balogun, S. A., Oluwaseyi, A. A. and Omeike, S. O. (2020) Improved biodiesel from palm oil using lipase immobilized calcium alginate and *Irvingia gabonensis* matrices. Beni-Suef Univ. J Basic Appl Sci, 9, 1-8.

31. Modi, M.K., Reddy, J. R. S., Rao, B. V. S. K. and Prasad, R. B. N. (2007) Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor. Bioresour Technol, 98(6), 1260–1264.

32. Arumugam, A. and Ponnusami, V. (2017) Production of biodiesel by enzymatic transesterification of waste sardine oil and evaluation of its engine performance, Heliyon, 3(12), e00486.

33. Fatima, S., Muhammad, W. M., Hamid, M., et al. (2020) Response Surface Methodology Approach for Optimized Biodiesel Production from Waste Chicken Fat Oil. Catalysts, 10(6), 1-12.

34. Gangadhara, R. and Prasad, N. (2016) Studies on optimization of transesterification of certain oils to produce biodiesel. Chem Int, 2, 59-69.

35. Srikanth, H. V., Venkatesh, J. & Sharanappa, G. (2018) Box-Behnken Response Surface Methodology for Optimization of Process Parameters for Dairy Washed Milk Scum Biodiesel Production. Biofuels 10(1), 113-123.

36. Bobade, S. N. and Khyade, V. B. (2012) Detail study on the Properties of *Pongamia pinnata* (Karanja) for the Production of Biofuel. Res J Chem Sci, 2(7), 16-20.

37. Peng, X., Yang, G., Shi, Y., Zhou, Y., Zhang, M. & Li, S. (2020) Box–Behnken design based statistical modeling for the extraction and physicochemical properties of pectin from sunflower heads and the comparison with commercial low-methoxyl pectin. Sci Rep, 10(1), 3595.

38. Panichikkal, A. F., Prakasan, P., Kizhakkepowathial, N. U. et al., (2018) Optimization of parameters for the production of biodiesel from rubber seed oil using onsite lipase by response surface methodology. 3 Biotech, 8, 1-14.